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TECHNICAL REPORT

Literature review on the state of biocide resistance in wild vector populations in the EU and neighbouring countries

March 2023

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Abbreviations

AF	Allele frequency
AIM-COST	*Aedes* Invasive Mosquito – European Cooperation in Science & Technology
BR	Biocide resistance
Bti	*Bacillus thuringiensis israelensis*
CDC	US Centers for Disease Control and Prevention
DC	Discriminating concentration
DEF	S,S,S-tributyl phosphorotrithioate
ECDC	European Centre for Disease Prevention and Control
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
EU	European Union
Kdr	Knock-down resistance
LC	Lethal concentration
MR	Mortality rate
PBO	Piperonyl butoxide
RR	Resistance ratio
VEN	VectorNet Entomological Network
VGSC	Voltage-gated sodium channel
WHO	World Health Organization
WIN	Worldwide Insecticide resistance Network
WHOPES	WHO Pest Evaluation Scheme.

Glossary

Term	Definition
Acaricide	Active substance against ticks and mites (Acari)
Biocide or biocidal active substance	The biocidal active substances are mostly chemical compounds, but can also be microorganisms (e.g. bacteria).
Biocidal product	Biocidal products contain one or more biocidal active substances and may contain other non-active co-formulants that ensure the effectiveness as well as the desired pH, viscosity, colour, odour, etc. of the final product. Biocidal products are used to control unwanted organisms that are harmful to human or animal health or to the environment, or that cause damage to human activities. These harmful organisms include pests (e.g. insects, rats or mice) and microorganisms (e.g. bacteria, viruses, mould). The term refers to the product as placed on the market with specified formulation, instructions for use, including purpose, time and frequency of application, dose rate and protective equipment.
Biocide resistance	The ability of an organism to withstand the effects of biocide exposure, usually as a result of a behavioural or physiological adaptation.
Discriminating concentration	A fixed concentration of an active substance dissolved in a solvent, used as a biocide, with the intention of discriminating between susceptible and resistant individuals from a population.
EU-MediLabSecure	A health network focussing on the prevention of vector-borne diseases around the Mediterranean and Sahel regions to ultimately promote the integrated surveillance of emerging arboviruses.
IR-Mapper	An interactive tool displaying World Health Organization (WHO) and United States Centers for Disease Control and Prevention susceptibility bioassay results, indicating insecticide resistance of *Anopheles* mosquitoes, *Aedes albopictus* and *Aedes aegypti*.
Insecticide	Active substance against insects. Insecticides are governed by Regulation (EU) No 528/2012, with the exception of those used for plant protection purposes which are governed by Regulation (EU) No 1107/2009.
LC_{50}	Concentration of a biocide that will kill 50% of the sample population under study.
RR_{50}	Dividing the LC_{50} of a wild population with that of a susceptible one providing a measure of resistance. This is interpreted by the WHO guidelines as follows: <5, the population is considered to be susceptible; between 5−10, the resistance in the population is considered to be moderate; >10, the resistance in the population is high.
Synergist	A substance which is used in combination with an active ingredient to enhance its efficacy. The synergist does not have biocidal properties by itself, but often acts by inhibiting the enzyme which normally detoxifies the biocide.
Vector	An arthropod capable of transmitting a pathogen.
VectorBase	Part of The Eukaryotic Pathogen, Vector and Host Informatics Resource (VEuPathDB), which is one of two Bioinformatics Resource Centers (BRCs). VectorBase is the fraction that focuses on invertebrate vectors of infectious diseases and the collection of data on it. It has global coverage.
Vector control	Measures of any kind against pathogen-transmitting arthropods (vectors) intended to limit their presence or abundance or their ability to transmit the pathogen.
Vector surveillance	Continuous, systematic collection, analysis and interpretation of vector-specific data that can be used in planning, implementing and evaluating public or veterinary health practice.
Worldwide Insecticidal resistance Network (WIN)	Collaboration of 19 worldwide recognised institutions in vector research, with the aim of identifying countries/regions vulnerable to insecticide resistance and providing WHO and Member States with recommendations on how to handle this resistance.

Executive summary

In the light of (re-)emerging vector-borne pathogens, European Union/European Economic Area (EU/EEA) countries are increasingly implementing intervention strategies in an attempt to decrease their public and veterinary health burden. In many cases, vector control is essential for preventing and controlling the transmission of vector-borne pathogens, as (prophylactic) drugs and/or vaccines are often unavailable. There are a wide array of control measures available, often using a biocide in their approach. The extensive use of these chemicals is known to give rise to resistance in the target vector populations, undermining the efficacy of the biocide and the control efforts. The aim of this report is to assess the state of biocide resistance among vectors present in the EU/EEA region through a literature search and contacting the members of the VectorNet Entomological Network and their intermediaries.

A literature review was carried out covering the period 2000−2021. Biocide resistance (BR) assessment studies were included when performed on wild vector populations (mosquitoes, sand flies, biting midges and ticks) from the EU countries and selected neighbouring countries. Additional data were obtained through a questionnaire sent to the members of the VectorNet Entomological Network. Studies of species belonging to one of the four vector groups were eligible for inclusion and there was no limit to the number of species that could be included. Studies on species not belonging to these vector groups were excluded. There were no exclusion criteria for the type of biocide tested.

We found that biocide resistance assessment studies have mainly been conducted in countries around the Mediterranean region and on mosquito species such as *Culex pipiens* and *Aedes albopictus*. Fewer studies have been done on ticks and sand flies, and no studies on biting midges were identified in this review. Resistance has been confirmed in multiple species and regions and for multiple biocide classes. *Culex pipiens* and *Aedes albopictus* were found to be resistant against biocides from the pyrethroid, organophosphate, organochlorine and carbamate classes. Knock-down resistance (Kdr) allele mutations have also been detected in these two species. A large amount of data was found through the literature search. However, the totality of studies does not provide a good insight into the status of biocide resistance in Europe due to the fact that the studies are largely fragmented in time and space and the results are not always reported according to predefined criteria. Predictions based on these results are premature, but biocide resistance is emerging and is likely to hinder current and future vector control efforts. We recommend further research, using consistent and standardised methods, to monitor the spread and development of resistance in vector species.

Based on literature review results and the data provided by the VectorNet Entomological Network members, the implications for public health practice can be summarised as follows:

- In countries where authorities consider deploying vector control, biocide resistance monitoring results in the target vector species could inform estimates of expected biocide effectiveness and the use of biocides in vector control activities.
- The biocide assessment studies would need consistent and standardised methods to facilitate an EU-wide view of the vector species' biocide resistance status.
- Where availability study results warrant this, it could be of value to explore (in the context of EU biocide legislation) vector control intervention mixes that are not (or less) based on biocides.
- There is a need for further investigation to improve the use of molecular assays as resistance detecting methods.
- Cooperation between biocide resistance researchers and professionals from the public and veterinary health services involved in vector control could be useful to update biocide resistance monitoring data, generate risk maps, provide scientific and technical expertise to policy makers, and disseminate information among actors and countries.

1 Background

Vector-borne diseases are a specific group of infections that represent a (re-)emerging threat to health in Europe requiring particular attention [1-8]. Diseases such as dengue, Zika virus disease, West Nile fever, leishmaniasis, Lyme borreliosis, bluetongue and Schmallenberg disease impose health and veterinary burdens on communities. In the past, many vector-borne diseases affected tropical and subtropical regions [9]. However, due to globalisation and climate change, previously unaffected regions may now also be at risk as a result of the introduction and settlement of new vector species [10]. Vector-borne diseases therefore pose a special challenge for national public health and veterinary authorities, the European Centre for Disease Prevention and Control (ECDC) and the European Food Safety Authority (EFSA).

In many cases, vector control is essential to preventing and controlling transmission of vector-borne pathogens, as (prophylactic) drugs and/or vaccines are often unavailable [11,12]. To mitigate the impact of vector-borne diseases and break the cycle of pathogen transmission, a comprehensive approach to vector control is needed [13]. There is a large array of mosquito vector control intervention methods, ranging from larvivorous fish to odour-baited traps. However, most interventions use some sort of biocide. These are products containing an active substance which are used to kill, deter or render unwanted organisms harmless. Biocides against mosquitoes are sprayed or vaporised, applied to water surfaces or used to impregnate materials such as curtains or bed nets. They can also be used to target different life stages of the mosquito species in question [14]. Control of sand fly vectors is often based on the use of biocides on bed nets, indoor residual spraying or the application of reservoir feed-through insecticides [13,15]. Vector control methods of *Culicoides* include mechanical, biological, and genetic control where the use of biocides has a prominent role [16]. The current measures to prevent human tick-borne disease burden include a range of personal protection measures, with biocides playing a prominent role in impregnated clothing. In the veterinary health sector, the control of ticks is largely based on biocides used directly on livestock [17].

In 2021, a questionnaire-based survey was conducted on the organisation of vector surveillance and control in and around Europe [18]. Of the 31 countries implementing mosquito control, 21 used biocides in their control efforts. Though vector control efforts targeting biting midges, sand flies or ticks are implemented less often, biocide-based interventions are also frequently used to control these vector groups [18].

The abundant use of biocides has resulted in vector populations gradually becoming resistant to one or more biocidal active substances [19]. Due to the build-up of resistance, the biocide in question will gradually lose its effectiveness, defeating its purpose as a means of decreasing the public or veterinary health burden. A good understanding of the status of biocide resistance in wild populations can be very useful for local authorities organising vector control campaigns, helping them to adopt mitigation measures and readjust the control strategy.

Resistance to most of the WHO-approved public health biocides has been reported around the globe [20,21]. The primary mechanisms of resistance to biocides, predominantly in adult vectors, are generally divided into two categories [14,22]:

1) Target-site resistance: a mutation in the protein receptor which the biocide attacks, causing the active substance to no longer be able to bind to the target site of the receptor. Therefore, the vector is no longer (or less) affected by the biocide. Two commonly studied mutations are mutations in the sodium channel receptor resulting in so-called 'knockdown resistance', caused by pyrethroid and DDT use [23]; and a mutation in the protein acetylcholinesterase (a neurotransmitter), resulting in so-called 'Ace-1 resistance', caused by organophosphate and carbamate use [24].
2) Metabolic resistance: changes in the insects' enzyme systems resulting in a more rapid detoxification of the active substance of the biocide. Resistance arises because the detoxification prevents the biocide from reaching the intended action site.

In addition, changes in the behaviour of the vector species could potentially have a detrimental effect on the efficacy of biocides used in vector control. This type of resistance cannot be studied as easily as physiological resistance. Possible ways in which behavioural resistance could develop are by avoiding contact with biocides or changing feeding times to the evening and morning, when humans are not protected by bed nets or indoor residual treatments. Behavioural adaptations have already been detected in some mosquito species [25]. Finally, cuticular resistance is an underexplored mechanism detected in some mosquito vectors, which may also lead to phenotypic resistance [26]. The thickening of the cuticle or the change in its composition may reduce the absorption rate of the biocide, reducing its efficacy.

In order to inform public and veterinary health officials and those working in vector control, a literature review was conducted to assess the state of biocide resistance in wild vector populations (as opposed to insect colonies maintained in insectaries) in and around the EU/EEA (excluding the EU/EEA outermost regions[1] and overseas countries and territories) and to provide an overview of detected biocide resistance mechanisms in these vector populations.

[1] The nine EU outermost regions, which are geographically very distant from the European continent, are French Guiana, Guadeloupe, Martinique, Mayotte, Réunion and Saint-Martin (France), Azores and Madeira (Portugal), and the Canary Islands (Spain).

2 Methods

Literature review

The main question posed for this review was: 'What is the biocidal resistance status of field populations of arthropod vectors of public and veterinary health importance in Europe?' An area of further interest were the underlying mechanisms of biocide resistance present in populations where resistance is detected.

To review biocide resistance in a standardised manner, data were collected in line with predefined eligibility criteria.

Population

Biocide resistance (BR) assessment studies were included if they were performed on wild vector populations from countries covered by the VectorNet geographical mapping area (see 'Geography' section, Annex 1).

Biocide resistance assessments were included for wild populations of the following vector groups:

- mosquitoes;
- sand flies;
- biting midges;
- ticks.

Studies of all species belonging to one of the four vector groups were eligible for inclusion and there was no limit on the number of species that could be included. Studies on species not belonging to these vector groups were excluded. There were no exclusion criteria for the type of biocide used.

Assessment

The biocide resistance assessments of mosquitoes, sand flies and biting midges included were:

- WHO adult susceptibility bioassay;
- CDC bottle bioassay;
- WHO larvicide susceptibility bioassay;
- WHO insect growth regulators test.

Biocide resistance assessment studies by means of synergist bioassay (metabolic resistance) or biochemical/molecular assay (target site resistance) were also included.

For ticks, the biocide resistance assessments included were:

- larval packet test;
- adult immersion test;
- larval immersion micro assay;
- larval tarsal test.

In ticks, assessments of biocide resistance by means of synergist bioassay (metabolic resistance) or biochemical/molecular assay (target site resistance) were also included.

Resistance assessments are usually performed using a discriminating concentration (DC) assay (Annex 2). In these assays, individuals from wild populations are subjected to a predetermined biocide concentration and the mortality rate (MR) is measured [22]. For WHO bioassays, populations are considered as susceptible if MR >98%, possibly resistant if MR is between 90−98% and resistant if MR <90%, while for CDC bottle bioassays MR <80% confirms resistance [27]. Another way to measure the efficacy of a biocide is by calculating the concentration which is lethal for 50 or 90% of the wild population (LC_{50}; LC_{90}). Dividing the lethal concentration of a wild population by that of a susceptible one produces a resistance ratio (RR) which indicates whether a population is resistant or not (RR <5: low resistance; 5 < RR <10: moderate resistance; RR >10: high resistance). When discriminating concentrations were not predetermined, a comparison is often made with a known susceptible population. This method was also eligible.

Temporal delineation

As this review aims to gather knowledge on the biocide resistance status (of the vector groups studied) relevant for current vector control operations, studies were only included that assessed the biocide status from 2000 onwards. Where reviews were available covering a broader period of biocide resistance assessment for the target vector groups in Europe, we only included the data within the time frame of this review (i.e. from 2000 till January 2022.)

Search strategy

The following search engines were used to find relevant publications:

- Web of Science;
- PubMed.

The search terms comprised three categories (the complete list can be found in Annex 1):

- biocide resistance terms;
- geography (all included countries and a selection of regions);
- vector terms (common names, synonyms and genera).

Grey literature was obtained by contacting the VectorNet Entomological Network (VEN) members directly via a questionnaire (Annex 3) and sending them the data extraction form. The enquiry was limited to the last ten years (2011-2021) and was based on a two-step approach.

1. VEN members were asked to take part in a survey about whether biocide resistance research had been conducted in their country, by whom and for which vector group. Participants were asked whether they were willing to share their data for this review. The initial survey was sent out on 11 November 2021 and three reminders were sent during the following month.
2. All researchers conducting research on biocide resistance were contacted, either as part of the VEN or following referral by a VEN member. We specifically requested data on biocide resistance assessment assays. The database template developed for the literature review was shared for the collection of the data.

Additional data sources were cross-checked and data available in these data sources but not found during the literature search were included. By cross-checking in these data sources we also obtained an idea of how effective the search strategy was. The additional data sources included:

- The Worldwide Insecticidal resistance Network (WIN);
- EU MediLabSecure;
- AIM-COST;
- IR-mapper;
- VectorBase.

Literature review

One researcher ruled out duplicate papers and removed studies conducted outside of the geographical scope. Two researchers then assessed the studies individually by title and abstract. Literature was formally screened for several eligibility criteria. Criteria for exclusion were:

- testing of new biocides or synergists;
- usage of lab colonies (i.e. populations not collected from the field);
- use of assays other than bioassays, synergist assays, molecular assays or biochemical assays;
- study of non-target species;
- researchers only examined (molecular) mechanisms of biocide resistance (and did not perform field assays to measure resistance);
- researchers handled a topic related to biocide resistance rather than assessments;
- no abstract was available, and the article did not seem useable based on the title;
- tests were conducted before the year 2000.

If both researchers opted to include (or exclude) a paper, no further discussion was needed. Disagreements on study eligibility were assessed and a consensus decision was made.

Next, the full text of the resulting literature was screened, based on the same eligibility criteria, to make a definitive literature selection from which data would be extracted. In addition, data records were not generated if:

- no distinction was made between species when conducting the assays;
- data were compiled in graphs and there were no numeric data to extract.

Data extraction

Data from literature and reports obtained through the literature search and the VEN survey were entered into a database (Annex 4). Whenever needed, data were converted to uniform units throughout the database (e.g. entering coordinates in decimal units.)

Data analysis

Rstudio was used to summarise data and make plots and maps. Packages used were the 'tidyverse' collection of packages, 'ggplot2', 'lwgeom', 'sf' and 'map'.

In the 'Results' section of the report we first present an overview of the extracted data to provide an overall impression of the research conducted during the period 2000–2021. Then in the second part we present the biocide resistance data per vector species.

The WHO guidelines for susceptibility tests demand at least four replications of 20–25 individuals. However, studies in which the sample size did not meet the WHO requirements were included in the graphs and tables to avoid excluding too many data points.

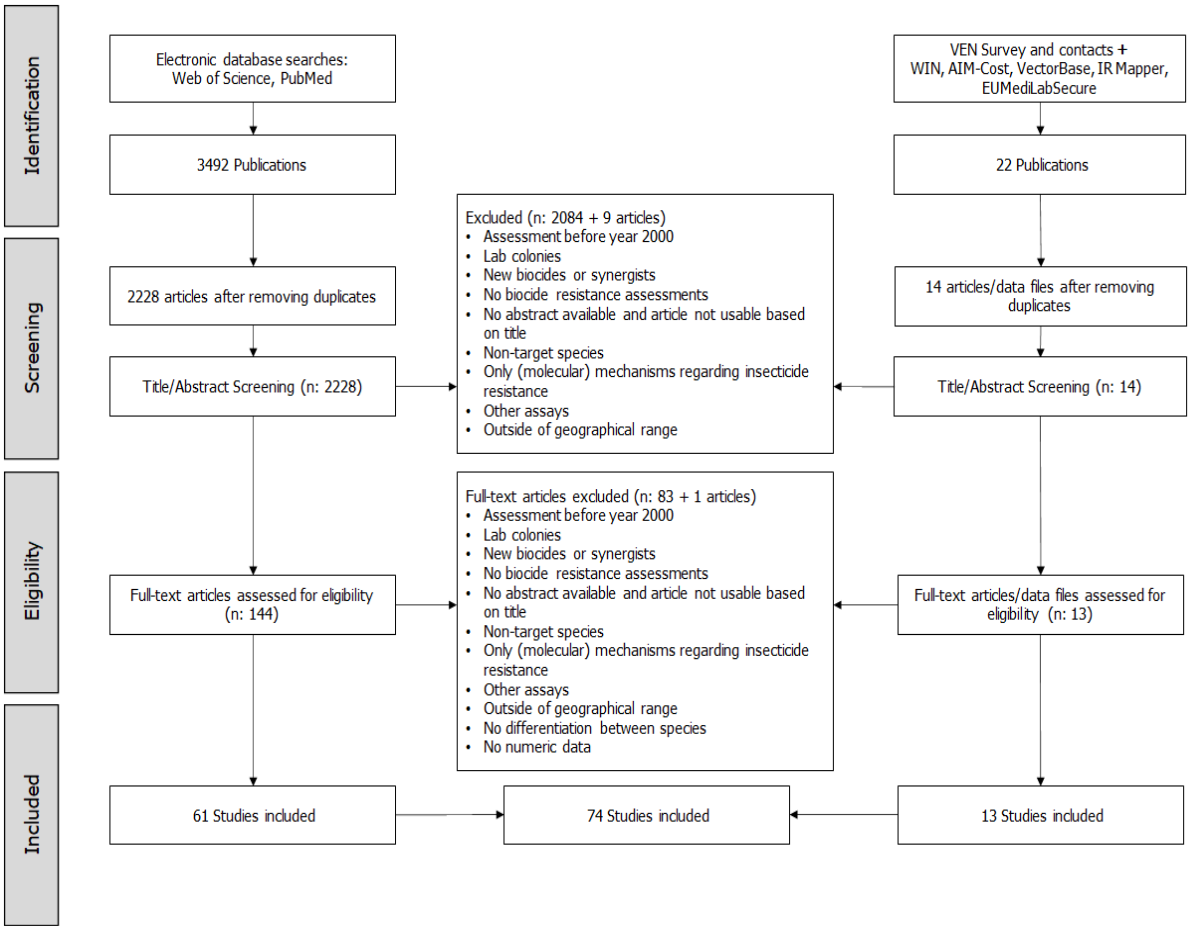
To summarise molecular assay results, resistance genes were grouped per type of mutation and the frequency of the mutated alleles was reported.

3 Results

Literature review

The initial search resulted in 3 492 publications, or 2 228 publications after removing the duplicates. Publications were subsequently screened for title and abstract by two independent reviewers, leaving 144 publications. After the full text screening, data were extracted from 61 publications (Figure 1).

Figure 1. Overview of the selection and screening process for the publications

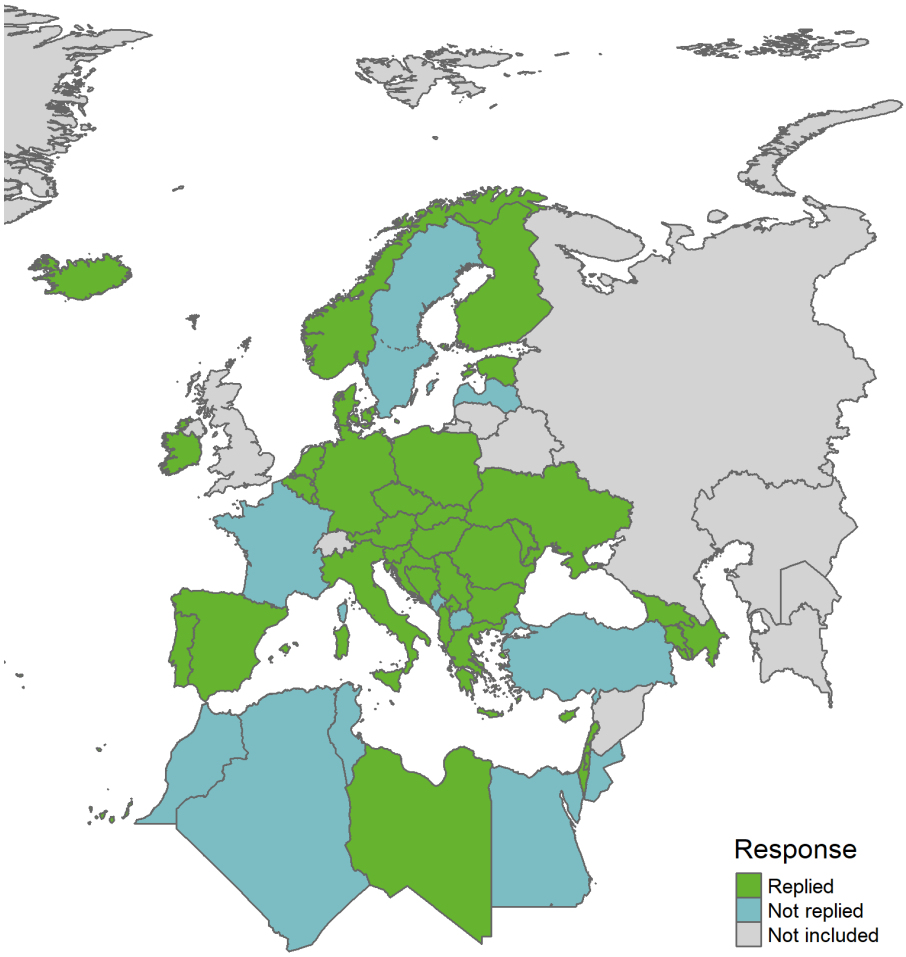


Publications found on IR-Mapper within the study area [28-32] were already included after scanning PubMed and Web of Science. In addition to publications already included in the search results, we found extra information in a supplementary file of a WIN publication [20]. This contained data by Toma et al. [33] and a master thesis by Seixas [34]. On the website of AIM-COST there was one publication on biocide resistance mentioned which was eligible for inclusion, but this was already in our database [31]. Scanning VectorBase did not result in additional literature. We also added data records from tests conducted through the Moroccan Ministry of Health and from the Malaria Threat Map on WHO's website [35]. Finally, we added data from two unpublished studies by Pichler et al., provided by VEN members; data from a French report by ANSES; an unpublished Belgian study, and data from two publications by Yavasoglu et al. [36,37]. The latter two publications were in our initial search, but were excluded because the data were not presented numerically. We received the data through contact with the author.

VEN survey

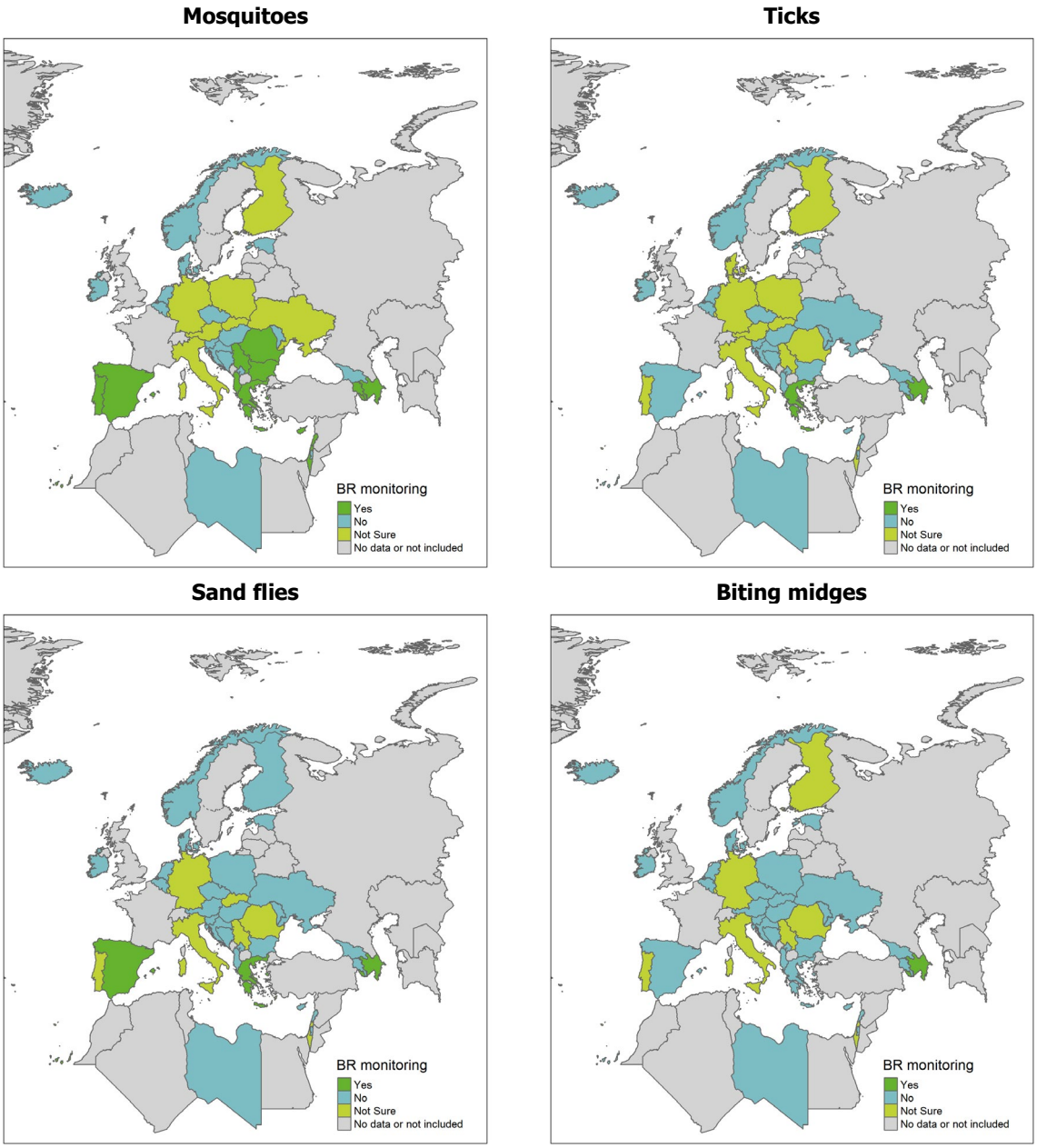
Of the 55 VEN members contacted, representing 50 countries, a total of 38 completed the survey (Figure 2), 17 VEN members completed it after the initial request, and 14 after the first reminder. After the final reminder we received seven more completed surveys.

Figure 1. Overview of the response to the questionnaire sent to the VEN members



Ten of the responding countries stated that biocide resistance is monitored in mosquitoes, making this the most studied vector group. The biocide resistance monitoring of mosquitoes in Cyprus, Portugal, Romania, Serbia and Spain is based on both government and institutionally-based assignments (i.e. in the context of a research project). In Azerbaijan, monitoring of biocide resistance in mosquitoes is exclusively the result of government assignments, whereas in Albania, Armenia, Bulgaria, Greece, Lebanon and Romania, the monitoring is solely institutionally-based (Figure 3, Table 1). Two countries implement biocide resistance monitoring of ticks: in Azerbaijan it is governmentally-assigned and in Greece it is institutionally-based. Eleven VEN members were not sure whether biocide resistance monitoring of ticks was performed in their country (Figure 3, Table 1). Biocide resistance monitoring of sand flies was conducted in Azerbaijan, Greece and Spain, where it is institutionally-based. Finally, based on the questionnaire, biocide resistance monitoring of biting midges is only conducted in Azerbaijan (Figure 3, Table 1).

Figure 2. Overview of countries implementing biocide resistance (BR) monitoring, according to the VEN survey



Note: *these are results from the question: 'Is biocide resistance of wild populations of the following vector groups (mosquitoes, ticks, sand flies, biting midges) assessed in your country? The assessment can be done as part of a governmental assignment or as part of a research project at national, regional or local level.'*

Table 1. Number of countries implementing biocide resistance monitoring per vector group (based on 38 countries)

Biocide resistance monitoring commissioned by	Mosquitoes	Ticks	Sand flies	Biting midges
Government assigned (national, regional and/or local)	7	1	1	1
Institutionally-based (in the context of a research project).	11	1	2	0

Thirteen VEN members were willing to share data and 23 additional researchers were referred to. Eight of these forwarded data, six were unable to contribute to our study and 16 did not respond to our requests. In the end, four additional studies, whether still ongoing or unpublished, were added to the database. This, plus the nine additional studies we obtained and the 61 publications we found through the literature search, brought the total number of studies in the database to 74 (Figure 1).

Overview of the extracted data

General overview

Data extraction from the literature review resulted in 1 738 records being entered in the database. Adding 345 records from the studies obtained from the VEN members, other contacts and the additional databases brought the total to 1 972 data records (Table 2). A large number of bioassays (n=242) in Tunisia were performed using the method devised by Raymond et al. [38]. These data were included in the database despite not being fully in line with the predefined criteria. Not all studies using bioassays to calculate lethal concentrations and resistance ratios are displayed here but they all appear in the section 'Biocide resistance status per vector species'.

Most of the data records include mosquito species (n=1 855), with *Culex pipiens* being the species most often investigated (n=853), followed by *Aedes albopictus* (n=453) and *Anopheles sacharovi* (n=235). In total, we found data on 13 mosquito species. Unless specifically noted, mosquitoes from the *Anopheles maculipennis* complex were defined as 'sensu lato'. The sand fly species tested for resistance all belonged to the *Phlebotomus* genus (n=36), with *Phlebotomus sergenti* (n=14) and *Phlebotomus papatasi* (n=12) being the most frequently tested species of the six sand fly species included. With regard to ticks, two species were tested for resistance: *Rhipicephalus sanguineus* (n=45) and *Rhipicephalus annulatus* (n=36). We did not find any publications relating to biting midges.

Table 2. Overview of number of data records per species and test compiled from the literature search

Vector species	Data records	Bioassay*	Synergist assay	Molecular assay	Biochemical assay	Bioassay lethal concentration
MOSQUITOES						
Aedes aegypti	13	7	0	6	0	0
Aedes albopictus	453	124	0	281	0	48
Aedes caspius	2	2	0	0	0	0
*Aedes detritus***	4	3	1	0	0	0
Anopheles hyrcanus	1	0	0	1	0	0
Anopheles labranchiae	62	26	0	0	0	36
Anopheles maculipennis sensu lato	74	68	0	0	6	0
Anopheles maculipennis sensu stricto	1	0	0	1	0	0
Anopheles sacharovi	235	218	0	17	0	0
Anopheles sergentii	22	2	0	2	0	18
Anopheles superpictus	127	127	0	0	0	0
Culex modestus	2	0	0	2	0	0
*Culex pipiens****	853	199	4	399	1	250
Culex torrentium	6	0	0	6	0	0
SAND FLIES						
Phlebotomus neglectus	2	0	0	2	0	0
Phlebotomus papatasi	12	11	0	1	0	0
Phlebotomus perfiliewi	4	0	0	4	0	0
Phlebotomus sergenti	14	14	0	0	0	0
Phlebotomus simici	2	0	0	2	0	0
Phlebotomus tobbi	2	0	0	2	0	0
TICKS						
Rhipicephalus annulatus	36	33	0	3	0	0
Rhipicephalus sanguineus	45	0	0	0	0	45
TOTAL	**1 972**	**834**	**5**	**729**	**7**	**397**

Note:
* *Bioassays include the WHO adult susceptibility bioassay, CDC bottle bioassay and WHO larvicide susceptibility bioassay.*
**Aedes detritus *was named* Ochlerotatus detritus *in the original publication.*
*** Culex pipiens *refers to data on* Culex pipiens *and the biotypes* Culex pipiens *biotype* pipiens, Culex pipiens *biotype* molestus *and the* Culex pipiens pipiens-molestus *hybrid. The biotypes accounted for 271 data records, of which 206 were records from Tunisia in which lethal concentration bioassays were conducted.*

The majority of biocide resistance data records originated from Türkiye (n=589), followed by Italy (n=33) and Tunisia (n=287) (Table 3, Figure 4). In total, data from 26 countries were included. Yavaşoglu et al. accounted for the majority of the data entries (n=361) [36,37], followed by Pichler et al., including unpublished data (n=212) [32,39,40], and Tabbabi et al. (n=149) [41-55]. In 124 of the records from Tabbabi et al. bioassays were performed according to the method proposed by Raymond et al. [38].

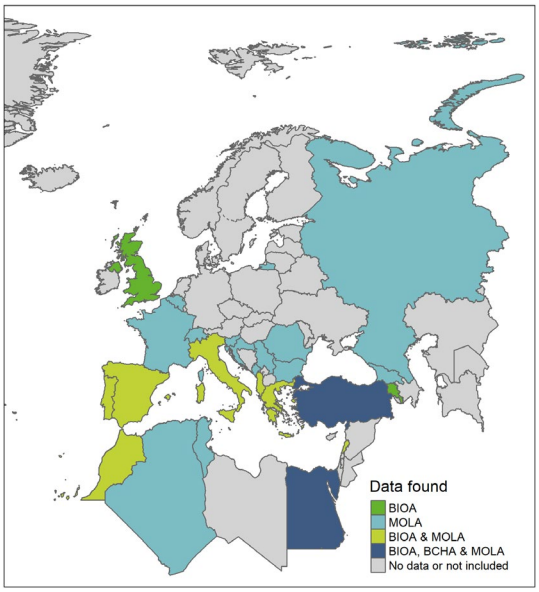
Table 3. Overview of number of data records per country and tests compiled in the biocide resistance database

Country	Data records	Bioassay	Synergist assay	Molecular assay	Biochemical assay	Bioassay lethal concentration
Albania	7	5	0	2	0	0
Algeria	18	0	0	18	0	0
Armenia	7	7	0	0	0	0
Belgium	26	0	0	26	0	0
Bulgaria	2	0	0	2	0	0
Croatia	3	0	0	3	0	0
Cyprus	22	0	0	0	0	22
Egypt	84	78	0	4	1	1
France	37	0	0	5	0	32
Georgia	3	0	0	3	0	0
Greece	223	54	0	169	0	0
Italy	334	86	0	219	0	29
Lebanon	9	5	0	4	0	0
Malta	1	0	0	1	0	0
Montenegro	3	0	0	3	0	0
Morocco	129	58	0	63	0	8
Portugal	31	13	0	18	0	0
Romania	4	0	0	4	0	0
Russia	4	0	0	4	0	0
Serbia	8	0	0	8	0	0
Slovenia	1	0	0	1	0	0
Spain	130	72	0	13	0	45
Switzerland	6	0	0	6	0	0
Tunisia	287	0	0	27	0	260
Türkiye	589	453	4	126	6	0
United Kingdom	4	3	1	0	0	0
TOTAL	**1 972**	**834**	**5**	**729**	**7**	**397**

Biocide resistance data on mosquitoes was retrieved from 26 countries, while biocide resistance monitoring on ticks was limited to Egypt and research on sandflies to Morocco, Greece and Türkiye (Table 3, Figure 4).

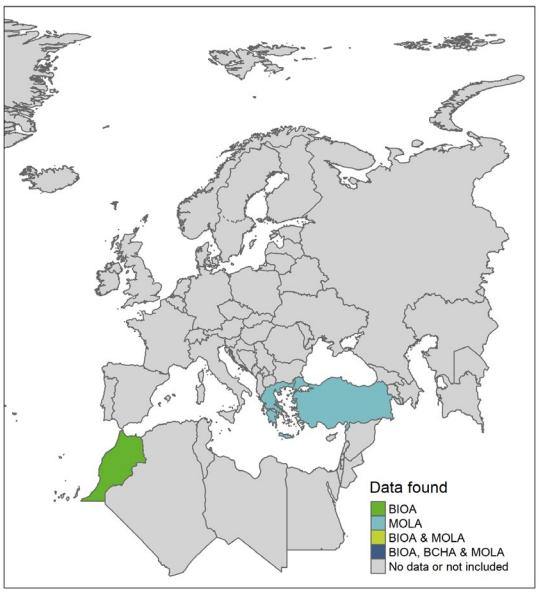
Figure 3. Overview of biocide resistance data retrieved by vector group

Mosquitoes



Ticks

Sand flies



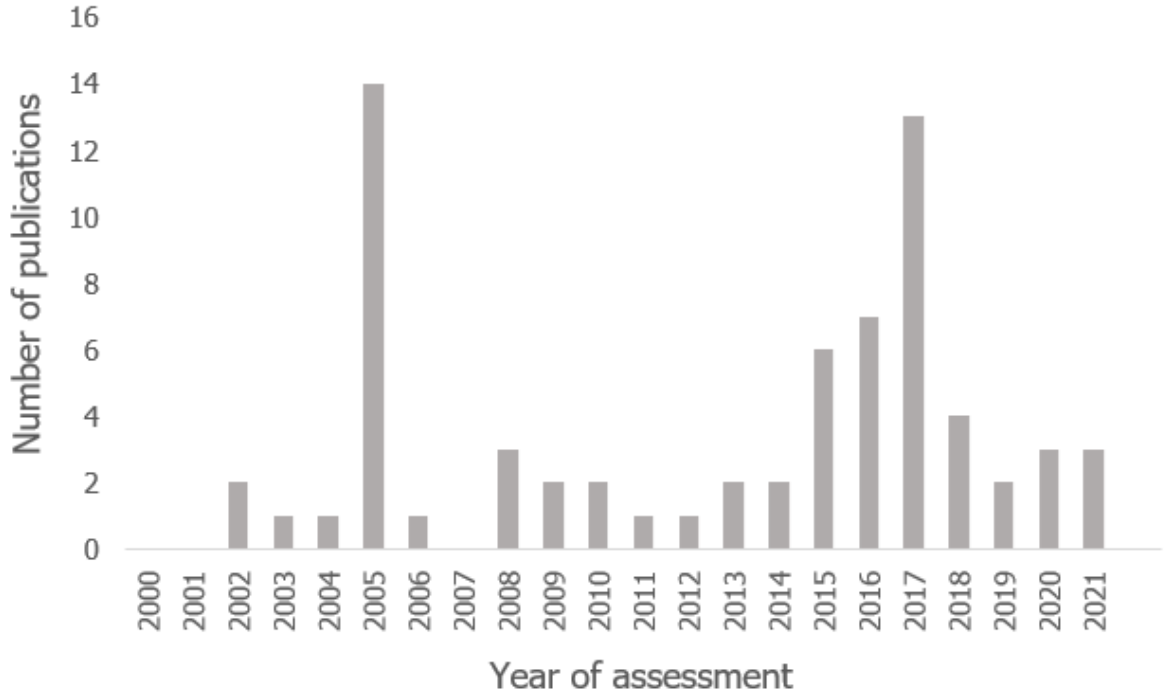
Biting midges

No data available in the BR-database

***Note**: BIOA, bioassays including the standard WHO bioassays and CDC bottle tests; MOLA, molecular assays; BCHA, biochemical assays. For Cyprus only data on LC_{50} of* Culex pipiens *were found in the literature.*

Between 2000–2021, on average one to three studies per year were conducted, with the exception of 2005 and the period 2015–2017. The high numbers of studies in 2005 is due to Tababbi et al. publishing results from a large study in separate reports (Figure 5).

Figure 4. Number of publications on biocide resistance included in the database, by year in which tests were conducted



Note: *in publications where the assessments were made over multiple years, we used the last year in which the assessments were done. Four studies did not report when their tests were conducted and are not included in this figure [43,56-58]*

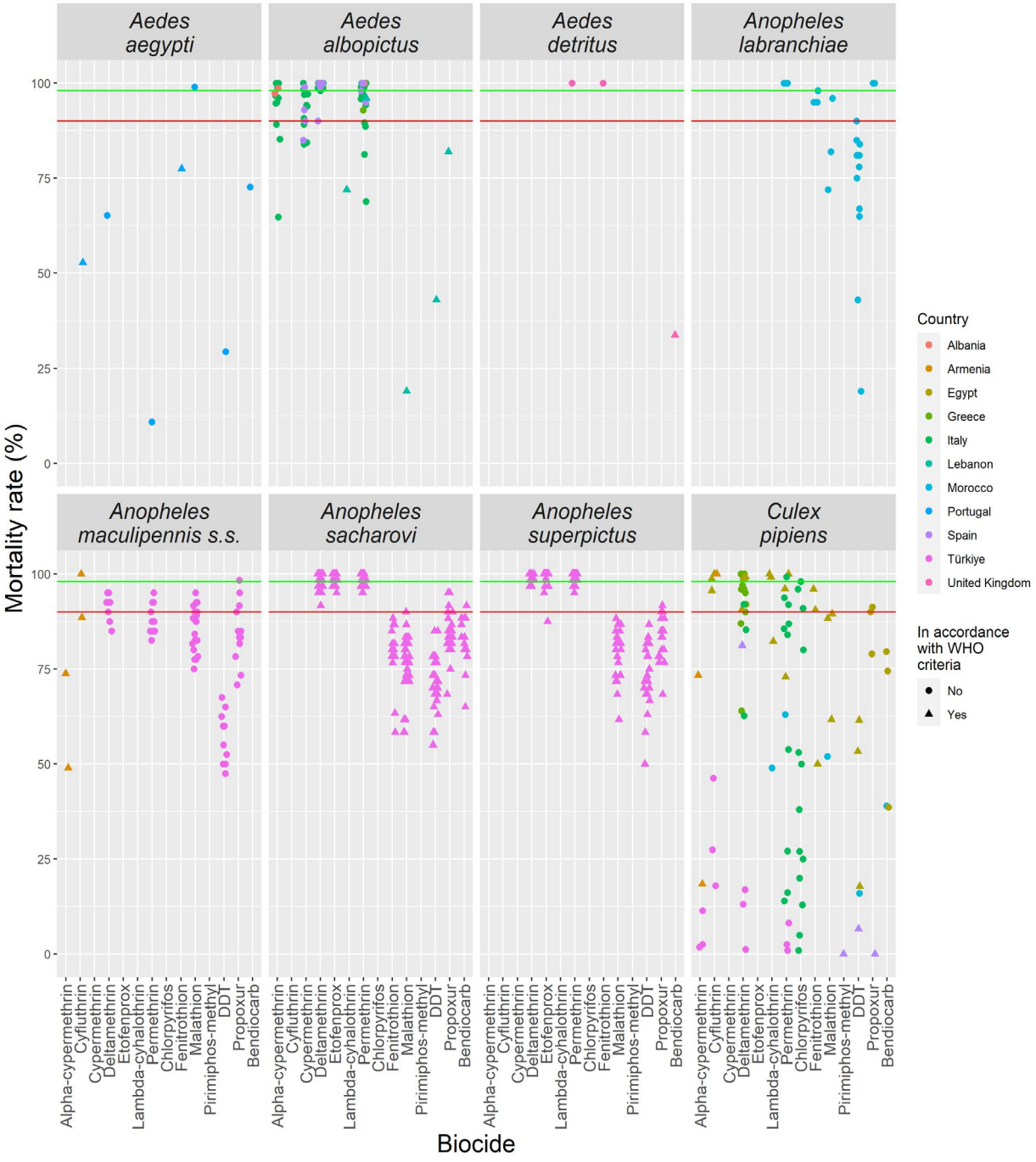
Data extraction of bioassay results

WHO adult susceptibility test

Figure 6 provides an overview of the WHO adult susceptibility test available in the database. Studies are split according to their compliance with the WHO criteria.

- In the studies, which complied fully with the WHO criteria, mosquitoes were the only vector group tested and *Anopheles sacharovi* was the species most tested. These included studies from Armenia (x records: 7), Egypt (n= 21), Lebanon (n= 5), Portugal (n= 2), Spain (n= 4), Türkiye (n= 345) and the United Kingdom (n= 1). Biocides used in these studies were pyrethroids (alpha-cypermethrin, cyfluthrin, deltamethrin, etofenprox, lambda-cyhalothrin, permethrin), organophosphates (fenitrothion, malathion, pirimiphos-methyl), organochlorines (DDT), and carbamates (bendiocarb, propoxur). The lowest mortality rates were found for pirimiphos-methyl and propoxur in a *Culex pipiens* population in Spain, both biocides had zero effect (MR: 0%). Resistance was confirmed in 213 cases (MR: <90%) and possibly present in 54 cases (MR: 90–97%), while in 118 cases, populations were susceptible (MR: >98%).
- Data from studies which did not fully comply with the WHO criteria originated from Albania (x records: 3), Greece (n= 4), Italy (n= 26), Morocco (n= 36), Spain (n= 4), United Kingdom (n= 2). The majority of tests were performed on mosquitoes (n= 50), but also on the sand fly species *Phlebotomus papatasi* (n= 11) and *Phlebotomus sergenti* (n= 14).

Figure 5. Overview of biocide resistance status of mosquito species, based on WHO bioassay test



***Note**: this is a compilation of the data for the period 2000–2021. It is possible that there are multiple data points from the same region, but from a different year. Studies that did not comply with WHO criteria were studies in which (1) the mortality rate of the control group was not reported, (2) the exposure time was not in accordance with the WHO guidelines, or (3) the WHO standard discriminating concentration was not used. Data on species where the mortality rate was 100% (i.e.* Anopheles sergentii, Phlebotomus papatasi *and* Phlebotomus sergenti*) were excluded from this graph. The green horizontal line indicates the threshold between susceptible and possible resistance, the red line the threshold between possible resistance and resistance.*

There were three publications in which not all data were in accordance with the WHO criteria: Zayed et al. (2006) did not report the mortality rate of the control group when testing carbamate biocides [59]; Brown et al. (2018) did not report the mortality rate in three of the four cases [60] and Seixas [61] used discriminating concentrations of *Anopheles* mosquitoes when assessing *Aedes* populations, which in four of six cases differed from those of the *Aedes* mosquitoes. In the other two cases the concentration was the same for *Aedes* and *Anopheles* mosquitoes, so these were in line with the WHO criteria. These data are discussed in the section 'Biocide resistance status per vector species'.

Data from Pichler et al. (2018, 2019) were not fully in line with the WHO criteria because the concentrations used for the assays differed from the discriminating concentrations and were higher than recommended [31,32]. Their results are described when discussing results by species, as low mortality rates in higher concentration assays may indicate strong levels of resistance. Assays from Toma et al. (2010) were excluded here because a discriminating concentration for chlorpyrifos has not yet been established and no mortality rates for the control group have been described [62]. In eleven of the thirteen excluded studies the mortality of the control group was not reported [31,59,60,62-69]. Some publications noted that they applied Abbott's formula, however they were still excluded because they did not report the mortality rates. In three of the thirteen studies, the exposure time was not in line with the tests requirements or was not reported.

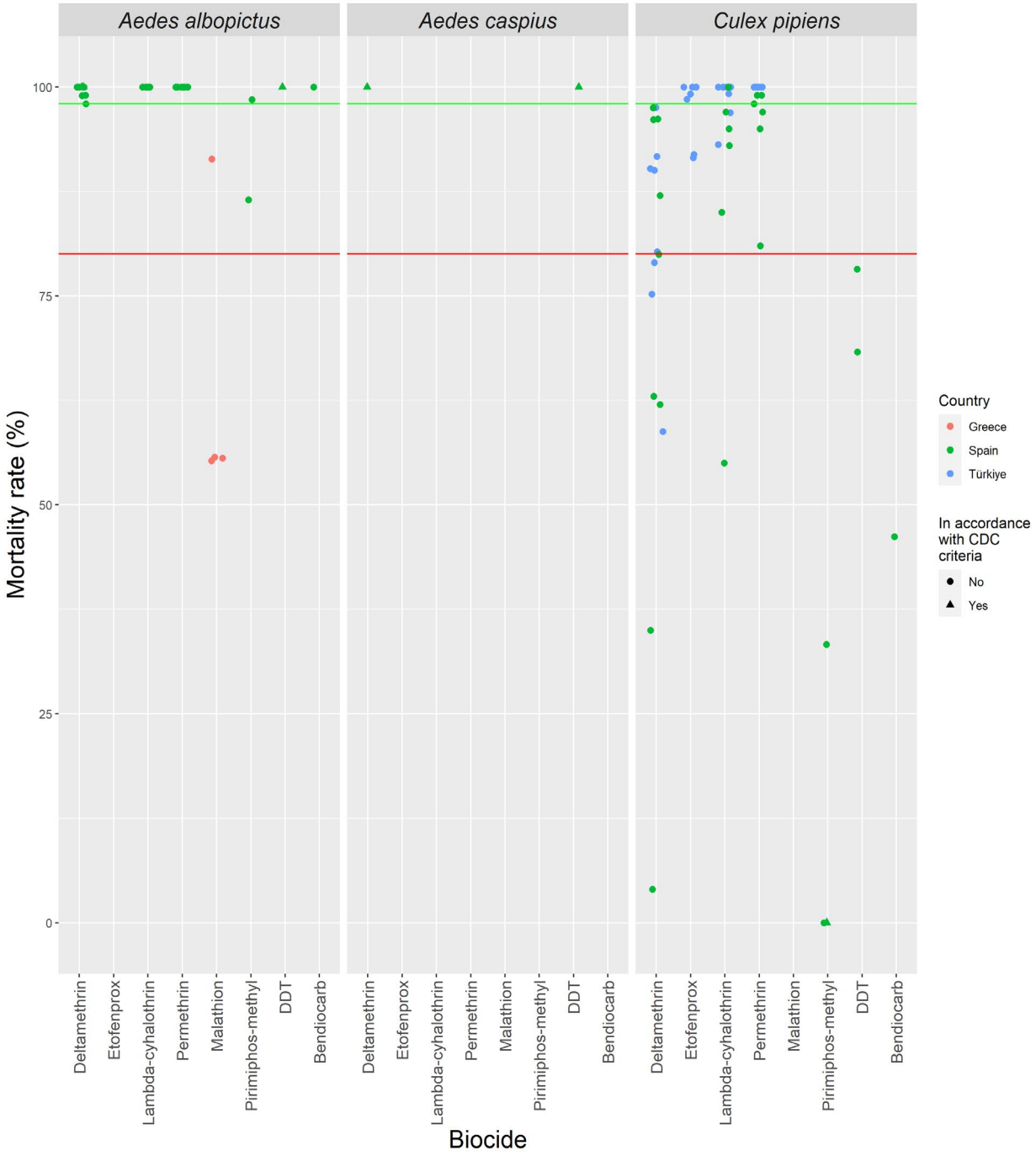
Bengoa et al. [70] and Kioulos et al. [71] reported the mortality rate of their control group, but used a susceptible population for their assays. The mortality rates for these groups were 100%, which meant that the biocide was applied correctly. However, control groups in biocide resistance assays are primarily used to verify whether the test environment exerts an influence on the results of the assays and should involve individuals from the wild population. If the mortality rate in a control group is high when no biocide is applied, researchers should investigate whether there is an external influence on the mortality rate. As it was impossible whether the mortality rate was entirely reliable, we chose to exclude these studies.

CDC bottle bioassay

One publication using CDC bottle bioassays met the CDC criteria [72] (Figure 7). The results confirmed the susceptibility of two Spanish *Aedes albopictus* and an *Aedes caspius* population to deltamethrin (MR: 100%) and one population of both species to DDT (MR: 100%). One Spanish *Culex pipiens* population was not affected by pirimiphos-methyl (MR: 0%).

Data not in line with the pre-set criteria include data from Paaijmans et al. [72] regarding *Aedes albopictus* and *Culex pipiens* populations in Spain. Furthermore, a Greek study by Balaska et al. [73] exposed a susceptible group to the biocide, resulting in a 100% mortality rate. They did not mention a control group that was not exposed to the biocides. In the other study that did not fully meet the CDC criteria, tests were conducted on adult *Culex pipiens* from Türkiye, but the concentrations used were for WHO adult susceptibility bioassays, which are higher than the recommended concentrations for CDC bottle bioassays [74].

Figure 6. Overview of biocide resistance status of mosquito species, based on the CDC bottle bioassay results

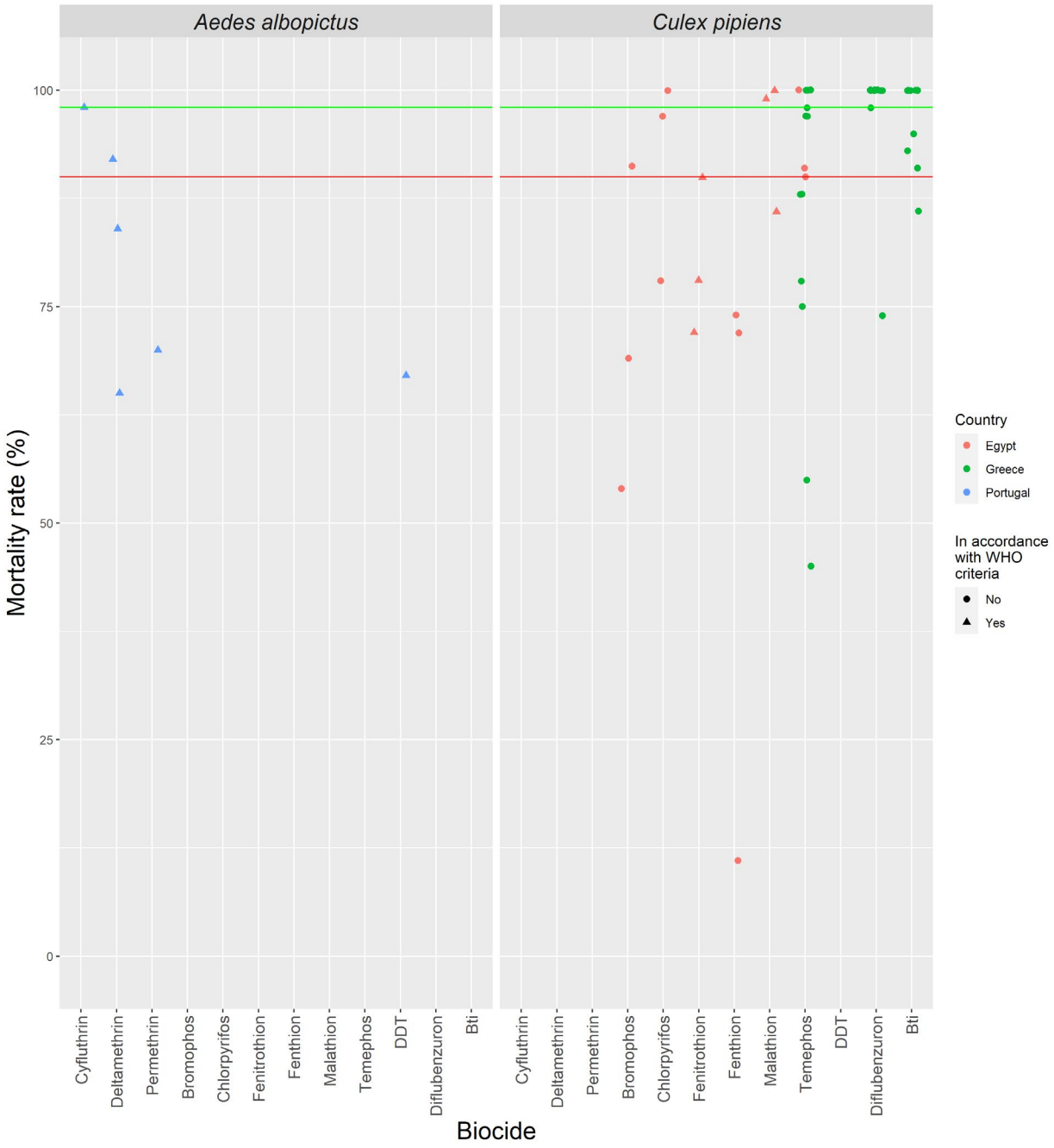


Note: *this is a compilation of the data assayed during the period 2000–2021. It is possible that there are multiple data points in the same region, but from a different year. The green horizontal line indicates the threshold between susceptible and possible resistance, the red line the threshold between possible resistance and resistance.*

WHO larvicide susceptibility bioassay

Tests were conducted on *Aedes albopictus* populations from Portugal, for which we acquired unpublished data through the VEN, and on *Culex pipiens* populations from Egypt [59]. A study from Kioulos et al. [71] did not meet WHO criteria because the mortality rates of the control group were not reported using the correct method.

Figure 7. Mortality rates for *Aedes albopictus* and *Culex pipiens* larvae using WHO larvicide susceptibility bioassay



Note: *this is a compilation of the data assayed during the period 2000–2021. It is possible that there are multiple data points in the same region, but from a different year. The green horizontal line indicates the threshold between susceptible and possible resistance, the red line the threshold between possible resistance and resistance.*

Synergist assay

Synergist assays were conducted in two studies, from Türkiye and the United Kingdom. Guntay et al. [64] tested for the efficacy of permethrin and deltamethrin in combination with piperonyl butoxide (PBO) on individuals from *Culex pipiens* populations at two different locations. Brown et al. [60] tested for the efficacy of bendiocarb in combination with PBO on *Aedes detritus*. Results could not be reliably interpreted because these studies did not meet the WHO criteria [22] - i.e. in synergist studies assays should be performed in four arms: (1) biocide, (2) synergist, (3) pre-exposure to synergist, then exposure to biocide, (4) control group. Neither of the studies reported assays conducted solely with the synergist.

Data extraction of molecular assay results

Results from the molecular assays were summarised by type of mutation and by vector group, as different mutations affect the vector's metabolism in a variety of ways (Table 4). Since there are several ways to note resistance-related allele mutations, we standardised the notation. In our notation of alleles, the number describes the codon where the mutation occurred, while the letters describe which amino acid has been replaced by which in the mutated form. It should be noted that not all allele mutations are directly linked to the expression of resistance and these associations need to be validated.

Table 4. Overview of different types of mutations linked to biocide resistance with the mutated alleles investigated

Mutation class	Description	Alleles included in database	Validation status*	Reference
Knock-down resistance (Kdr)	Mutations altering the function of voltage-sensitive sodium channels in nerve membranes, presumably selected by DDT and pyrethroid use [23]	C190A	Possibly associated with resistance	[56]
		F1534C	Associated with resistance	[75]
		F1534L	Possibly associated with resistance	[76]
		F1534S	Functionally validated	[29]
		F1534W	Possibly associated with resistance	[76]
		I1011M	Possibly associated with resistance	[77]
		I1011V	Possibly associated with resistance	[20]
		I1532T	Associated with resistance	[78]
		L1014C	Associated with resistance	[79]
		L1014F	Functionally validated	[80]
		L1014S	Associated with resistance	[81]
		S989P	Associated with resistance when in combination with other mutations	[20]
		S989Y	Associated with resistance when in combination with other mutations	[76]
		V1016G	Functionally validated	[20]
		V1016I	Associated with resistance when in combination with other mutations	[20]
		V410A	Associated with resistance	[82]
		V410G	Associated with resistance	[82]
		V410L	Associated with resistance	[82]
Acetylcholin-esterase (Ace-1)	Mutations in a neurotransmitter, affecting the organophosphate and carbamate susceptibility [24]	F290V	Functionally validated	[83]
		G119S	Functionally validated	[20]
Ester²	Hydrolase enzymes, including biocide detoxifying enzymes [84]	Ester²	Associated with resistance	[85]
Chitin synthase	Enzyme responsible for chitin synthesis in insect's cuticle. Diflubenzuron inhibits its working [86].	I1043F	Functionally validated	[87]
		I1043L	Functionally validated	[88]
		I1043M	Functionally validated	[88]
Resistant to dieldrin gene (Rdl)	Encodes GABA receptor, playing a central role in neuronal signalling. Mutations cause resistance to dieldrin [89]	A302S	Functionally validated	[90]

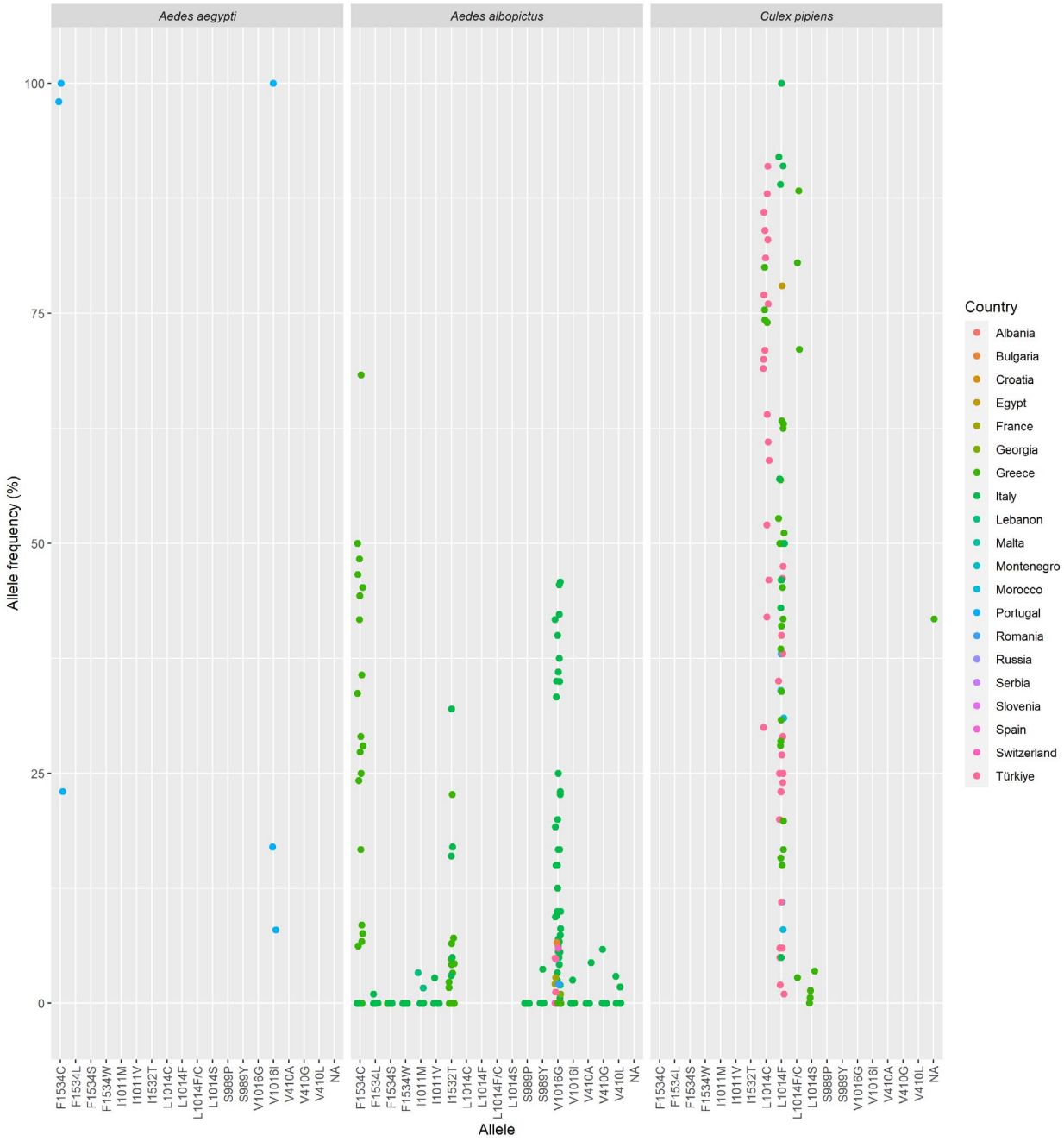
Note: **Functionally validated: in vitro or in vivo functional proof for the involvement of the mutation in resistance phenotype. Associated with resistance: several molecular studies report associations between the presence of this mutation and resistance phenotypes: Possibly associated: in one or a few studies, an association has been reported.*

Knock-down resistance

Mosquitoes

Kdr-related allele mutations have primarily been found in *Aedes albopictus* and *Culex pipiens* following studies in Egypt, Greece, Italy, Lebanon, Morocco, Portugal and Türkiye (Figure 9). In total, eighteen mutated alleles linked to knock-down resistance were detected (Figure 9, Annex 5).

Figure 9. Frequencies of mutated alleles linked to knock-down resistance in *Aedes* and *Culex* mosquitoes



Note: *This is a compilation of the data assayed during the period 2000–2021. It is possible that there are multiple data points in the same region, but from a different year. Details are described in Annex 5.*

Sand flies

For sand flies, allele frequencies of knock-down resistance were published in one Greek and one Turkish study [91,92]. In the Greek study, mutations of the L1014 allele were not found in any of the investigated sand fly species (*Phlebotomus neglectus, Phlebotomus perfiliewi, Phlebotomus simici* and *Phlebotomus tobbi*). In the Turkish *Phlebotomus papatasi* population investigated, the L1014F mutation was present with an allele frequency of 48%.

Ticks

Kdr mutations in ticks were investigated in an Egyptian study by Arafa et al. [56]. A C190A mutation was found in one of the three populations investigated (AF: 44.2%) (Table 5).

Table 5. Allele frequencies of the C190A allele, related to knock-down resistance, in the tick species *Rhipicephalus annulatus*

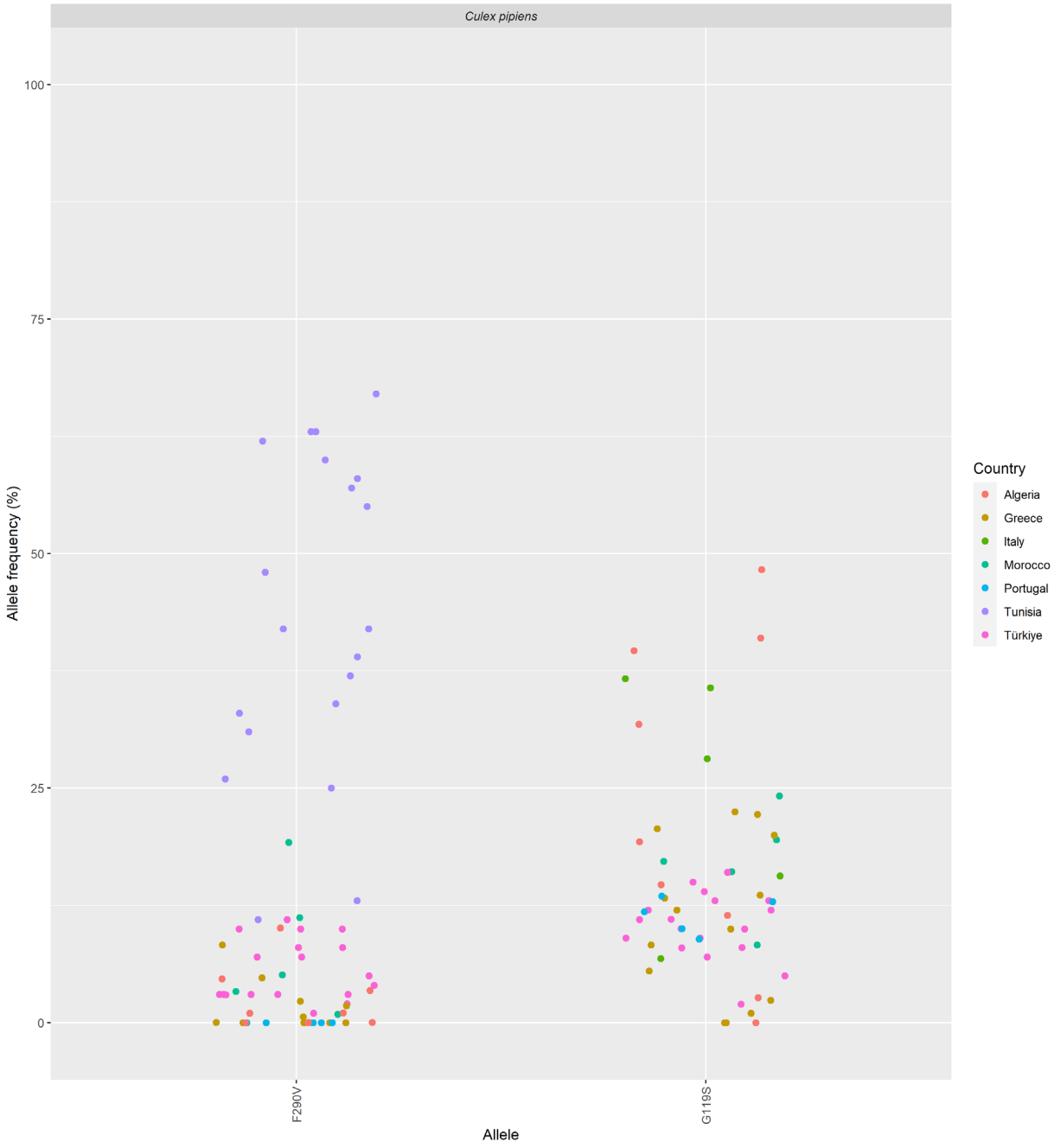
Vector species	***Rhipicephalus annulatus***		
Country	Egypt		
Location	El Wasta – A	El Wasta – B	El Hakamna
Year of assessment	2020	2020	2020
C190A	0%	44.18%	3.85%

Note: *In this study the two locations at El Wasta were separated into two areas A and B.*

Ace-1

In the search for Ace-1 mutations, researchers have mainly focused on *Culex pipiens* populations. The two exceptions are a study in which researchers confirmed the absence of the G119S allele in one Moroccan *Anopheles sergentii* population [63], and a Belgian study in which G119S was detected in the one *Culex modestus* population tested (AF: 1.95%) while it was absent in the three *Culex torrentium* populations tested [93]. In *Culex pipiens* populations, the G119S mutation was present in all the countries studied. Allele frequencies ranged from 0–50%. F290V allele mutations were detected in some, but not all countries studied, with Tunisian populations having the highest allele frequencies, up to 67% (Figure 10).

Figure 8. Allele frequencies of Ace-1 related mutations in *Culex pipiens*



Note: *this is a compilation of the data assayed during the period 2000–2021. It is possible that there are multiple data points in the same region, but from a different year.*

Esterases

The occurrence of mutations in the Ester[2] allele was investigated in one recent Moroccan study on five *Culex pipiens* populations [94], with allele frequencies ranging from 32.8–73.7% (Table 6).

Table 6. Allele frequencies of the mutated Ester[2] allele in *Culex pipiens* populations in five Moroccan regions

Vector species	***Culex pipiens***				
Country	Morocco				
Region	Tangier	Larache	Mohammedia	Marrakech	Agadir
Year of assessment	2020	2020	2020	2020	2020
Ester[2]	32.8%	58.3%	73.7%	63.6%	48.2%

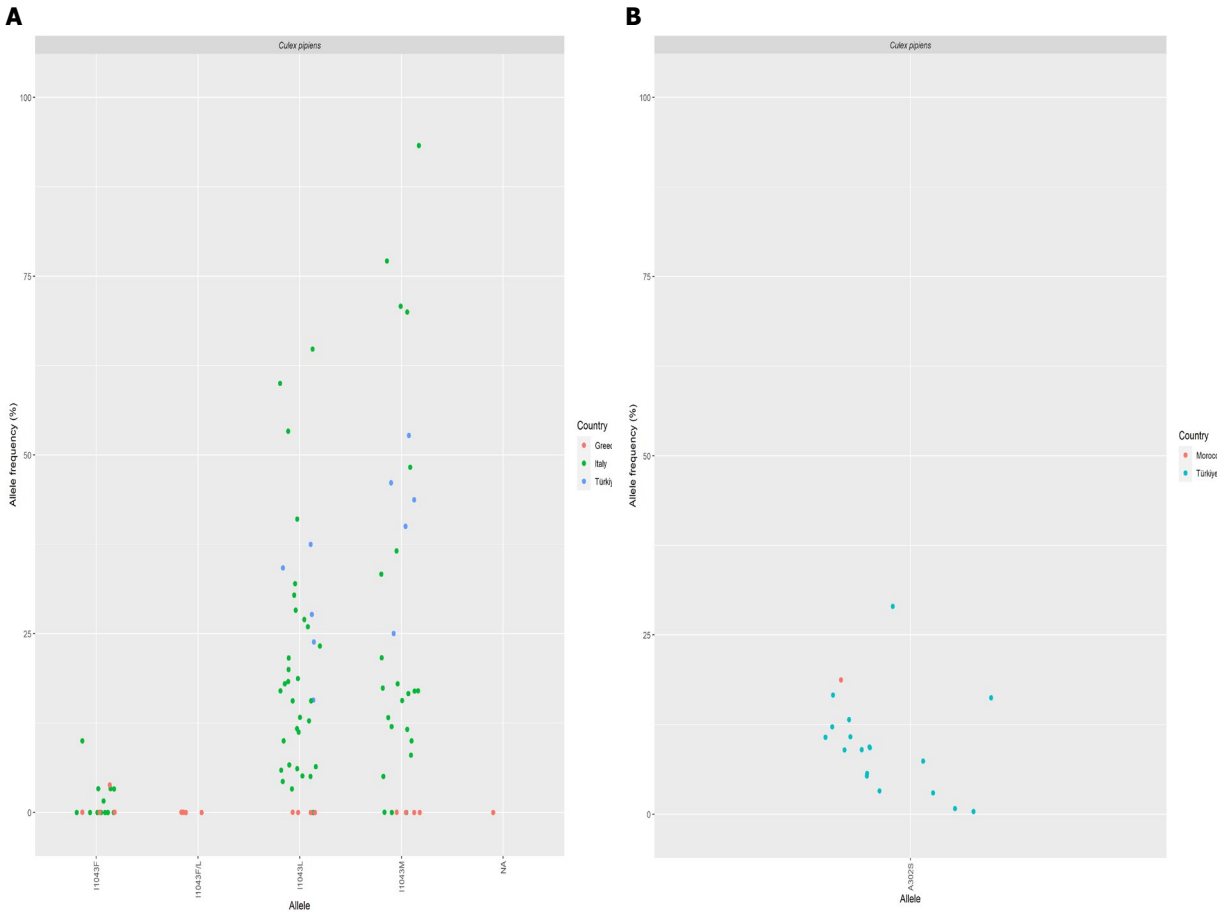
Chitin synthase

Of the three mutations of chitin synthase investigated in *Culex pipiens* populations, I1043F had the lowest allele frequency, while I1043L and I1043M mutations appeared more frequently and seemed to be more widespread in Italian and Turkish populations [87,88,95,96]. Unpublished data acquired through the VEN survey were also included which involved attempts to detect I1043M and I1043L alleles in Greece. Although neither of these alleles were detected, I1043F was detected in one population (AF: 3.84%) (Figure 11) [97,98].

Rdl

The Rdl-related A302S allele has been mainly found in *Culex pipiens* populations, with an allele frequency consistently below 20%, except in one population in Izmir, Türkiye (AF: 29%) (Figure 11) [94,99]. In Lebanon, one of two *Aedes albopictus* populations investigated included individuals with this mutation (AF: 7.1%). These data were acquired through the VEN survey.

Figure 9. (A) Allele frequencies for chitin synthase-related mutations in *Culex pipiens* (B) Allele frequencies for Rdl-related mutations in *Culex pipiens*



Note: *this is a compilation of the data assayed during the period 2000–2021. It is possible that there are multiple data points in the same region, but from a different year. In Greece, tests were undertaken for some mutations related to knock-down resistance, but researchers did not specify which alleles were involved, so these data points were entered in the NA or the I1043F/L column.*

Data extraction of biochemical assay results

Data from two studies involving biochemical assays were entered in the database. Fahmy et al. [57] found a significant upregulation of the P450 complex' enzymatic activity in *Culex pipiens* individuals from Egyptian populations, compared to a susceptible population ($P<0.05$). Akiner et al. [66] found that in five of the six Turkish regions investigated, acetylcholinesterase sensitivity decreased significantly ($P<0.05$) over the course of two years in populations for which resistance to malathion and propoxur rose.

Biocide resistance status per vector species

Mosquitoes

Aedes aegypti

All data on this species stems from research in Madeira (Portugal) by Seixas et al. [34,61,100]. Using WHO adult susceptibility bioassays with higher concentrations than recommended, resistance to deltamethrin (concentration: 0.05%, MR: 65.2%) was found, but not to malathion (concentration: 5%, MR: 99%). For DDT, there was no DC for *Aedes* species, so the 4% concentration for *Anopheles* species was used (MR: 29.4%). In the same region and year (Funchal, 2009), the Kdr-mutated allele F1534C was dominant in the tested population (AF: 98%) and the Kdr allele V1016I had an allele frequency of 8%.

In 2013, the allele frequencies were calculated in another study and bioassays were conducted again [34]. Using the discriminating concentrations, resistance was found against cyfluthrin (MR: 52.8%) and fenitrothion (MR: 77.5%); higher concentrations were used for malathion (concentration: 5%, MR: 99%) and permethrin (concentration: 4%, MR: 10.9%), indicating strong levels of resistance to permethrin. In Funchal, the allele frequency of mutation F1534C had decreased (AF: 23%), but had increased for the V1016I mutation (AF: 17%). In Paúl do Mar, both mutations were found in all individuals tested (AF: 100%).

Aedes albopictus

Biocide resistance has been tested in line with the study criteria in three studies for this species, with assessments conducted during the period 2016–2021: once using the WHO adult susceptibility bioassay in Lebanon, once using the WHO larvicide susceptibility bioassay in Portugal, and once in Spain using CDC bottle bioassays. Data from Lebanon and Portugal were acquired through the VEN survey. With the exception of the deltamethrin and cyfluthrin assay in Portugal, sample sizes were less than 100 individuals in all the assays described below (n=53-96) (Table 8).

- **Pyrethroids**
  - The species was susceptible for cyfluthrin (MR: 98%).
  - For deltamethrin, both Spanish populations were completely susceptible (MR: 100%), while in Portugal two out of the three populations showed resistance (MR_1: 65%, MR_2: 84%, MR_3: 92%).
  - Resistance to lambda-cyhalothrin was detected in the one Lebanese population investigated, from Fanar-El Metn (MR: 72%).
  - For permethrin, the Lebanese population may be resistant (MR: 96%), while resistance in the Portuguese population was confirmed (MR: 70%).
- **Organochlorides**
  - Resistance to **DDT** was discovered in Fanar-El Metn, Lebanon (MR: 43%) and in Almancil, Portugal (MR: 67%), while the Spanish population from Cornella de Llobregat was completely susceptible (MR: 100%).
- **Organophosphates**
  - Resistance to malathion was also detected in the same population from Lebanon (MR: 19%).
- **Carbamate**
  - Resistance to propoxur has also been confirmed in Fanar-El Metn, Lebanon (MR: 82%).

In WHO adult susceptibility bioassays conducted by Pichler et al. [31,39] and CDC bottle assays by Paaijmans et al. [72] a higher discriminating concentration than the WHO criteria was used, except for the assays with bendiocarb and pirimiphos-methyl. When using high concentrations, low mortality rates imply higher levels of resistance. Bengoa et al. [70] used WHO adult susceptibility assays and Balaska et al. [73] used CDC bottle bioassays, but neither reported the mortality of the control group correctly. All assessments from these studies were made during the period 2012–2019 and their results were as follows:

- **Pyrethroids**
  - For alpha-cypermethrin results varied. In Albania, results bordered on susceptible and possibly resistant (MR_1: 98.6%, MR_2: 97.3%). In Italy, three of eleven populations tested showed resistance to this biocide, with the population in Lido di Spina, Emilia Romagna showing the highest level of resistance (MR_1: 64.8%, MR_2: 85.3%, MR_3: 89.2%). The concentration used in all these assays was 0.05%, while the discriminating concentration is 0.03% [31,32].
  - Results from using a 0.05% concentration cypermethrin on Italian populations varied from fully susceptible to resistant populations (MR: 84.4-100%). The most resistant population was found in Talenti, Lazio. Bengoa et al. [70] used a 0.10% concentration and found mortality rates of 85–99% in four Spanish populations.
  - Populations from Italy, Spain and Albania were susceptible in the assays where deltamethrin was applied (MR: 98–100%), in all but one case. The mortality rate in Peñıscola, Spain, was 90%. Pichler et al. [31,32] used a 0.05% concentration in their WHO adult susceptibility bioassays, while Paaijmans et al. [72] used a 1 000 µg/bottle concentration, both of which are higher than the 0.03% and 10 µg/bottle concentrations respectively recommended for these tests. Results from CDC bottle bioassays in four Greek regions showed full susceptibility to deltamethrin [73].

  - For lambda-cyhalothrin, all Spanish populations were fully susceptible in assays where the concentration was 1 000 µg/bottle, although the recommended concentration is 10 µg/bottle.
  - When using permethrin results varied among populations and countries. Paaijmans et al. [72] used a 3 125 µg/bottle concentration instead of the recommended 15 µg/bottle concentration, resulting in Spanish populations showing high mortality rates. Pichler et al. [31,32] used a 0.75% concentration instead of the recommended 0.25%. Bengoa et al. [70] used a 1% concentration on four Spanish populations and found mortality rates of 95–100%. Both Albanian populations showed full susceptibility (MR: 100%), while the Greek population was possibly resistant (MR: 93%). Resistance was confirmed in four of the 28 Italian populations tested, with mortality rates lowest in Lido di Volana, Emilia Romagna (MR_1: 68.9%, MR_2: 81.3%, MR_3: 88.7%, MR_4: 89.6%). In five other populations, resistance was possibly present (MR: 94.3–97.7%). The other nineteen populations showed susceptibility (MR: 98.9–100%), with fourteen of them being fully susceptible (MR: 100%).

- **Organophosphates**
  - In Greece, resistance to malathion was confirmed in populations from Aghios Stefanos (MR: 55.7%), Kefalonia (MR: 55.6%) and Heraklion (MR:55.3%). The Patras population was possibly resistant (MR: 91.4%) [73].
  - For pirimiphos-methyl, no discriminating concentration has been established yet. When conducting a 20 µg/bottle concentration CDC bottle bioassay, mortality rates were 86.5% and 98.5% in the two Spanish populations investigated, confirming resistance in the population from Cornella de Llobregat and pointing at possible resistance in the population from El Prat de Llobregat.
- **Carbamate**
  - For bendiocarb, Paaijmans [72] used a 10 µg/bottle concentration for their CDC bottle bioassay, which is lower than the recommended 12.5 µg/bottle concentration (Annex 2). The Spanish population exposed to bendiocarb was fully susceptible (MR: 100%) to this concentration.

In France, when measuring resistance during the period 2016–2019, bioassays were used to calculate the resistance ratio. WHO larvicide susceptibility bioassays did not detect resistance to Bti (RR_{50} <5) and WHO adult susceptibility bioassays did not detect resistance to deltamethrin (RR_{50} <5) [101]. Toma et al. [33] also used WHO larvicide bioassays to calculate LC_{50} values, but did not use a susceptible group to calculate RR_{50} values.

Several Kdr-related allele mutations have been detected in *Aedes albopictus*, often with varying allele frequencies among populations and countries. The data refer to the period 2011–2020 and include unpublished data acquired through the VEN survey or other contacts (Table 7).

Table 7. Overview of Kdr-related allele mutations detected in *Aedes albopictus*

Allele	Description	Reference
F1534C	Was not detected in Italian populations, but was present in 20 of 32 Greek populations where the allele frequency ranged from 6.2–68.3%, with the population from Attica having the highest frequency.	[29,31,73,76,97]
F1534L	Has been detected once, in Arco, Italy with a 1% allele frequency in 2015. In 2018, it was no longer detected.	[29]
F1534S, F134W	Investigated in Italy by Tancredi et al. (2020), but not detected.	[76]
I1011M	Was present in the two Lebanese populations investigated (AF_1: 3.3%, AF_2: 1.7%), but not in the Italian ones.	Data obtained through the VEN survey
I1011V	Of the seven Italian populations tested, this allele was only detected in Modena (AF: 2.8%).	[76]
I1532T	Was detected in all four Italian populations investigated (AF: 3-32%) and was present in nine of nineteen Greek populations tested (AF: 2.3-22.7%).	[31,73]
S898P	Was not detected in Italian populations.	[76]
S898Y	Was detected in Comacchio, Italy (AF: 3.71%).	[76]
V1016G	Was present in 38 of the 51 Italian populations investigated, with allele frequencies ranging from 0 to 45.8%, and the population in Porto Garibalda having the highest allele frequency. It has also been detected in several other countries (Bulgaria, France, Georgia, Italy, Malta, Romania, Slovenia, Switzerland and Türkiye), with allele frequencies up to 8%. In Albania, Croatia, Greece, Montenegro, Portugal, Russia and Serbia, this mutation has not been detected. In some of the ongoing assays by Pichler et al. the sample size was as low as four individuals.	[31,39,73,76,97]
V1016I	Has been detected once, in Cosenza, Italy (AF: 2.5%).	[76]
V410A	Has been detected once, in Arco, Italy (AF: 4.4%).	[76]
V410G	Has been detected once, in Cosenza, Italy (AF: 5.9%)	[76]
V410L	Has been detected twice; in Turin (AF: 1.8%) and Cosenza, Italy (AF: 2.9%).	[76]

Chitin synthase mutations I1043F and I1043L were not detected in 2017 in the Greek populations investigated [97] and Rdl mutation A302S was detected in one of two Lebanese populations in 2021 (AF: 7.2%), according to data acquired through the VEN.

Aedes caspius

Resistance was tested once in Spain in 2017 [72], using CDC bottle bioassays. In this study, DDT and deltamethrin were tested. For DDT, no discriminating concentration has been established for *Aedes* mosquitoes, while for deltamethrin a higher concentration was used than that recommended. When using a 0.75% DDT concentration and a 0.1% deltamethrin concentration, all individuals succumbed (MR: 100%).

Aedes detritus

A 2018 study by Brown et al. [60] investigated the efficacy of three biocides and a synergist against a population from Little Neston, United Kingdom, using the WHO adult susceptibility bioassay. They found that the individuals sampled from the population were completely susceptible to fenitrothion (concentration: 1%, sample size: 64, MR: 100%) and permethrin (concentration: 0.75%, sample size: 11, MR: 100%), but showed resistance to bendiocarb (concentration: 0.10%, sample size: 158; MR: 33.7%). Using bendiocarb in combination with synergist PBO resulted in a 97.7% mortality rate (sample size: 87). However, the effect of the synergist should be interpreted with care, because the test did not meet the WHO criteria of synergist bioassays, requiring that there should also be an assay using the synergist alone.

Anopheles hyrcanus

Bioassays have not been conducted to test for resistance. In Greece 2017, researchers searched for Kdr mutated L1014 alleles but found neither the L1014F, L1014S, or the L1014C mutation (sample size: 4) [87].

Anopheles labranchiae

WHO adult susceptibility tests were conducted once in Morocco in 2005, to test for the efficacy of DDT, fenitrothion, malathion, permethrin and propoxur, using the discriminating concentrations [35,69]. Resistance to DDT was confirmed in five of six populations (MR_{1-6}: 78-90%) and became more prevalent a couple of years later, when measurements between 2011 and 2014 showed mortality rates to be 47−71.9%. There may have been resistance to fenitrothion, as three populations tested in 2005 all had a 95% mortality rate. For malathion, results from 2017 varied among the populations investigated, from confirmed resistance to possible resistance (MR_1: 72%, MR_2: 82%, MR_3: 96%). All populations showed full susceptibility to permethrin and propoxur (MR: 100%). The mortality rates of the susceptible groups were not reported for either of the studies, nor was a correction method on the mortality rates of the populations, so these results should be interpreted with care.

Tests for resistance to deltamethrin and permethrin were also conducted in Tunisia, 2016, by using bioassays in which lethal concentrations and the resistance ratio were calculated (see Glossary) [45,48].

- High resistance was found against deltamethrin in all six populations, from 12.5 for RR_{50} in Ben Arous to 72.5 in Monastir. When using deltamethrin in combination with synergist DEF (S,S,S-tributyl phosphorotrithioate), moderate resistance was present in Ben Arous and high resistance in other populations. The highest RR_{50} came from the Ben Arous population (RR_{50}: 50). When DEF was added, the results did not differ significantly from those for use of deltamethrin alone, so the effects of DEF are probably limited and should be investigated further. When using deltamethrin in combination with synergist PBO, susceptibility was confirmed in Kairouan, moderate resistance was detected in Ben Arous (RR_{50}: 6) and Monastir (RR_{50}: 7.3), and high resistance was found in Ariana (RR_{50}: 14), Beja (RR_{50}: 18) and Jendoub (RR_{50}: 32.7). In the susceptible population, the effect of PBO was significant, indicating that PBO is an effective synergist. In the other populations, the results did not differ significantly from those for use of deltamethrin alone.
- For temephos, moderate resistance was detected in Beja (RR_{50}: 6.2). In Ben Arous, the resistance detected was very high (RR_{50}: 624) and in the other four populations there was also high resistance (RR_{50}: 10.2-25.4). When using temephos in combination with synergist DEF, the RR_{50} for Ben Arous was 263.9, marking very high resistance. The Monastir population was susceptible when it was exposed to the DEF combination (RR_{50}: 2.2) and in the other four populations, high resistance was found (RR_{50}: 10.4-24.8). The results from the tests adding DEF were significantly lower in four of the six populations, indicating that the addition of this synergist to temephos could help restore susceptibility in resistant populations. In combination with PBO, Ben Arous again proved to be highly resistant (RR_{50}: 874.6), as did the other populations (RR_{50}: 12.3-27.3). Even though the RR_{50} was higher in some populations when using PBO, these results did not differ significantly and can be explained by the laboratory population having a lower LC_{50} in the tests with PBO, which resulted in a higher ratio. The counterintuitive low LC_{50} in the laboratory strain may be linked to the experimental set-up, but is not discussed in the study.

Anopheles maculipennis sensu lato

Results from a study by Paronyan et al., acquired through the VEN survey, using WHO adult susceptibility tests in 2021 with the discriminating concentrations showed resistance to alphacypermethrin in both Armenian populations researched (MR_1: 48.9%; MR_2: 73.8%), while resistance to cyfluthrin was confirmed in just one of the two populations (MR_1: 88.5%; MR_2 100%). The sample sizes in these tests (n: 30-44) were below WHO's requirements of 80−100 individuals. In the same study, researchers found that in five of the six regions investigated, Ace-1 activity was significantly higher than in a susceptible population (P<0.05).

Using the discriminating concentrations, but doubling the recommended exposure time (120 min), Akiner et al. [67] were still able to confirm resistance to DDT in five Turkish populations in 2007 (MR: 47.5-65%) and again in 2008 (MR: 50-67.5%). The same assays were conducted with deltamethrin, using the recommended exposure time. These results showed that the mortality rates using deltamethrin had dropped over the course of one year (MR_{2007}: 90-95%; MR_{2008}: 85-92.5%). For malathion, additional results using the discriminating concentration were published by Akiner et al. [66]. Taken together with the results from Akiner et al. (2013), mortality rates in 2007 ranged between 90−92.5% and between 75−83% in 2008, confirming the presence of resistance to malathion in all populations in 2008. For permethrin, Akiner et al. (2013) reported mortality rates of 85−95% in 2007 and 82.5−87.5% in 2008 for the five populations tested, also confirming resistance in 2008. For propoxur, double the exposure time was used, and mortality rates were between 83.3% and 95% in 2007 and 70.8% and 85% in 2008, confirming resistance for all populations in 2008.

All results described by Akiner et al. [66,67] should be interpreted with care, because the mortality rates of the susceptible groups were not reported and no correction method was applied to the mortality rates of the investigated populations. Furthermore, the sample size in Akiner et al. (2013) only involved 40 individuals in all assays.

Anopheles maculipennis sensu stricto

In Greece in 2017, the Kdr allele mutations L1014F, L1014S and L1014C were not found in the only population studied [87].

Anopheles sacharovi

In 2014 and during the period 2017−2018, Yavaşoglu et al. (2019) tested the efficacy of six biocides on multiple Turkish populations using the discriminating concentrations [37] (Table 8).

- In 2014, resistance was confirmed in all 19 populations for DDT (MR: 55−78.3%) and 18 of 19 for malathion (MR: 58.3-90%), while for propoxur, 15 out of 18 populations were resistant (MR: 68.3−90%). For the pyrethroids tested, resistance could not be fully confirmed. Five of 19 populations were possibly resistant against deltamethrin (MR: 95−96.7%), four of 19 for etofenprox (MR: 95−98.3%) and three of 19 for permethrin (MR: 95−96.7%), while all the others showed susceptibility.
- During the period 2017−2018, resistance was confirmed in fourteen of fifteen populations tested for bendiocarb (MR: 65−91.7%) and in all populations for DDT (MR: 55−85%), fenitrothion (MR: 58.3−88.3%) and malathion (MR: 58.3−86.7%). Of 15 populations tested for the efficacy of propoxur, resistance was possibly present in two (Abbaslar and Afyon) (MR_1: 95%; MR_2: 91.7%). In all other populations, resistance was confirmed (MR: 68.3−86.7%). For deltamethrin, eight populations were possibly resistant and seven showed susceptibility. Finally, seven populations were possibly resistant against permethrin, while the eight others were susceptible.

Yavasoglu et al. [36] also conducted molecular assays between 2017 and 2018. Kdr mutation L1014F was present in Hatay, Bektaslı (AF: 70%) and in K.maras, Abbaslar (AF: 30%) (sample size: 12). Mutation L1014S had an allele frequency of at least 50% in seven out of eight populations tested and occurred in all individuals from the Mersin and Osmaniye population (AF: 100%, sample size: 12). Mutation was not detected in Hatay. A molecular assay was conducted in Greece in 2017, during which the Kdr mutations L1014F, L1014S and L1014C were not detected in the Lagadikia population [87].

Anopheles sergentii

In 2015, Filali et al. [63] conducted WHO adult susceptibility bioassays, using the discriminating concentrations, to test for the efficacy of DDT and malathion against one Moroccan population. Although the control group showed complete susceptibility, details of mortality were not given, and therefore these results need to be interpreted with care (Table 8). In the same study, the absence of the Kdr allele L1014F and the Ace-1 allele G119S was confirmed.

In 2016, bioassays were conducted in Tunisia by Tabbabi et al. using the resistance ratio to test for resistance [44].

- All three populations were susceptible to deltamethrin (RR_{50} <5). Moderate resistance was detected in Tozeur (RR_{50}: 5.8) and in one of the two Tataouine populations (RR_{50}: 6) when using the biocide together with synergist DEF, however the results did not significantly differ from those without DEF. It is therefore possible that DEF has no additional effect, but this should be investigated further before conclusions can be drawn. The other Tataouine population was susceptible (RR_{50}: 4.4). In combination with PBO, two populations tested as susceptible (RR_{50} <5) and one Tataouine population tested as moderately resistant. The addition of PBO lowered the resistance ratio in all three populations, but it only differed significantly in one of them. Therefore, further testing is required to prove the efficacy of PBO as a synergist.

- For permethrin, two populations were susceptible (RR_{50} <5). The second Tataouine population was moderately resistant (RR_{50}: 5.8). Results were similar when adding synergists DEF and PBO, with the second Tatouine population showing moderate resistance for the combination with DEF (RR_{50}: 6.1) and the combination with PBO (RR_{50}: 7.1), while the others were susceptible.

Anopheles superpictus

In 2014, Yavaşoglu et al. [37] tested the efficacy of the same six biocides in Türkiye. Resistance to DDT was confirmed in all populations (MR: 50–86.7%), as was resistance to malathion (MR: 61.7–88.3%). Resistance to propoxur varied (MR: 68.3–91.7%): nineteen out of 22 populations were resistant to propoxur, the other three were possibly resistant. Resistance to deltamethrin may have been present in four populations, all with a mortality rate of 96.7%, while the other populations were susceptible. The Birecik population was resistant to etofenprox (MR: 87.5%), three other populations showed possible resistance (MR_1: 95%, MR_2: 96.7%, MR_3: 96.7%) and the others were susceptible. Resistance to permethrin was possibly present in three populations (MR_1: 95%, MR_2: 96.7%, MR_3: 96.7%), while the other populations were susceptible (Table 8).

Culex pipiens

In line with the pre-set criteria, WHO adult and larvicide susceptibility bioassays were conducted in Egypt in 2003 [59], Spain in 2015-2016 [72] and in Armenia in 2021 by Paronyan et al. (acquired through the VEN survey).

- **Pyrethroids**
  - Resistance to alpha-cypermethrin was in both Armenian populations: Katnaghbyur (MR: 18.4%, sample size: 30) and Benyamin (MR: 73.3%, sample size: 32). In Egypt, all three tested populations showed resistance to this biocide (MR_1: 1.8%, MR_2: 2.6%, MR_3: 11.4%), but the mortality rates of the control group were not reported here.
  - Mortality rates for cyfluthrin ranged from 95.6-100% in Egypt. The population from Armenia was completely susceptible (MR: 100%, sample size: 35).
  - Resistance to deltamethrin was possibly present Qalubiya, Egypt (MR: 90.6%), while the other two populations tested were susceptible. In Spain, resistance was also confirmed in Torrelles de Llobregat (MR: 81.2%). Additionally, Kioulos et al. [71] found two resistant populations (MR_1: 64%, MR_2: 87%) out of 13 Greek populations, but did not correctly report the mortality rates of the control groups.
  - Resistance to lambda-cyhalothrin was confirmed in Qalubiya, Egypt (MR: 82.3%). Populations from Sharkiya and Assiut were susceptible (MR_1: 100%; MR_2: 99).
  - For permethrin, resistance was confirmed in Qalubiya (MR: 72.9%), possibly present in Assiut (MR: 96.1%) and absent in Sharkiya (MR: 100%).
- **Organochlorides**
  - Resistance to DDT has been confirmed in all tested populations from Egypt (MR_1: 17.8%, MR_2: 53.3%, MR_3: 61.5%) and Spain (MR: 6.6%).
- **Organophosphates**
  - For fenitrothion, WHO adult (A) and larvicide (L) susceptibility bioassays have been used in three areas of Egypt. According to the adult bioassay, resistance was possibly present and confirmed with the larvicide tests in Sharkiya (MR_A: 90.5%; MR_L: 89.9%) and in Assiut (MR_A: 50%; MR_L: 78%), while in Qalubiya, resistance was confirmed only by the larvicide bioassay (MR_A: 96%; MR_L: 72%).
  - Results for malathion differed in Sharkiya (MR_A: 89.5%; MR_L: 100%) and Qalubiya (MR_A: 88.3%; MR_L: 99%). The Assiut population was resistant according to both assays (MR_A: 61.7%; MR_L: 86%).
  - Pirimiphos-methyl had no effect on a population from Torrelles de Llobregat, Spain, both when using an adult susceptibility bioassay (MR: 0%) and a CDC bottle bioassay (MR: 0%).
- **Carbamate**
  - For bendiocarb in Egypt, the mortality rates were also not reported. All three tested populations showed confirmed resistance (MR: 86%) against this biocide (MR_1: 74.5%, MR_2: 79.6%, MR_3: 38.6%).
  - When using propoxur in the same population from Torrelles de Llobregat, Spain, the mortality rate was also 0%. In Egypt, resistance was also confirmed in Sharkiya (MR: 90%) and possibly present in Qalubiya (MR: 79%) and Assiut (91.3%), but these results should be interpreted with care, because the mortality rate of the control group was not reported.

Results from CDC bottle bioassays, conducted by Ser et al. [74] in 2017 showed complete susceptibility of all Turkish populations to permethrin (concentration: 0.75%) and to etofenprox (concentration: 0.5%) and lambda-cyhalothrin (concentration: 0.05%) in almost all the Turkish populations investigated. Resistance to deltamethrin (concentration: 0.05%) was found in three populations and is possibly present in the other five. The three resistant populations were from Döşemealtı (MR: 75.2%), Mangavat (MR: 79%) and Alanya (MR: 80.3%). For all these assays, a concentration higher than the recommended discriminating concentration was used.

Paaijmans et al. [72] also used CDC bottle bioassays to test for the efficacy of six biocides against Spanish populations during the period 2012–2017, although they used higher concentrations than recommended in all but two assays.

- **Pyrethroids**
  - For deltamethrin, three concentrations deviating from the discriminating concentration (12.5 µg/bottle) were used in assays. Using a 10 µg/bottle concentration, the mortality rate was 96% in Torrelles de Llobregat. Using a 25 µg/bottle concentration, the mortality rates were 97.5% in Torrelles de Llobregat and 96.2% in El Prat de Llobregat. Using a 250 µg/bottle concentration in six other populations, mortality rates ranged from 4% in Bellvis to 87% in Vic, confirming resistance in all six populations.
  - Using 250 µg/bottle lambda-cyhalothrin concentrations instead of the recommended 12.5 µg/bottle, mortality rates ranged from 55−100%, confirming resistance in the Bellvis (MR: 55%) and Gava population (MR: 85%).
  - For permethrin, 2 500 µg/bottle concentrations were used instead of 21.5 µg/bottle concentrations, resulting in mortality rates of 81−99%, confirming resistance in the Bellvis population and the possible presence of resistance in Empuriabrava (MR: 95%) and Bellaterra (MR: 97%).
- **Organochlorides**
  - With a 75 µg/bottle DDT concentration, instead of the recommended 100 µg/bottle concentration, the mortality rate in the Torrelles de Llobregat population was 78.2%. A 200 µg/bottle concentration resulted in a 68.9% mortality rate in El Prat de Llobregat.
- **Organophosphates**
  - Assays with pirimiphos-methyl were conducted with a 40 µg/bottle instead of 20 µg/bottle concentration. Resistance was confirmed in both the Torrelles de Llobregat (MR: 0%, sample size: 49) and the El Prat de Llobregat population (MR: 33.3%, sample size: 90).
- **Carbamate**
  - Using a 25 µg/bottle bendiocarb concentration, the mortality rate in the El Prat de Llobregat population was 46.20%, confirming resistance. The recommended concentration is 12.5 µg/bottle.

Guntay et al. [64] reported results using WHO adult susceptibility bioassays in three Turkish populations in 2017, but did not report the mortality of the control group, meaning that the results should be interpreted with care. Resistance to alpha-cypermethrin, cyfluthrin, deltamethrin and permethrin was confirmed in all three populations and the discriminating concentrations were used in all assays.

- Mortality rates using alpha-cypermethrin were 1.8% in Menemen, 2.6% in Çiğl and 11.4% in Bornova.
- For cyfluthrin, mortality rates were 18% in Menemen, 27.4% in Çiğl and 46.3% in Bornova.
- For deltamethrin, mortality rates were 1.2% in Menemen, 13.1% in Çiğl and 17% in Bornova.
- For permethrin, mortality rates were 1% in Menemen, 2.5% in Çiğl and 8.1% in Bornova.

Results from Tmimi et al. [65] and data from an unpublished study by Pichler et al. were not fully compliant with the pre-set criteria, for the same reason as Guntay et al. [64]. In the study by Tmimi et al. resistance was reported in Mohammadia in 2016 using bendiocarb (MR: 16%), DDT (MR: 39%), lambda-cyhalothrin (MR: 49%), malathion (MR: 52%) and permethrin (MR: 63%). Resistance was confirmed in eleven of thirteen populations using this concentration.

Pichler et al. did not report the mortality rates of the control group either, although they mentioned that they applied Abbott's formula whenever necessary. These unpublished results are from 2016−2017 and were obtained through additional contacts. Seven of ten Italian populations tested as resistant to permethrin (MR: 14-86.9%) and the three others were possibly resistant. Two of three populations were resistant to deltamethrin (MR_1: 62.7%; MR_2: 85.4%) and one was possibly resistant (MR: 92%).

Assays have also been conducted with biocides for which no discriminating concentration has yet been established. Between 2002 and 2006, Toma et al. [62] used a 0.01 mg/l (=0.000001%) chlorpyrifos concentration to investigate 13 Italian populations and found mortality rates from 1% in Iesolo, Veneto to 98% in Abbazia di Pomposa, Emilia Romagna. In 2003, Zayed et al. [59] also used biocides lacking a discriminating concentration in their study of three Egyptian populations. They found resistance to chlorpyrifos in Qalubiya (MR: 78%), using a 0.01% concentration. Using a 0.02% temephos concentration, resistance was found in Qalubiya (MR: 90%). Resistance to bromophos was also found in Qalubiya (MR: 54%) and Assiut (MR: 69%), using a 0.01% concentration. Finally, resistance to fenthion was found in all three populations using a 0.05% concentration. In Sharkiya, the mortality rate was 72%, in Qalubiya 74% and in Assiut 11%.

Between 2008-2010 Kioulos et al. [71] used WHO larvicide susceptibility bioassays to test for the efficacy of Bti, diflubenzuron and temephos on Greek populations, but did not report the mortality rates of the control groups. Moreover, no discriminating concentrations have been established for these biocides. For Bti, the concentration used was 0.000008% (MR: 86-100%, 10 populations), for diflubenzuron (MR: 74-100%, 10 populations) and for temephos (MR: 45-100%, 12 populations) concentrations of 0.000002% were used.

A number of studies have used the resistance ratio to measure resistance in *Culex pipiens* populations in Cyprus (2002−2004), Egypt (year not reported), Italy (2002−2006), Morocco (2017) and Tunisia (2003−2005).

- Resistance to chlorpyrifos has been investigated by Vasque et al. [102] and Aboulfadl [103]. In Cyprus, high resistance was present in Agros (RR_{50}: 25.2) and Episkopi (RR_{50}: 35.2). Moderate resistance was present in Agia Eirini (RR_{50}: 7) and the populations from Marki, Persisterona and Platanistasa were susceptible (RR_{50} <5). In Morocco, calculating LC_{90} values and the RR_{90}, resistance was found in all four populations tested (RR_{90}: 19.5-82.5). The highest RR_{90} was found in Skhirate.
- For deltamethrin, Fahmy et al. [57] found moderate resistance in the population from the Mansourya canal in the Giza governate, Egypt (RR_{50}: 7.4). This was the only population tested.
- Vasque et al. [102] also tested the efficacy of permethrin against Cyprian populations. They found high resistance in Episkopi (RR_{50}: 54), Akrotiri (RR_{50}: 41) and Marki (RR_{50}: 11.9). The other five populations were susceptible (RR_{50} <5).
- Studies have been conducted in three countries on resistance to temephos. In Italy, the population of S. Anna showed moderate resistance, while the other twelve populations showed high resistance (RR_{50}: 14-182) [62]. The populations showing the most resistance were those from Lignano Sabbiadoro (RR_{50}: 182) and Ravenna (RR_{50}: 146) In Cyprus, high resistance was detected in Akrotiri (RR_{50}: 26.7) and moderate resistance in Agros (RR_{50}: 7.9). Six other populations were susceptible (RR_{50} <5) [102]. In Morocco, high resistance was detected in Mohammedia (RR_{90}: 40.1) and Skhirate (RR_{90}: 20.1) [103]. Moderate resistance was detected in Salé (RR_{90}: 8.4) and Beslimane (RR_{90}: 5.9).

In 2012, using molecular assays, researchers demonstrated that in Türkiye the allele frequency of Kdr-related mutation L1014C was above 50% in the majority of populations [99]. In Greece, during the period 2018−2020 the allele frequency ranged from 74−80% in the four populations tested. Between 2008 and 2020, the L1014F allele was detected in multiple populations in different countries, with allele frequencies up to 100% in an Italian population (sample size: 9) [57,71,87,94,95,98,99]. Individuals from an Egyptian population with an allele frequency of 78% also showed a significant up-regulation in the P450 complex, compared to a control group (P<0.001). In Greece, the L1014S mutation was detected in three of four populations tested (AF: 0.6−3.5%) between 2018 and 2020. Data on these L1014 allele mutations include data acquired through the VEN survey.

During the period 2005−2018, tests were conducted for the presence of Ace-1 allele mutation F290V in six countries (Armenia, Greece, Morocco, Portugal, Tunisia and Türkiye), with allele frequencies ranging from 0−15% [71,91,94,99,104-106]. Tunisian *Culex pipiens* populations form the exception as the majority of populations had an allele frequency of 25−65%. In Portugal, this allele was not present. The other Ace-1 allele mutation, G119S, has been found more frequently in populations of this species, with allele frequencies ranging from 0−50%. Searches were undertaken in Algeria, Greece, Italy, Morocco, Portugal and Türkiye between 2002 and 2018.

The occurrences of esterase mutations in the Ester² allele have been investigated once, in a recent Moroccan study on five *Culex pipiens* populations [94], with allele frequencies ranging from 32.8% to 73.7%.

Chitin synthase mutations are linked to mutations of the I1043 allele. Of the three mutations investigated between 2016 and 2020, I1043F had the lowest frequency, while I1043L and I1043M mutations appeared more often and were more widespread in Italian and Turkish populations [87,88,95,96]. Tests were conducted for I1043F and I1043L alleles in Greece however they were not detected [87].

In 2012, the Rdl mutation A302S was detected in multiple Turkish *Culex pipiens* populations, with the allele frequency always being below 20%, except for the population in Izmir (AF: 29%) [94,99].

In the publications described above, the *Culex pipiens* biotype was not mentioned. The following information is available on resistance in *Culex pipiens* by biotype:

- *Culex pipiens molestus.* In Morocco, between 2015 and 2016, the Kdr allele mutation L1014F was detected in six populations, (AF: 0.01−50%) and the Ace-1 G119S in eight populations (AF: 0−39%) [65,107,108].
- *Culex pipiens pipiens.* Bioassays done in Tunisia between 2003 and 2005 showed: high resistance (RR >10) to chlorpyrifos, deltamethrin, fenitrothion, permethrin and pirimiphos-methyl, on their own and in combination with the synergists DEF and PBO. High resistance to DDT and temephos was also detected [41-43,46,49-55,58,109,110]. In addition, between 2015 and 2016, Ace-1 allele mutation G119S was detected in Morocco in eight populations (AF: 0−36%) [65,108] and also in Belgium between 2019 and 2021, in nine populations (AF: 12.5−28.5%) [93].
- *Culex pipiens pipiens molestus* hybrid. Between 2015 and 2016, Ace-1 mutation G119S was detected in Morocco in six populations, (AF: 0−50%) [65,108].

Table 8. Number of populations tested and their biocide resistance status in bioassays (on adults) per species and country

Species	Country	Method	Full compliance to strandard protocol	Alpha-Cyper-methrin			Cyfluthrin			Cyper-methrin			Deltamethrin			Etofenprox*			Lambda-cyhalothrin			Permethrin			DDT*			Chlorpyrifos*			Fenitrothion*			Malathion*			Pirimiphos-methyl*			Bendiocarb			Propoxur*		
				S	P	R	S	P	R	S	P	R	S	P	R	S	P	R	S	P	R	S	P	R	S	P	R	S	P	R	S	P	R	S	P	R	S	P	R	S	P	R	S	P	R
Aedes aegypti																																												
	Portugal	WHO	N												1									1																					
	Portugal	WHO	Y						1																							1												
Aedes albopictus																																												
	Albania	WHO	N	1	1								1									2																						
	Greece	CDC	N										4																					1	3									
	Greece	WHO	N																				1																					
	Italy	WHO	N	4	4	3				2	5	3	11									19	5	4																				
	Lebanon	WHO	Y																		1		1				1							1										1
	Spain	CDC	N										7						7			7														1	1		1					
	Spain	CDC	Y										2												1																				
	Spain	WHO	N							1	2	1	3	1								3	1																					
Aedes caspius																																												
	Spain	CDC	Y										1												1																				
Aedes detritus																																												
	U.K.	WHO	N																			1									1													
	U.K.	WHO	Y																																									
Anopheles labranchiae																																												
	Morocco	WHO	N																			5			1	10				1	2		1	2						4			
Anopheles maculipennis s.l.																																												
	Armenia	WHO	Y			2	1		1																																		
	Türkiye	WHO	N											8	2								4	6		10								8	14						1	3	8
Anopheles sacharovi																																												
	Türkiye	WHO	Y										21	##		15	4					23	11			34					15		1	33				1	14		5	28	
Anopheles sergentii																									1								1											
	Morocco	WHO	N																																									
Anopheles superpictus																																												
	Türkiye	WHO	Y										14	4		17	3	1				19	3			22								22							3	19	
Culex pipiens																																												
	Armenia	WHO	Y			2	1																																				
	Egypt	WHO	N																																						3		2	1
	Egypt	WHO	Y				2	1					2	1					2		1	1	1	1		3				2	1			3									
	Greece	WHO	N										5	6	2																												
	Italy	WHO	N											1	2							1	2	7				1	2	10													
	Morocco	WHO	N																		1			1		1								1						1			
	Spain	CDC	N											5	4				1	4	1	3	3			2											2			1			
	Spain	CDC	Y																																		1							
	Spain	WHO	Y												1											1										1						1	
	Türkiye	CDC	N											5	3	6	2		6	2		8																					
	Türkiye	WHO	N			3			3						3									3																				

Note. *S = susceptible (blue); P = Possible resistance (yellow); R = Resistant (red). * DDT, chlorpyrifos, fenitrothion, malathion, pirimiphos-methyl and propoxur are not registered in the EU/EEA. etofenprox is not registered for mosquitoes, only for spot treatment against cockroaches.*

Sand flies

Phlebotomus neglectus

Data was acquired solely from Fotakis et al. [97]. Kdr-related mutations L1014F, L1014S and L1014C were not detected in the 26 individuals investigated from two Greek populations in 2017.

Phlebotomus papatasi

Faraj et al. [68] tested for the efficacy of DDT, lambda-cyhalothrin and malathion in Morocco, 2011, and found all populations to be fully susceptible to these biocides (MR: 100%). Discriminating concentrations for *Anopheles* mosquitoes were used, which are tentative for sand flies. The results also have to be interpreted with care because the mortality rates of the susceptible groups were not reported and there was no mention of applying a correction method to the mortality rates of the investigated populations. In Türkiye, the Kdr-related L1014F mutation was found in one population, tested in 2014, with an allele frequency of 48% [92].

Phlebotomus perfiliewi

In 2017, molecular assays were conducted in Greece to look for Kdr mutations L1014F, L1014S and L1014C in four different populations, but these mutations were not detected [97].

Phlebotomus sergenti

In 2011, Faraj et al. (2012) tested the efficacy of DDT, lambda-cyhalothrin and malathion in Morocco and found all tested populations to be fully susceptible to these biocides (MR: 100%) [68]. However, these results have to be interpreted and discussed with care because the mortality rates of the susceptible groups were not reported and there was no mention of applying a correction method to the mortality rates for the populations investigated. The concentrations used were the discriminating concentrations for *Anopheles* mosquitoes, which are tentative for sand flies.

Phlebotomus simici

Tests were conducted for Kdr mutations L1014F, L1014S and L1014C in two Greek populations in 2017, but they were not detected in the 13 individuals tested (Fotakis et al. 2020).

Phlebotomus tobbi

Tests were conducted for Kdr mutations L1014F, L1014S and L1014C in two Greek populations in 2017, but they were not detected in the 60 individuals tested [97].

Ticks

Rhipicephalus annulatus

In two recent Egyptian studies, bioassays were conducted to test the efficacy of deltamethrin, although they used different concentrations. Between 2013 and 2015, Aboelhadid et al. [111] found low mortality rates in all but one tick population tested, for both the 0.02% and the 0.04% concentration (MR: 33.3–76.6%). Arafa et al. [56] confirmed the presence of deltamethrin resistance in four of six populations tested, acquiring similar results with adult immersion tests (concentration: 0.2%) and larval packet tests (concentration: 0.01%), although they did not report the year in which the assays were conducted. The allele frequency of Kdr-related mutation C190A was also measured in this study and reported as low in the two susceptible populations (AF: 0%; 3.9%) and higher in a population where resistance was confirmed (AF: 44.2%).

Rhipicephalus sanguineus

In 2002, Estrada-Pena et al. (2005) used larval packet tests to detect resistance of this tick species in Spanish populations by calculating lethal concentrations and the resistance ratio [112]. They found that none of the five populations was resistant to amitraz and propoxur (RR_{50}<5). The populations in Barcelona and Madrid were sensitive to deltamethrin (RR_{50} <5), while moderate resistance was detected in Castellón (RR_{50}: 5.9), Tarragona (RR_{50}: 7.7) and Zaragoza (RR_{50}: 7.7). For amitraz, the researchers used the Miller adaptation of the larval packet test [113].

Biting midges

Neither the literature search nor via the VEN consultation yielded data on biocide resistance in biting midges.

4 Discussion

Biocide resistance monitoring and reporting

This literature review on biocide resistance in disease vectors across the VectorNet geographical mapping area from 2000 till January 2022 identified 74 studies from which data were extracted. The monitoring of biocide resistance generally amounted to one or two studies per year, with the exception of 2005 and the period 2015–2017, in which the number was noticeably higher. Most studies focused on biocide resistance in mosquitoes and data came from a total of 26 countries (Albania, Algeria, Armenia, Belgium, Bulgaria, Croatia, Cyprus, Egypt, France, Georgia, Greece, Italy, Lebanon, Malta, Montenegro, Morocco, Portugal, Romania, Russia, Serbia, Slovenia, Spain, Switzerland, Tunisia, Türkiye and United Kingdom.) For sand flies, data were found from two countries (Türkiye and Morocco) and for ticks data were only available from Egypt. For biting midges, no data were available about the current state of biocide resistance in the EU and its neighbouring countries.

We did not find biocide resistance data for all countries implementing vector control in accordance with ECDC's technical report 'Organisation of vector surveillance and control in Europe' [18]. For mosquitoes, no biocide resistance data were found for the following countries implementing vector control using biocides: Austria, Czechia, Germany, Hungary, Kosovo, the Netherlands, North Macedonia, Poland, Sweden, and Ukraine. For sand flies, no biocide resistance data were available for Armenia, Greece, Spain, or Tunisia. For ticks, no biocide resistance data were found for Bulgaria, Egypt, Georgia, Greece, Kosovo, Romania, Portugal, Spain, Serbia, or Ukraine, despite the fact that these countries implement vector control [18]. Of the nine countries (Bulgaria, Croatia, Egypt, Greece, Hungary, Morocco, Spain, Serbia, and Tunisia) conducting biting midge control, five use biocides in their approach and none of the responding VEN members had knowledge of biocide resistance monitoring in their countries. Through the questionnaire, we learned that biocide resistance monitoring of biting midges was conducted in Azerbaijan, but we were unable to obtain any data [18].

Even for countries with reported biocide resistance data, the data were sparse in terms of time and space. Therefore, the results reported in this review may not fully reflect the current state of resistance in certain areas or against certain biocides. Despite these limitations, the current database is a first step towards obtaining an integrated view of the biocide resistance of vector species in the VectorNet geographical mapping area and assessing its implications for vector control in Europe. In mosquitoes, resistance was detected to biocides from the pyrethroid, organophosphate, organochlorine and the carbamate classes. In *Ae. aegypti, Ae. albopictus*, *An. labranchiae*, *An. maculipennis s.l.*, *An. sacharovi,* and *Cx. pipiens* populations, resistance to one or multiple biocides was found. Kdr mutations were detected in *Ae. aegypti*, *Ae. albopictus*, *An. sacharovi,* and *Cx. pipiens* populations. Rdl mutations were found in *Ae. albopictus* and *Cx. pipiens* populations. Ace-1, esterase and chitin synthase mutations were only been detected in *Cx. pipiens* populations. Tests on sand fly species have been conducted in Morocco using a pyrethroid (lambda-cyhalothrin), an organochlorine (DDT) and an organophosphate (malathion). Both *Ph. papatasi* and *Ph. sergenti* were susceptible to the biocides of these classes. A Kdr mutation was also found in Morocco. In Greece, Kdr mutations were not found in *Ph. neglectus, Ph. perfiliewi, Ph. simici* or *Ph. tobbi* [97]. Two Egyptian studies found resistance in various populations of *R. annulatus* to a pyrethroid (deltamethrin). In resistant populations, Kdr mutation C190A was present. In research on Spanish *R. sanguineus* populations, resistance to a pyrethroid (deltamethrin) was confirmed, while all populations were susceptible to a formamidine (amitraz) and a carbamate (propoxur).

Based on these results, biocide resistance in vector species seems to be a problem that needs to be monitored more closely to inform public and veterinary health authorities. Outside of Europe, biocide resistance in vector mosquito species is a growing problem in *Ae. aegypti* and *Ae. albopictus* [20], as well as in *Anopheles* species [35,114]. Balaska et al. [15] compiled biocide resistance data on sand fly vectors worldwide and identified biocide resistance in sand flies in the Indian Subcontinent, Iran and Sudan.

Biocide resistance in vector species does not only result from vector control activities, but also from the use of products with active substances against arthropods in agriculture, industry, and households. Evidence of an association between use of plant protection products and the emergence of resistance in malaria vectors has been repeatedly reported [115-117]. In the EU/EEA, there is currently no detailed overview of the extent to which products with active substances against arthropods are used and or the sources of these substances (households, industry, public and veterinary health action, agriculture, etc.). This information could be used to search for associations between the resistance observed in vector populations and biocide use [118]. Such information could be collected to inform biocide resistance management options. Furthermore, according to the data available for this review, testing of the phenotypic resistance of vectors against active substances from different classes provides information on possible alternative biocide products that can be used in vector control. This also gives an initial indication of the possible resistance mechanism, information which is essential for the development of informed biocide resistance management strategies (e.g. rotation of biocides from different classes).

Adhering to the Biocidal Products Regulation (EU) No 528/2012 (BPR), a biocidal product must be authorised before it can be made available on the market or used in the European Economic Area (EEA) and Switzerland. This takes place in two consecutive steps. First, the active substance is evaluated and, provided the criteria are fulfilled, it is then approved in a specified product-type. The second step is the authorisation of each product consisting of, containing, or generating the approved active substance(s). With the authorisation system, the BPR aims to harmonise the European market, while offering a high level of protection for humans, animals and the environment. Notably, the BPR considers that active substances with 'the worst hazard profiles' should not be approved, 'except in specific situations'. There are nine articles of law that provide for these 'specific situations' [119].

Many of the studies referenced (both in the EU/EEA and in neighbouring countries) describe resistance to active substances which are not approved in the specific Product Type (PT) 18 for use in vector control (PT 18 – Insecticides, acaricides and products to control other arthropods) in the EU. An overview of the active substances used in larval and adult vector control (Product Type 18) and approval status can be consulted at the following link: https://echa.europa.eu/information-on-chemicals/biocidal-active-substances. Two active substances commonly used for mosquito larval control, diflubenzuron and two Bti strains, are approved for PT 18. In our review, the only study [71] found to test their efficacy was conducted on Greek *Culex pipiens* populations, using WHO larvicide susceptibility assays. Resistance to diflubenzuron was detected in one of ten Greek populations tested and resistance to Bti in three of the ten Greek populations. For adult control, multiple studies have already confirmed resistance to deltamethrin and permethrin, two active substances approved in PT 18. Biocide resistance monitoring of the approved active substances registered for use in the EU will be vital for assessing whether their continued use in vector control activities is still justified.

Implications for vector control

As in many cases vector control is key to preventing and controlling transmission of vector-borne pathogens, due to the lack of adequate medication and/or vaccines, the development of biocide resistance might jeopardise disease prevention efforts. However, the effect of biocide resistance on the outcome of vector control interventions is often complex. For example, Long-Lasting Insecticide Nets (LLINs) used as a vector control tool against malaria seemed to provide users with some protection against biocide-resistant *Anopheles* mosquitoes, whereas community protection (i.e. the indirect protection of non-users through the killing of mosquitoes by those using LLINs) appears to have decreased due to biocide resistance [120]. Furthermore, for a number of vector control interventions primarily targeting arbovirus-vectors, the epidemiological impact of the interventions is not well known [121,122], complicating our understanding of the impact of biocide resistance on the outcome of the interventions.

Ticks are the main group of ectoparasites infesting cattle, sheep, horses and companion animals (dogs, cats), transmitting pathogens, threatening animal health, production and reproduction [123]. The implications of biocide resistance for tick control are presumably rather limited in the context of public health, where protection against ticks is primarily taken at a personal level, in the form of protective clothing which may be impregnated with a biocide, while control on a larger environmental scale is less common than in countries such as the USA [124].

Despite the uncertainties of the exact impact of biocide resistance on the outcome of vector control interventions, it is reasonable to assume that biocide resistance will have a negative impact. Therefore, it will be important to reduce and manage biocide resistance. Up-to-date monitoring information could inform decision-making and adjust vector control plans, where necessary. In addition, research is needed to develop vector control interventions that reduce reliance on biocides.

Limitations

This literature review provided a first view of the available data on biocide resistance for four vector groups among the disease vectors in the VectorNet geographical mapping area, from 2000 till January 2022. The current data are limited in terms of time and space and some data have had to be interpreted with caution, because the mortality rate of the control group was not reported. Overall, there seems to be a general lack of standardised study approaches and reporting, with researchers using different methods and concentrations, making it difficult to compare the state of biocide resistance between studies in the EU and neighbouring countries.

Biocide resistance monitoring is confounded by the fact that discriminating concentrations have not been established for all biocides and that they are not available for all vector species (leading to ad hoc solutions, such as the concentrations for *Anopheles* species being used for sand fly biocide resistance monitoring). Recently, WHO conducted a large, multi-centre study to establish new discriminating concentrations for *Ae. aegypti* and *Ae. albopictus* and *Anopheles* species, which will help to further standardise biocide resistance testing for these genera [125]. The most recent discriminative concentration for the CDC bottle tests can be found in the publication by the US Centers for Disease Control and Prevention 'CONUS manual for evaluating insecticide resistance in mosquitoes using the CDC bottle bioassay kit' [126].

5 Conclusions and potential implications

This literature review, covering the period from 2000 to January 2022, assessed the state of knowledge on biocide resistance in wild vector populations across the EU/EEA and neighbouring countries. Resistance to pyrethroids, organochlorines, organophosphates and carbamates has been confirmed in a number of vector species such as *Cx. pipiens* and *Ae. albopictus* in the countries where the majority of assessments were conducted. Resistance has also been observed in other *Aedes* and *Anopheles* species and in two tick species. However, our knowledge on biocide resistance for the four vector groups is still limited and patchy. Since biocide resistance is expected to reduce the impact of vector control, the biocide resistance status of key vectors should be further monitored using standard methods such as WHO and CDC bioassays. In addition, research is needed into insecticide resistant management in the context of the EU biocide legislation.

The following implications for public and veterinary health practice are suggested:

- In countries where vector control is considered, biocide resistance monitoring in the target vector species could inform estimates of expected biocide effectiveness. Up-to-date monitoring information could inform decision-making and help with the adjustment of vector control plans, where necessary.
- The biocide assessment studies would need consistent and standardised reporting. For mosquitoes, WHO guidelines exist on test procedures for insecticide resistance monitoring. The use of standard testing and reporting in studies performed across the EU/EEA could provide the necessary base for comparison between regions, countries and vector species and would facilitate insecticide resistance management. In addition, data on insecticide use in vector control, agriculture, industry and households could give insight into biocide pressure which, in turn, could inform management options in a 'One-Health' context.
- In the EU, a small number of active substances are available for vector control. Therefore, limited options exist for the implementation of resistance management – i.e. by alternating different classes of biocides. Therefore, the development of insecticide resistance could have a large impact on vector control programmes that rely heavily on biocides. Consequently, exploration of vector control intervention mixes that are not, or less, based on biocides could be useful, if warranted by available study results.
- Since the relationships between allele mutations and their putative phenotypic expression of resistance have not always been validated, further investigation to improve the use of molecular assays as resistance detecting methods would be of value.
- The monitoring of biocide resistance in Europe can be improved by establishing links between scientists in biocide resistance research and professionals from the public and veterinary health services involved in vector control. These collaborations could be useful to update biocide resistance monitoring data, generate risk maps, provide scientific and technical expertise to policy makers and disseminate information among actors and countries. This literature review could be a starting point for achieving this goal.

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Annex 1. Search strategy of the literature review

Search strategy per category

Resistance

(((Insecticid* OR acaricide* OR biocid* OR pyrethroid* OR organiphosphat* OR organochlorin* OR carbamate* OR neonicotinoid*OR DDT OR allethrin OR bifenthrin OR cyfluthrin OR cypermethrin OR cyphenothrin OR deltamethrin OR pyriproxyfen OR PBO OR Bti) AND resistan*) OR ((Insecticid* OR acaricide* OR biocid* OR pyrethroid* OR organiphosphat* OR organochlorin* OR carbamate* OR neonicotinoid*OR DDT OR allethrin OR bifenthrin OR cyfluthrin OR cypermethrin OR cyphenothrin OR deltamethrin OR pyriproxyfen OR PBO OR Bti) AND susceptib*) OR "kdr resistance)

Geography

Europe* OR "Mediterranean Basin" OR "Mediterranean area" OR Balkan* OR Scandinavia* OR "Iberian peninsula" OR Aland OR Albania* OR Andorra* OR Atlantic OR Austria* OR Belgi* OR "Black sea" OR Bosnia* OR Herzegovina OR Bulgaria* OR Croatia* OR Cypr* OR "Czech Republic" OR Czec* OR Denmark OR Greenland OR German* OR Spain OR Estonia* OR Finland OR "Faroe islands" OR France OR Corsica* OR Greece OR Gibraltar OR Hungary OR Iceland* OR Ireland OR Italy OR Sicil* OR Sardinia* OR Kosov* OR Latvia* OR Liechtenstein OR Lithuania* OR Luxembourg OR Macedonia* OR FYROM OR Malta OR Monac* OR Monegasqu* OR Montenegr* OR Netherlands OR Norway OR Poland OR Portug* OR Slovenia* OR Romania* OR "San Marino" ORSerbia* OR Slovakia* OR Switzerland OR Sweden OR "United Kingdom" OR "British Isles" OR "Great Britain" OR Wales OR England OR Scotland OR Turk* OR "Vatican city" OR Svalbard OR Israel* OR Palestin* OR Jordan* OR Leban* OR Syria* OR Morocc* OR Algeria* OR Tunisia* OR Libya* OR Egypt* OR "Western Sahara*" OR Armenia* OR Azerba* OR Belarus OR Bielorussia* OR Georgia* OR Moldov* OR Russia* OR Yugoslavia* OR Ukrain* OR Ukrayin* OR USSR OR SSSR OR "Soviet Union" OR British OR Irish OR Scottish OR Welsh OR "Channel Islands" OR Jersey OR Guernsey OR Sark OR French OR Greek OR Italian OR Spanish OR Swiss OR Swedish OR Transcaucasia* OR Caucasus OR Danish OR Finnish OR Norwegian OR Baltic OR Czech* OR Hungarian OR Polish OR Mediterranean OR Sahara OR OR Majorca* OR Mallorca* OR Minorca* OR Ibiza OR "North Africa*" OR Kazakh*OR Uzbek* OR Karakalpakstan* OR Crimea* OR Nakhchivan* OR Alsac* OR Abkhazia* OR Adjara* OR Ossetia OR Athos OR Aosta* Friuli* OR Giulia* OR Trentin* OR Adige OR Südtirol* OR "South Tyrol*" OR Găgăuzia* OR Transnistria* OR Madeira* OR Catalan OR Catalonia* OR Adygea* OR Bashkortostan* OR Chechnya* OR Chuvashia* OR Dagestan* OR Ingushetia* OR Karbardin* OR Kalmykia* OR Karachay OR Komi* OR Karelia OR "Mari El"* OR Mordovia* OR Tatarstan* OR Udmurtia* OR Nenets OR Vojvodin* OR Metohija* OR Srpska* OR Andalusia* OR Aragon* OR Asturias* OR Basque OR Cantabria* OR Castile* OR Mancha* OR León OR Extremadura* OR Galicia* OR Rioja* OR Madrid OR Murcia* OR Navarr* OR Valencia* OR Ceuta* OR Melilla* OR "Isle of Man"

Vectors

Ticks OR Ornithodoros OR Ixodes OR Rhipicephalus OR Dermacentor OR mosquito* OR Aedes OR Anopheles OR Culex OR Stegomyia OR Ochlerotatus OR Culicidae OR Culiseta OR Coquillettidia OR sandfly OR sandflies OR "sand flies" OR "sand fly" OR Phlebotominae OR Phlebotomus OR Culicoides OR "biting midges"

Annex 2. WHO and US CDC discriminating concentrations

WHO susceptibility bioassay – adult *Anopheles*
(http://apps.who.int/iris/bitstream/10665/250677/1/9789241511575-eng.pdf?ua=1)

Biocide class	Biocide	Discriminating concentration (%)	Remarks
Pyrethroids	Alpha-cypermethrin	0.05	Tentative, to be confirmed by WHOPES
	Cyfluthrin	0.15	
	Cypermethrin	N/A	
	Deltamethrin	0.05	
	Etofenprox	0.5	
	Lambda-cyhalothrin	0.05	
	Permethrin	0.75	
Organochlorines	DDT	4	
Organophosphates	Bromophos	N/A	
	Chloropyrifos	N/A	
	Fenitrothion	1	
	Fenthion	N/A	
	Malathion	5	
	Pirimiphos-methyl	0.25	Tentative and based on unpublished industry data, 2006; to be confirmed by WHOPES
	Temephos	N/A	
Carbamate	Bendiocarb	0.1	
	Propoxur	0.1	

Note: also used for *Culex pipiens* and sand flies.

WHO susceptibility bioassay – adult *Aedes*
(https://apps.who.int/iris/bitstream/handle/10665/204588/WHO_ZIKV_VC_16.1_eng.pdf)

Biocide class	Biocide	Discriminating concentration (%)	Remarks
Pyrethroids	Alpha-cypermethrin	0.03	Tentative
	Cyfluthrin	0.15	Determined for *Anopheles* mosquitoes, tentative for *Aedes*
	Cypermethrin	N/A	
	Deltamethrin	0.03	Tentative
	Etofenprox	0.5	Determined for *Anopheles* mosquitoes, tentative for *Aedes*
	Lambda-cyhalothrin	0.03	
	Permethrin	0.25	
Organochlorines	DDT	N/A	
Organophosphates	Bromophos	N/A	
	Chlorpyrifos	N/A	
	Fenitrothion	1	
	Fenthion	N/A	
	Malathion	0.8	
	Pirimiphos-methyl	0.21	Determined for *Anopheles* mosquitoes, tentative for *Aedes*
	Temephos	N/A	
Carbamate	Bendiocarb	0.1	

US CDC bottle bioassay (http://apps.who.int/iris/bitstream/10665/250677/1/9789241511575-eng.pdf?ua=1)

Biocide class		Biocide concentration *Aedes* (µg/250 ml*)	Biocide concentration *Anopheles* (µg/250 ml*)
Pyrethroids	Alpha-cypermethrin	N/A	N/A
	Cyfluthrin	10	12.5
	Cypermethrin	10	12.5
	Deltamethrin	10	12.5
	Etofenprox	N/A	N/A
	Lambda-cyhalothrin	10	12.5
	Permethrin	15	21.5

Biocide class		Biocide concentration *Aedes* (µg/250 ml*)	Biocide concentration *Anopheles* (µg/250 ml*)
Organochlorines	DDT	75	100
Organophosphates	Bromophos	N/A	N/A
	Chlorpyrifos	N/A	N/A
	Fenitrothion	50	50
	Fenthion	N/A	N/A
	Malathion	50	50
	Pirimiphos-methyl	N/A	20
	Temephos	N/A	N/A
Carbamate	Bendiocarb	12.5	12.5
	Propoxur	N/A	N/A

***Note.** Assays are conducted using 250 ml Wheaton bottles.

Annex 1. Questionnaire sent to VEN

In the context of the VectorNet project, we are reviewing the biocide resistance status of wild populations of mosquitoes, ticks, sand flies and biting midges in the EU and its neighbouring countries (VEN countries). In general, we are searching for data on biocide resistance obtained by performing WHO bioassays or CDC bottle tests, synergist assays, molecular assays and biochemical assays.

Through this questionnaire we would like to know whether the biocide resistance status of the four vector groups is assessed in your country and if data is available and can be shared with VectorNet.

This questionnaire is being sent to the VectorNet Entomology Network (VEN) members of all EU/EEA countries, the EU candidate countries and potential candidates, and the European Neighbourhood Policy partner countries.

The completion of the questionnaire will take around 15 minutes.

Full Name: Country you are based in:	
1.	Is biocide resistance of wild populations of the following vector groups (mosquitoes, ticks, sand flies, biting midges) assessed in your country? The assessment can be done as part of a governmental assignment or as part of e.g. research project at national, regional or local level.
	1.1. Government-assigned (national, regional and/or local): yes, no, not sure 1.2. Institutionally-based (e.g. in the context of a research project): yes, no, not sure.
2.	Who in your country conducts the assessment of biocide resistance? Please list the contact details of all these researchers below, so we can contact them as well if deemed feasible. (Name + e-mail + other contact details (optional))
	2.1. Mosquitoes 2.2. Ticks 2.3. Sand flies 2.4. Biting midges
3.	If you have access to data from biocide resistance assessments, could you share the data with VectorNet? If so, we will contact you once more through e-mail with the specific information we are looking for.
	Yes, I am willing to share the data with VectorNet No, I cannot share the data with VectorNet.
4.	Here you can provide any additional remark (not the data) related to the assessment of biocide resistance of the four vector groups that you want to share.

Annex 2. Database structure

Sheet 1. Includes data from bio-assays, resistance, synergist, molecular and biochemical assays

Code name	Explanation
First Author	Name of the researcher or institution
Year	Publication year
Country	Country of research
Vector_species	Investigated vector species
Location_Adress	Research location
Latitude_dec	Latitude (in decimal degrees)
Longitude_dec	Longitude (in decimal degrees)
Year_assessment	Year of insecticide resistance assessment
Bioassay	**Bioassay used in the research? (Y/N)**
Method	Method of bioassay applied
Biocide	Biocide used for the assessments
Discriminative dose	Did the researchers use the discriminate dose (DD) for the assessment? (Y/N)
Dose_perc	Biocide dose used for the assessment (%)
Exposure_time	Time vector species got exposed to the biocide
Bioassay_sample_size	Number of individuals the biocide was tested on
Bioassay_mortality_perc	Mortality number of the field population
Correction_method	Correction for percentage of mortality (e.g. Abbott's formula)
Mort_control_group_perc	Mortality percentage of the control group
Quality_check	Were the data in line with the pre-set criteria of the test?
Resistance_intensity	Did the researchers test the intensity of the biocide resistance? (Y/N)
5_DD_mortality_perc	Sample size of the 5x DD assay
5_ DD_samplesize	Mortality percentage when using five times the discriminating dose
10_DD_mortality_perc	Sample size of the 10× DD assay
10_DD_samplesize	Mortality percentage when using 10× the DD
Synergist_bioassay	Was the effect of a synergist on the resistance phenotype assessed? (Y/N)
Synergist	Synergist used
Sample_size	Number of vector individuals tested for the synergist bioassay
Syn_mortality_perc	Mortality number when performing the synergist bioassay
Molecular_assay	Did the researchers use the molecular assay to assess resistance alleles? (Y/N)
Allele	Which allele was tested for resistance?
Mutation	The group of mutations the mutated allele belongs t
Sample_size_MA	Number of individuals tested for the molecular assay
Allele_frequency_perc	Mutated allele percentage
Biochemical_assay	Did the researchers use the biochemical assay to assess resistance alleles? (Y/N)
Enzyme	Which enzyme was tested for resistance?
Enzyme_activity_field_population	Enzyme activity of the field population
Enzyme_activity_susceptible_population	Enzyme activity of the susceptible population
P_value	Did the enzyme activity of the wild population differ significantly from that of the susceptible population?
Comment	**Additional remarks**

Sheet 2. Data on bioassays in which LC and RR values were calculated to mark resistance

Code name	Explanation
First author	Name of the first author of the scientific article
Year	Publication year
Country	Country of research
Vector_species	Investigated vector species
Biocide	Biocide used for the assessments
Location_Adress	Research location
Latitude_dec	Latitude (in decimal degrees)
Longitude_dec	Longitude (in decimal degrees)
Year_assessment	Year of IR assessment
Method	Method of bioassay applied
LC_{50} sus (mg/l)	Biocide concentration which causes 50% mortality of the susceptible population (LC=Lethal Concentration)
$SSLC_{50}$ field	Sample size used to calculate the LC_{50} of the field population
LC_{50} field (mg/l)	Biocide concentration which causes 50% mortality of the field population
$SSLC_{50}$ sus	Sample size used to calculate the LC_{50} of the field population
RR_{50} field_sus	Resistance ratio: LC_{50} field / LC_{50} sus
LC_{90} sus (mg/l)	Biocide concentration which causes 90% mortality of the susceptible population
$SSLC_{90}$ sus	Sample size used to calculate the LC_{90} of the field population
LC_{90} field (mg/l)	Biocide concentration which causes 90% mortality of the field population
$SSLC_{90}$ field	Sample size used to calculate the LC_{90} of the field population
RR_{90} field/sus	Resistance ratio: LC_{90} field / LC_{90} sus
Comment	Additional remarks

Annex 3. Detailed description of the Kdr data in the database

Note: additional, unpublished data was acquired via the VEN from Pichler et al., Fotakis et al. and Haddad et al. for the allele mutations F1534C, I1011M, I1532T, L1014C, L1014F, L1014S and V1016G.

- Mutated allele F1534C has been detected in two countries. In Madeira, Portugal, it was dominant in the *Aedes aegypti* population in Funchal in 2009 (AF: 98%), but had declined to 23% by 2013. In Paúl do Mar, it was present in all *Aedes aegypti* individuals tested (AF: 100%) [61,100]. In Greek *Aedes albopictus* populations, F1534C was detected in most populations with an allele frequency ranging from 0–50%, with one exception (AF: 68.3% in Attica) [29,73,76,97]. In Italy, it was investigated in *Aedes albopictus* populations, but not detected [31,76].
- Kdr-related allele F1534L was investigated in Italian *Aedes albopictus* populations and was absent in all but one population, that of Arco, where its allele frequency was 1% [29] [76].
- Mutation F1534S was not detected in the seven Italian *Aedes albopictus* populations investigated [76].
- Mutation F1534W was not detected in the seven Italian *Aedes albopictus* populations investigated [76].
- The I1011M mutation was investigated in two Lebanese *Aedes albopictus* populations, with reported allele frequencies of 1.66% in Fanar-El Metn and 3.33% in Chehim. In Italy, it was not detected in the seven populations tested [76].
- Mutation I1011V was detected in Italy. Of the seven populations tested it was detected in the *Aedes albopictus* population from Modena (AF: 2.78%) [76].
- Allele mutation I1532T was only investigated for *Aedes albopictus* [31,73,97]. Whenever present, the allele frequency was lower than 10% in more than half of the populations.
- Presence of L1014 allele mutations was only tested for in *Culex pipiens* populations. In Türkiye, L1014C was detected in all tested populations, with allele frequencies of at least 27% in each population, and up to 91% [91,97,99]. In Greece, this mutation was also detected in the four tested populations, with allele frequencies from 74–80%.
- The presence of L1014F in *Culex pipiens* populations was investigated in Egypt, Greece, Italy, Morocco and Türkiye, and was detected with varying degrees of prevalence [36,57,65,71,94,95,97-99,107]. The mutation was detected with the highest allele frequencies in Italy, but in these tests the sample size was always below 25 individuals (Figure 9). In Türkiye, L1014F was detected in two of eight *Anopheles sacharovi* populations, with allele frequencies of 25% and 71% (sample size: 12). In Belgium, this mutation was detected with an allele frequency of 21.5% in the only *Culex modestus* population tested and was not detected in three *Culex torrentium* populations, from which a total of eight mosquitoes were examined [93].
- Mutation L1014S was detected in seven of eight Turkish *Anopheles sacharovi* populations (sample size: 12 [36]). In two of them, it was present in all individuals (AF: 100%). In Greece, it was detected in three of four *Culex pipiens* populations (AF: 0.6-3.5%).
- Mutation S989P was only investigated in Italian *Aedes albopictus* populations, but was not detected [76].
- Mutation S989Y was only present in the *Aedes albopictus* population of Comacchio of the seven Italian populations investigated (AF: 3.71%) [76].
- In Morocco, one *Anopheles sergentii* population was tested for the presence of allele L1014F, but this mutation was not detected [63].
- The V1016G allele was not detected in any of the Greek *Aedes albopictus* populations, while in Italy the allele frequencies in *Aedes albopictus* populations varied from 0–45.8% [31,39,73,76,97]. It has also been detected in Bulgaria, France, Georgia, Italy, Malta, Romania, Slovenia, Switzerland and Türkiye, with allele frequencies up to 8%. In some of these assays, the sample size was as low as four individuals.
- V1016I was detected in all three *Aedes aegypti* populations tested in Madeira, Portugal, and was present in all individuals of the population in Paúl do Mar (AF: 100%) [61,100]. In Italy, of the seven populations tested, it was detected in the *Aedes albopictus* population from Cosenza [76].
- Mutated allele V410A was detected in one of seven Italian *Aedes albopictus* populations tested, that of Arco (AF: 4.4%) [76].
- Mutated allele V410G was detected in one of seven Italian *Aedes albopictus* populations tested, that of Cosenza (AF: 5.6%) [76].
- Mutated allele V410G was detected in two of seven Italian *Aedes albopictus* populations tested, those of Turin (AF: 1.8%) and Cosenza (AF: 2.9%).

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