Determining discriminating concentrations of insecticides for monitoring resistance in mosquitoes

Report of a multi-centre laboratory study and WHO expert consultations

World Health Organization

Determining discriminating concentrations of insecticides for monitoring resistance in mosquitoes

Report of a multi-centre laboratory study and WHO expert consultations

World Health Organization

Determining discriminating concentrations of insecticides for monitoring resistance in mosquitoes: report of a multi-centre laboratory study and WHO expert consultations

ISBN 978-92-4-004520-0 (electronic version)
ISBN 978-92-4-004521-7 (print version)

Suggested citation. Determining discriminating concentrations of insecticides for monitoring resistance in mosquitoes: report of a multi-centre laboratory study and WHO expert consultations. Geneva: World Health Organization; 2022. Licence: CC BY-NC-SA 3.0 IGO.

Cataloguing-in-Publication (CIP) data. CIP data are available at http://apps.who.int/iris.

Contents

Acknowledgements v

Abbreviations and acronyms vii

Key outcomes ix

Recommendations made to WHO x

1 Introduction 1

2 Objectives of the study 4

3 Participating laboratories 5

3.1 Lead coordinating institution 5

3.2 Collaborators 5

4 Test compounds 7

5 Mosquito species 10

6 General framework of the study 11

6.1 Development of bottle bioassay method for group B compounds 13

6.1.1 Method development 13

6.1.2 Cross-validation of bottle bioassay method 13

6.2 Multi-centre testing of serial concentrations of test compounds (groups A and B) to establish concentration–response curves (steps 1 and 2) 14

6.3 Multi-centre validation of tentative discriminating concentrations against various mosquito species (step 3) 17

6.4 Data analysis and reporting 19

6.4.1 Data analysis, validation and interpretation 19

6.4.2 Data reporting and monitoring of progress 20

7 Results 21

7.1 WHO tube tests 21

7.1.1 Test completion rates 21

7.1.2 Concentration–response curves and estimated LC_{99} and LC_{100} 22

7.1.3 Validation of tentative discriminating concentrations in filter paper tests (step 3) 39

7.1.4 Main constraints encountered 42

7.2 WHO bottle bioassays 43

7.2.1 Development and validation of the WHO bottle bioassay protocol 43

7.2.2 Bioassay completion rates 55

7.2.3 Concentration–response curves and estimate of LC_{99} and LC_{100} 57

7.2.4 Validation of tentative discriminating concentrations in step 3 for WHO bottle bioassays 76

7.2.5 Main constraints encountered 81

8 Conclusions 83

8.1 WHO tube tests 83

8.2 WHO bottle bioassays 84

8.3 Challenges and the way forward 87

8.4 Research priorities for WHO tube tests 89

8.5 Research priorities for WHO bottle bioassays 89

References 90

Annex. Participating institutions and mosquito strains available 92

Acknowledgements

The World Health Organization (WHO) Department of Control of Neglected Tropical Diseases thanks the following people who participated in the study and in the WHO consultations to review results, assisted in the data validation and finalized this report.

Study investigators (organized alphabetically by country): Corine Ngufor (Collaborative Research Programme, London School of Hygiene & Tropical Medicine, Cotonou, Benin); Ademir Martins Jr (Fiocruz, Rio de Janeiro, Brazil); Bayili Bazoma (Institut de Recherche en Sciences de la Santé, Bobo-Dioulasso, Burkina Faso); Severin N'Do, Institut de Recherche en Sciences de la Santé, Cameroon; Josiane Etang and Nwane Philippe (Organisation de coordination et de coopération pour la lutte contre les grandes endémies en Afrique Centrale, Yaoundé, Cameroon); Zhao Chunchun, Meng Fengxia, Liu Qiyong (National Institute for Communicable Disease Control and Prevention, Chinese Centre for Disease Control and Prevention, Beijing, China); Martha Liliana Ahumada (Instituto Nacional de Salud, Bogotá, Colombia); Martha Quinones (University of Colombia, Bogotá, Colombia); Raphael N'Guessan (London School of Hygiene & Tropical Medicine, Bouake, Côte d'Ivoire); Laura Andréo, Vincent Corbel, Stephane Duchon, Celine Montazeau (Institut de Recherche pour le Développement, Montpellier, France); Mamadou B. Coulibaly (Malaria Research and Training Centre, Bamako, Mali); Kasinathan Gunasekaran, Ashwani Kumar, Shriram Ananganallur Nagarajan (Indian Council of Medical Research–Vector Control Research Centre, Puducherry, India); Urugayala Sreehari (Indian Council of Medical Research–National Institute of Malaria Research, Bengaluru, India); Kamaraju Raghavendra, Vaishali Verma (Indian Council of Medical Research-National Institute of Malaria Research, New Delhi, India); Hamdan Ahmad, Adanan Che Rus (Vector Control Research Unit, Universiti Sains Malaysia, Penang, Malaysia); Adriana Flores (Universidad Autonoma de Nuevo Leon, Mexico); Jesús A. Pinto Caballero, Miriam Palomino (National Institute of Health, Lima, Peru); Cheon Huat Tan (Wilson), Agnes Koou Sin Ying (Environmental Health Institute, Singapore); Basil Brooke, Michael Samuel (Vector Control Reference Laboratory, National Institute for Communicable Diseases, Johannesburg, South Africa); Pie Müller (Swiss Tropical and Public Health Institute, Basel, Switzerland); Waraporn Juntarajumnong (Kasetsart University of Agriculture, Bangkok, Thailand); Sungsit Sungvornyothin (Mahidol University, Bangkok, Thailand); Rosemary Lees, Giorgio Praulins (Liverpool School of Tropical Medicine, Liverpool, United Kingdom); Mark Rowland (London School of Hygiene & Tropical Medicine, London, United Kingdom); Matt Kirby (Kilimanjaro Christian Medical University College and London School of Hygiene & Tropical Medicine, Moshi, United Republic of Tanzania); Audrey Lenhart (Entomology Branch, Centers for Disease Control and Prevention, Atlanta (GA), United States of America).

Experts who participated in WHO consultations[1]**:** Rajib Chowdhury (National Institute of Preventive and Social Medicine, Bangaldesh); Luc Djogbenou (Universite Abomey Calavi,

1 Experts who participated in the WHO consultations were those who were found to have no declared conflict of interests.

Benin); Ahmad Ali Enayati (Mazamdaran University of Medical Sciences, Islamic Republic of Iran); Eric Ochomo (Co-chair) (Kenya Medical Entomology Research Institute, Kenya); Intan Ishak (Universiti Science Malaysia, Penang, Malaysia); Gissella Vasquez (US Naval Medical Research Unit No. 6, Peru); João Pinto (Chair) (Instituto de Higiene e Medicina Tropical, Lisbon, Portugal); Thomas Churcher (Imperial College, London, United Kingdom); Steve Lindsay (Durham University, Durham, United Kingdom); Matthew Thomas (York Environmental Sustainability Institute, York, United Kingdom).

Other stakeholders[1]: Frederic Schmitt (Bayer CropScience, Lyon, France); Achim Reddig, Susanne Stutz (BASF SE, Limburgerhof, Germany); Sebastian Horstmann, Juergen Junkersdorf (Bayer AG Research & Development, CropScience, Monheim, Germany); Vimal Mahalingam, Rajesh Mathew (Tagros Chemicals Co. Ltd, Chennai, India); Yuki Ando, Mai Fukuzawa, Kunizo Mori (Mitsui Chemicals Agro, Inc., Tokyo, Japan); Kate Kolaczinski (Global Fund to Fight AIDS, Tuberculosis and Malaria, Geneva, Switzerland); Mark Hoppe, Clay Scherer (Syngenta, Basel, Switzerland); John Invest, John Lucas (Technical Consultants, Sumitomo Chemical Co. Ltd, London, United Kingdom); Dave Malone (Bill & Melinda Gates Foundation, Liverpool, United Kingdom); Angus Spiers (Innovation-2-Impact Project, Liverpool, United Kingdom); Richard Oxborough (London, United Kingdom); Barnabas Zogo (Sumitomo Chemical Co. Ltd., London, United Kingdom); Jennifer Armistead (President's Malaria Initiative, United States of America); James W. Austin (BASF Corporation, Durham (NC), United States of America); Ben Hamza, Rakim Turnipseed (FMC Corporation, Philadelphia (PA), United States of America); Helen Jamet (Bill & Melinda Gates Foundation, Seattle (WA), United States of America).

WHO regional and country office focal persons: Dr Emmanuel Chanda (Regional Office for Africa, Brazzaville, Congo); Haroldo Sergio da Silva Bezerra (Regional Office for the Americas, Washington (DC), United States of America); Aya Yajima (Regional Office for South-East Asia, New Delhi, India); Elkhan Gasimov (Regional Office for Europe, Copenhagen, Denmark); Dr Samira Al-Eryani (Regional Office for the Eastern Mediterranean, Cairo, Egypt); Amanda Kim Murphy (Country Office, Suva, Fiji); Tessa Knox (Country Office, Port Vila, Vanuatu).

WHO headquarters: Raman Velayudhan, Rajpal S. Yadav (Department of Control of Neglected Tropical Diseases); Jan Kolaczinski, Lucía Fernández Montoya, Jennifer Stevenson, Chunzhe Zhang (Global Malaria Programme); Dominic Schuler (Regulation and Prequalification); Florence Fouque (Special Programme for Research and Training in Tropical Diseases).

The study was led by Rajpal S. Yadav and Raman Velayudhan. Vincent Corbel coordinated the study, analysed the data and drafted and revised the report, with Rajpal S. Yadav.

The study was conducted as part of a WHO project supported financially by the Bill & Melinda Gates Foundation, Seattle (WA), United States of America.

1 The other stakeholders were representatives of the donor agencies and commercial entities who participated in the open sessions of the consultations with an Observer status and provided technical comments on the draft study report prepared by the WHO secretariat. Their technical comments and views were considered in the closed sessions limited to the WHO experts and the secretariat. Those stakeholders were not part of the final approval process for the report.

Abbreviations and acronyms

AI	active ingredient
CDC	Centers for Disease Control and Prevention (Atlanta (GA), United States of America)
CR	completion rate
CI	confidence interval
DC	discriminating concentration
IRD	Institut de Recherche pour le Développement (Montpellier, France)
MERO	rapeseed oil methyl ester
OI	oviposition inhibition
PBO	piperonyl butoxide
ppm	parts per million
SOP	standard operating procedure
TDC	tentative discriminating concentration
WHO	World Health Organization

Participating institutions and laboratories

CDC	Centers for Disease Control and Prevention, United States of America
FIOCRUZ	Fundaçao Oswaldo Cruz, Brazil
ICDC	International Center for Disease Control and Prevention, China
IRD	Institut de Recherche pour le Développement, France
KU	Kasetsart University of Agriculture, Thailand
LSHTM-CREC	London School of Hygiene & Tropical Medicine – Centres de Recherches Entomologiques de Cotonou, Benin
LSHTM-IPR	London School of Hygiene & Tropical Medicine – Institut Pierre Richet, Côte d'Ivoire
LSHTM-KCMC	London School of Hygiene & Tropical Medicine – Kilimanjaro Christian Medical University College, United Republic of Tanzania
LSTM	Liverpool School of Tropical Medicine, United Kingdom
MRTC	Malaria Research and Training Centre, Mali

MU	Mahidol University, Thailand
NEA	National Environmental Agency, Singapore
NICD	National Institute for Communicable Diseases, South Africa
NIH	National Institutes of Health, United States of America
NIH-Colombia	National Institutes of Health – Colombia
NIH-Peru	National Institutes of Health – Peru
NIMR-B	Indian Council of Medical Research – National Institute of Malaria Research – Benguluru, India
NIMR-D	Indian Council of Medical Research – National Institute of Malaria Research – New Delhi, India
OCEAC	Organisation de coordination et de coopération pour la lutte contre les grandes endémies en Afrique Centrale, Cameroon
Swiss TPH	Swiss Tropical and Public Health Institute, Switzerland
UANL	Universidad Autónoma de Nuevo León, Mexico
VCRC	Indian Council of Medical Research – Vector Control Research Centre, India
VCRU	Vector Control Reseach Unit, Universiti Sains Malaysia, Malaysia

Key outcomes

WHO conducted a multi-centre study in 2017–2021 involving 23 laboratories throughout the world to establish and validate discriminating concentrations (DCs) of insecticides in order to monitor the resistance to insecticides of the main *Anopheles* and *Aedes* spp. mosquito vectors of human diseases. A total of 18 insecticides in eight classes and one synergist, piperonyl butoxide (PBO), were tested in susceptible strains of the five main *Anopheles* spp., *Anopheles gambiae* s.s, *An. funestus, An. minimus, An. stephensi* and *An. albimanus;* and two *Aedes* spp., *Aedes aegypti* and *Ae. albopictus*.

The key outcomes of the study are:

- formation of an international network of 23 laboratories in five WHO regions that have susceptible colonized mosquito species for testing 18 insecticides and PBO according to standardized methods and test procedures;
- establishment and validation of 17 new insecticide DCs for *Aedes* spp. in either WHO filter paper bioassays or bottle bioassays (Tables 1 and 2);
- establishment and validation of 13 new DCs for *Anopheles* spp. in WHO filter paper or bottle bioassays (Tables 3 and 4);
- development and validation of a new standard bottle assay procedure, "the WHO bottle bioassay", for testing compounds with modes of action that are not suitable for impregnation on filter paper;
- development of comprehensive standard operating procedures (SOPs) for impregnation of filter papers with insecticides and conduct of WHO tube tests and WHO bottle bioassays for testing the susceptibility of mosquitoes to insecticides;
- development and validation of an SOP for testing the susceptibility of adult mosquitoes to the sterilizing properties of pyriproxyfen, a juvenile hormone analogue that acts as an insect growth regulator;
- development of a provisional SOP for determining the optimal concentration of PBO for synergist-insecticide bioassays with *Aedes* spp. mosquitoes;
- development of a centralized database of bioassay records for > 400 000 mosquitoes of concentration–response tests and assessments of the source of variation in mosquito mortality under different test conditions; and
- identification and recommendation of measures to improve laboratory testing procedures and to guide the selection of DCs of insecticides commonly used for vector control.

Recommendations made to WHO

After reviewing the results of the studies and discussions in WHO consultations, the following recommendations were made to WHO.

- Adopt new and revised DCs of insecticides impregnated on filter papers for monitoring insecticide resistance in *Anopheles* and *Aedes* spp. as listed in tables 1 and 2.
- Adopt the newly validated WHO bottle bioassay for monitoring insecticide resistance in *Anopheles* and *Aedes* spp. for compounds that are not amenable to impregnation on filter paper.
- Adopt new DCs in the WHO bottle bioassay for monitoring insecticide resistance in *Anopheles* and *Aedes* spp. as listed in tables 3 and 4;
- Adopt the SOPs for (i) impregnation of filter papers, (ii) WHO tube bioassays of insecticides impregnated on filter papers, (iii) WHO tube bioassays for determining synergistic insecticide–PBO action, (iv) WHO bottle bioassays for insecticides and (v) WHO bottle bioassays for sterilizing properties of pyriproxyfen.
- Continue to use the 4% concentration of PBO on filter papers for synergistic bioassays against *Anopheles* spp. and *Aedes* spp. until the test protocol and threshold for interpreting bioassay data are reviewed and updated as appropriate.
- Establish insecticide DCs for monitoring resistance of other *Anopheles* and *Aedes* mosquito species that may have a role in disease transmission in some settings and also insecticide DCs for *Culex* spp., in particular *Cx. quinquefasciatus* and *Cx. pipiens,* in view of their importance in transmission of lymphatic filariasis and West Nile virus, respectively. This should include re-evaluation of DCs of insecticides intended for larval control.
- As a requirement for WHO prequalification of new insecticides for which no DC has been established, make it mandatory for manufacturers to submit data on DCs as part of the dossier submitted to WHO.

Table 1. Insecticide discriminating concentrations for *Aedes* species in WHO tube tests

Insecticides	Species	Discriminating concentration	Carrier oil or solvent
Alpha-cypermethrin	*Ae. aegypti*	0.05%	Silicone oil
	Ae. albopictus	0.08%	
Bendiocarb	*Ae. aegypti* and *Ae. albopictus*	0.20%	Olive oil
Chlorpyrifos-ethyl	*Ae. aegypti* and *Ae. albopictus*	1%	Olive oil
Permethrin (40:60[a])	*Ae. aegypti* and *Ae. albopictus*	0.40%	Silicone oil
Deltamethrin	*Ae. aegypti* and *Ae. albopictus*	0.03%	Silicone oil
Lambda-cyhalothrin	*Ae. aegypti*	0.05%	Silicone oil
	Ae. albopictus	0.08%	
Malathion	*Ae. aegypti*	1.50%	Olive oil
	Ae. albopictus	5%	
Pirimiphos-methyl[b]	*Ae. aegypti* and *Ae. albopictus*	60 mg/m^2	Acetone alone

Mosquito exposure time, 1 h; holding/recording time, 24 h

[a] This is the cis:trans isomer ratio.

[b] DC is expressed as mg/m^2 as no carrier oil is used to treat filter papers with pirimiphos-methyl.

Table 2. Insecticide discriminating concentrations for *Aedes* species in WHO bottle bioassays

Insecticide	Species	Discriminating concentration (µg/bottle)	Solvent and surfactant
Clothianidin	*Ae. aegypti*	20	Acetone + MERO 1500 ppm
	Ae. albopictus	10	
Flupyradifurone	*Ae. aegypti* and *Ae. albopictus*	80	Acetone + MERO 1500 ppm
Metofluthrin	*Ae. aegypti* and *Ae. albopictus*	1	Acetone
Prallethrin	*Ae. aegypti* and *Ae. albopictus*	30	Acetone
Transfluthrin	*Ae. aegypti* and *Ae. albopictus*	3	Acetone

MERO: 81% rapeseed oil methyl ester.

Bottle drying time, 24 h; mosquito exposure time, 1 h; holding/recording time, 24 h.

Table 3. Insecticide discriminating concentrations for *Anopheles* species in WHO tube tests

Insecticide	Species	Discriminating concentration	Carrier oil or solvent
Alpha-cypermethrin	*An. albimanus* and *An. stephensi*	0.30%	Silicone oil
	An. funestus, *An. minimus* and *An. gambiae*	0.05%	
Pirimiphos-methyl[a]	*An. albimanus*, *An. stephensi*, *An. minimus* and *An. funestus*	100 mg/m^2	Acetone alone
	An. gambiae	170 mg/m^2	

Mosquito exposure time, 1 h; holding/recording time, 24 h

[a] Discriminating concentration expressed in mg/m^2 as no carrier oil is used to treat filter papers with pirimiphos-methyl.

Table 4. Insecticide discriminating concentrations for *Anopheles* species in WHO bottle bioassays and testing conditions (mosquito exposure time, 1 h)

Insecticide	Species	Discriminating concentration (μg/bottle)	Bottle drying time (h)	Recording/ holding time (h)	Solvent/ surfactant
Clothianidin	*An. albimanus*	10	24	24	Acetone + MERO 200 ppm
	An. stephensi	10	24	24	Acetone + MERO 800 ppm
	An. funestus and *An. gambiae*	4	24	24	
	An. minimus	6	24	24	
Flupyradifurone	*An. albimanus*	500	24	24	Acetone + MERO 200 ppm
	An. stephensi and *An. gambiae*	60	24	24	Acetone + MERO 800 ppm
	An. funestus and *An. minimus*	100	24	24	
Transfluthrin	*An. albimanus*, *An. stephensi*, *An. funestus*, *An. minimus* and *An. gambiae*	2	24	24	Acetone
Chlorfenapyr	*An. gambiae*, *An. stephensi*, *An. funestus* and *An. albimanus*	100	24	72	Acetone
Pyriproxyfen[a]	*An. gambiae*, *An. stephensi* and *An. funestus*	100	2	7 d[b]	Acetone

Mosquito exposure time, 1 h.

[a] Data not available for *An. albimanus* and *An. minimus*.

[b] The 7-day period includes a 72-h holding period in which mosquitoes are kept in paper cups to record mortality, followed by an additional 96 h of individual chambering of surviving females to record oviposition.

1 Introduction

Over 80% of the world's population lives in regions at risk of at least one vector-borne disease *(1)*, and vector control is a central element in their prevention, control or elimination *(2)*. The development and geographical expansion of insecticide resistance in vector mosquitoes has become a major threat to the control and prevention of vector-borne diseases *(3, 4)*. According to the latest reports, *Anopheles* spp. were found to be resistant to at least one class of insecticide in 84 countries with ongoing malaria transmission, resistance to the pyrethroids being the most common *(5)*. Regarding *Aedes* spp., a systematic review has indicated the presence of insecticide resistance in at least 30 countries, with evidence of widespread resistance in Asia and South America *(6, 7)*.

The aim of the Global Vector Control Response 2017–2030, adopted by the World Health Assembly in May 2017, is to reduce the burden and threat of vector-borne diseases through effective, locally adapted, sustainable vector control *(8)*. Monitoring of insecticide resistance is an essential component of the Response, as information on vector resistance to insecticides is a basic requirement to (i) guide the selection of insecticides and insecticidal products for vector control, (ii) provide data on susceptibility to insecticide products in use and (iii) provide evidence to plan insecticide resistance management strategies. Given the increasing resistance among malaria and dengue vectors worldwide, especially to the insecticides in the chemical classes most commonly used (e.g. pyrethroids, organophosphates and carbamates) and the potential risk of their cross-resistance to new public health pesticides, attention has been focused on more intensive, improved monitoring of insecticide resistance *(9)*.

In practice, insecticide resistance is monitored in field populations of mosquitoes by conducting bioassays of mosquito mortality in response to a standard concentration of the insecticide, known as the diagnostic or discriminating concentration (DC).[1] The concept of an insecticide DC has clear advantages in terms of the cost and efficiency of testing and has been adopted widely for the purposes of monitoring insecticide resistance in mosquitoes and other disease vectors *(10, 11)*. Although WHO had made recommendations

1 WHO has defined insecticide discriminating concentration (DC) as twice the lowest concentration that systematically results in 100% mortality after 60 min of exposure and a holding period of 24 h of a susceptible mosquito strain. The DC can also be defined as twice the LC_{99} value as determined in a log-probit statistical model against a susceptible strain of an insect.

on DCs before 1998, DCs for various pyrethroid insecticides were recommended for the first time in 1998 on the basis of a WHO multi-centre study involving nine institutions and 12 *Anopheles* species *(12)*. Thereafter, tentative DCs were adopted by WHO for some new insecticides (e.g. for alpha-cypermethrin and pirimiphos-methyl) for testing the resistance of *Anopheles* spp. *(10)*, but none was validated for *Aedes* spp. *(11)*. Furthermore, no DCs have been established or validated for the newer classes of public health insecticides, including those with distinct modes of action (e.g. flupyradifurone, clothianidin, pyriproxyfen) and/or slow killing action (e.g. chlorfenapyr). It is essential to determine DCs for new insecticides that are prequalified by WHO or are in an advanced stage of evaluation by WHO for vector control to establish the baseline susceptibility of vector populations and to detect any change in phenotypic resistance after their deployment.

A consultation was organized at WHO headquarters in Geneva on 13–14 February 2017 involving experts, researchers, industry repesentatives and other stakeholders to review the state of and gaps in knowledge about insecticide DCs for monitoring resistance in adult mosquitoes in the field and selecting test methods according to the insecticide chemistry. The participants discussed the selection of insecticides and mosquito species for multi-centre validation of DCs, identified institutions that could participate in a WHO multi-centre study and proposed a general framework for a suitable study design and test protocol. Concern was raised about the technical suitability of filter papers for evaluating some new insecticides, i.e. compounds with new modes of action and/or distinct chemical properties (e.g. compounds that cannot be dissolved in the currently used carrier oil or solvent (acetone) for impregnation on Whatman no. 1 filter paper). Given the suitability of glass bottles for testing insecticide resistance, these were explored for developing bottle bioassays for new insecticides in order to ensure the reproducibility of successive tests and high confidence in the results. As the bottle bioassay developed by the United States Centers for Disease Control and Prevention (CDC) provides an estimate of the time to knock down or incapacitate mosquitoes *(13)* but does not score mortality at 24 h (or later) after a 1-h exposure (which is the end-point measured in the WHO tube test with filter papers), the consultation agreed to adapt the CDC test to develop a "WHO bottle bioassay" for measuring mortality as an end-point at 24 h (or later for slow-acting compounds) after a 1-h exposure.

With feedback from the consultation and the participating institutions, geographically representative insecticide-susceptible strains of *Anopheles* and *Aedes* spp. were selected for the WHO multi-centre study at a meeting between WHO and the Institut de Recherche pour le Développement (IRD), Montpellier, France, on 18–19 May 2017. Using technical documents from industry partners, WHO and IRD jointly developed a generic study design, described the study objectives, listed the test compounds, solvent, carrier oil and surfactant to be used, the mosquito species, the institutions, the test methods and the bioassay end-points for determining the susceptibility of the selected test compounds. WHO then commissioned a study by 23 suitable laboratories to develop test protocols and determine DCs for 18 selected insecticides that are used in either vector control or in developing new vector control products for control of malaria and/or *Aedes*-borne diseases.

The participating laboratories were also asked to establish an optimum concentration of PBO to be used in synergist bioassays against *Aedes* spp. Each participating laboratory used the generic study design to develop their site-specific test protocol according to the mosquito species available, the compounds allocated to them by WHO and their staff capacity and resources. The study was launched in late 2017 and completed mainly in September 2021 with some tests completed in November 2021.

A WHO expert meeting was held in October 2019 to review the interim results of preliminary bioassays of the range of concentrations that killed 0–100% of the tested mosquitoes (step 1) and a complete set of bioassays (in triplicate) to estimate the goodness of fit (slope, *P*) and LC_{99} and LC_{100} with their 95% confidence intervals (CIs) (step 2) by the participating laboratories and to recommend selection of tentative DCs (TDCs) for further validation in step 3. They also discussed technical difficulties faced in testing some compounds, proposed possible improvements and solutions and recommended stopping testing of three compounds that showed conflicting results and/or high inter-laboratory variation.

The final results of the study were reviewed at a WHO consultation on 15–18 December 2020 with two additional sessions on 21 January and 1 October 2021, which made recommendations for DCs of individual compounds for monitoring insecticide resistance in *Anopheles* and *Aedes* spp. and proposed further work.

The present report presents the study objectives, design, results and conclusions and the recommendations of the expert consultations made to WHO on new DCs for filter paper and bottle bioassays and on further studies.

2 Objectives of the study

The objectives of the study were to:

- determine concentration–response curves in filter paper bioassays with tube tests or bottle bioassays for 19 selected compounds, comprising 9 compounds for the WHO tube tests and 10 compounds for bottle bioassays;
- develop and validate a bottle bioassay method for determining DCs for 10 selected compounds that are unstable or cannot be impregnated onto filter paper;
- establish and validate the DCs of the insecticides tested in the study against selected *Aedes* and *Anopheles* spp; and
- identify gaps and priority research for future work in determining insecticide DCs.

3 Participating laboratories

3.1 Lead coordinating institution

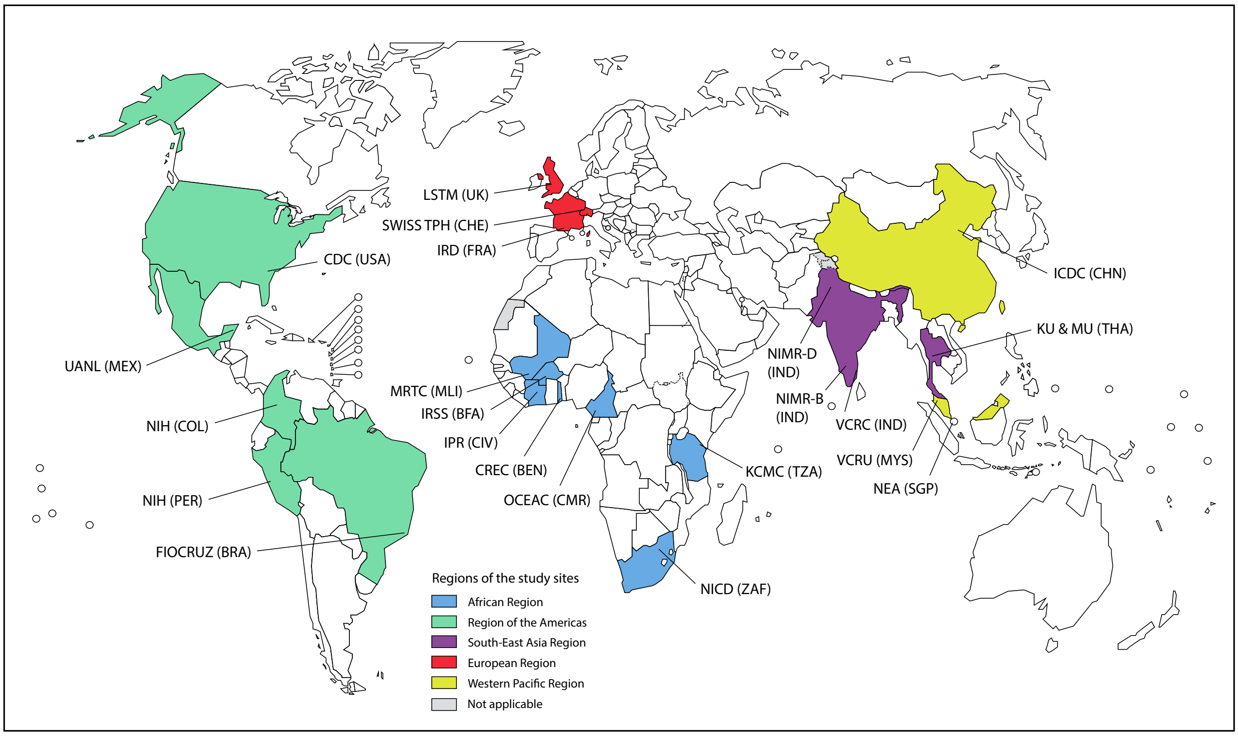
The Institut de Recherche pour le Développement (IRD), Montpellier, France,[1] was selected by WHO as the lead institution to coordinate the multi-centre study. IRD coordinated implementation of the study and maintained regular communication (through video conferences, e-mails and phone calls) with the participating centres to monitor progress, collect and analyse bioassay data and provide technical support if required. IRD also maintained regular exchanges with industry partners to discuss technical issues arising from the study, learn about any changes in test procedures (e.g. adjustment of the concentration in the surfactant assays) and obtain more information on product chemistry (composition, material data safety sheets, certificates of analysis, storage conditions).

3.2 Collaborators

A total of 23 internationally recognized laboratories with good entomological capacity in various regions agreed to participate in the study (Fig. 1). The institutions and the susceptible mosquito strains available at each are listed in the Annex. All the institutions maintain well-characterized susceptible mosquito colonies and have adequate facilities and capacity for laboratory testing. The institutions are either formally designated WHO collaborating centres or had the necessary capacity to test insecticides. The selected institutions were contracted directly by WHO and were asked to adhere strictly to the standardized study design developed jointly by IRD and WHO and to their site-specific protocols when agreed with WHO.

1 https://en.ird.fr and https://www.mivegec.ird.fr/fr/

Fig. 1. Locations of the 23 participating laboratories



Data Source: World Health Organization
Map Production: Control of Neglected Tropical Diseases (NTD)
World Health Organization

World Health Organization

The abbreviations are defined in the Annex.

4 Test compounds

Following the WHO expert consultation in 2017, 19 compounds (18 insecticides and PBO) were selected for high-priority testing for resistance in *Anopheles* spp. and/or *Aedes* spp. Of these, 9 were tested against *Anopheles* spp., and 15 insecticides and PBO were tested against *Aedes* spp. The compounds belong to nine different insecticide classes, and various formulations are currently in use or under evaluation for use in vector control, such as in indoor residual spraying, insecticide-treated nets, space spraying, household pest control and spatial repellents.

The compounds were categorized into two test groups:

- Group A: 9 compounds (8 insecticides and the synergist, PBO) for which the existing WHO filter paper test method is adequate for establishing and validating DCs (Table 5); and
- Group B: 10 compounds with distinct chemical properties and/or modes of action that are not technically suitable for impregnation on filter papers because of instability and for which glass bottle bioassays were considered suitable for establishing and validating DCs (Table 6).

With regard to group B, preliminary studies showed that some new insecticides tend to crystallize on filter paper if they are impregnated with an inappropriate carrier oil, hence limiting the bio-efficacy and duration of the treated papers. The CDC bottle bioassay is used to measure the time for an insecticide to penetrate the mosquito body, traverse its tissues, reach the target site and act on that site. Mosquitoes could be knocked down or killed. To be consistent with WHO procedures, in which mortality is the end-point of a susceptibility test, the CDC glass bottle bioassay method was modified to record mosquito mortality 24 h or later after a fixed period of exposure (e.g. 1 h) to an insecticide, similarly to susceptibility tests with impregnated papers. The test compounds were allocated to the participating laboratories according to the availability of mosquito species and laboratory capacity.

Bioassays were conducted with compounds in both groups to measure mosquito mortality (and other end-points where relevant) as compared with that of well-characterized susceptible mosquito strains. For pyriproxyfen, an insect growth regulator, the aim was to estimate its sterilizing properties (impact on fecundity) in adult female mosquitoes after exposure in treated bottles. For PBO, the aim was to determine the optimum percentage concentration to be used in synergist bioassays against *Aedes* spp. mosquitoes. The type of solvent and carrier oil used in the filter paper bioassays complied with the WHO guidelines for the filter paper test. For the WHO bottle bioassays, a surfactant (i.e. rapeseed oil methyl ester, MERO) was used to coat bottles when testing clothianidin and flupyradifurone as per the manufacturers' instructions, to prevent crystallization of the active ingredients and allow for adequate coating of bottles. All the test compounds and MERO were provided gratis to WHO by their respective manufacturers, with certificates of analysis and material safety data sheets. They were stored under appropriate conditions as per the manufacturers' instructions.

Table 5. Test compounds in group A, carrier oils and solvents used in filter paper tests

Class group	Insecticide	Test species		Carrier oil and solvent	Product or application
		Anopheles	*Aedes*		
Pyrethroid	Alpha-cypermethrin	✓	✓	Silicone oil + acetone	Space spray, insecticide-treated nets, indoor residual spraying
	Deltamethrin	–		Silicone oil + acetone	Space spray, insecticide treated nets
	Lambda-cyhalothrin	–	✓	Silicone oil + acetone	Indoor residual spraying
	Permethrin (40:60)	–	✓	Silicone oil + acetone	Space spray, indoor residual spraying, insecticide-treated nets
Organophosphate	Malathion	–	✓	Olive oil + acetone	Space spray, indoor residual spraying
	Pirimiphos-methyl	✓	✓	Acetone only[a]	Indoor residual spraying
	Chlorpyrifos-ethyl	–	✓	Olive oil + acetone	Space spray, indoor residual spraying
Carbamate	Bendiocarb		✓	Olive oil + acetone	Indoor residual spraying
Synergist	Piperonyl butoxide	–	✓	Silicone oil + acetone	Insecticide treated nets; synergist bioassays to detect presence of mixed-function oxidases

[a] No oil is used for paper impregnation as per the manufacturer's instructions.

Table 6. Test compounds in group B, solvents and surfactants used in bottle bioassays

Insecticide class	Insecticide	Test species		Surfactant and solvent	Product or application
		Anopheles	*Aedes*		
Pyrrole	Chlorfenapyr	✓	✓	Acetone	Insecticide-treated nets, indoor residual spraying
Neonicotinoid	Clothianidin	✓	✓	MERO + acetone	Indoor residual spraying
	Dinotefuran	✓	–	Acetone	Attractive targeted sugar baits
	Imidacloprid	–	✓	Acetone	Space spray
JH analogue	Pyriproxyfen	✓	–	Acetone	Insecticide-treated nets
Oxadiazine	Indoxacarb	✓	–	Acetone	Insecticide-treated nets
Butenolide	Flupyradifurone	✓	✓	MERO + acetone	Space spray
Pyrethroids	Transfluthrin	✓	✓	Acetone	Spatial repellent, space spray, household pesticide products
	Prallethrin	–	✓	Acetone	Space spray
	Metofluthrin	–	✓	Acetone	Spatial repellent

MERO: 81% rapeseed oil methyl ester (from Bayer CropScience).

5 Mosquito species

For logistical reasons and to keep the study size manageable, seven mosquito vector species (five *Anopheles* spp. and two *Aedes* spp.) were studied (Table 7). The criteria for selecting these species were that they:

- had a primary role in malaria or dengue transmission;
- are representative of a geographical region (Africa, Asia, Central and South America or the Middle East); and
- are maintained or colonized in at least three different laboratories to allow cross-validation tests at several sites.

Only fully susceptible mosquito strains (defined as susceptible to insecticides in all major insecticide classes with no detectable resistance mechanism) were tested. Some participating laboratories used different strains of a given species (e.g. Bora Bora, Rockefeller and New Orleans for *Ae. aegypti*); however, only one susceptible strain each of *An. gambiae* and *An. funestus* (i.e. Kisumu and Fang, respectively) was tested.

Table 7. Susceptible mosquito species and strains used for testing susceptibility

Genus	Species (strain)	Main geographical region of species distribution	Institutions hosting mosquito colonies[a]
Anopheles	*An. gambiae* s.s. (Kisumu)	Africa	IRD, LSHTM-IPR, Swiss TPH, IRSS, OCEAC, LSHTM-Benin, LSHTM-Tanzania, MRTC, CDC
	An. funestus s.s. (Fang)	Africa	LSTM, NICD, CDC
	An. stephensi (Nadiad, Puducherry)	South-East Asia	NIMR-D, NIMR-B, VCRC
	An. minimus s.s. (TM, MINIMUS1)	South-East Asia	KU, MU, CDC
	An. albimanus (Sanarate, Stecia, Buenaventura)	Americas	NIH-Peru, NIH-Colombia, CDC
Aedes	*Ae. Aegypti* (Bora Bora, New Orleans, Rockefeller)	All regions	IRD, LSTM, Swiss TPH, IRSS, LSHTM-Tanzania, MRTC, UANL, FIOCRUZ, VCRU, KU, VCRC, NIMR, ICDC, NEA, NIH-Peru
	Ae. albopictus (Chengdu, NEA-EHI, Perols, VCRU)	All regions	IRD, FIOCRUZ, VCRU, ICDC, NEA

[a] The acronyms of the names of the institutions are defined above and in the Annex.

NEA-EHI: National Environment Agency–Environmental Health Institute; TM: Tropical Medicine; VCRU: Vector Control Research Unit.

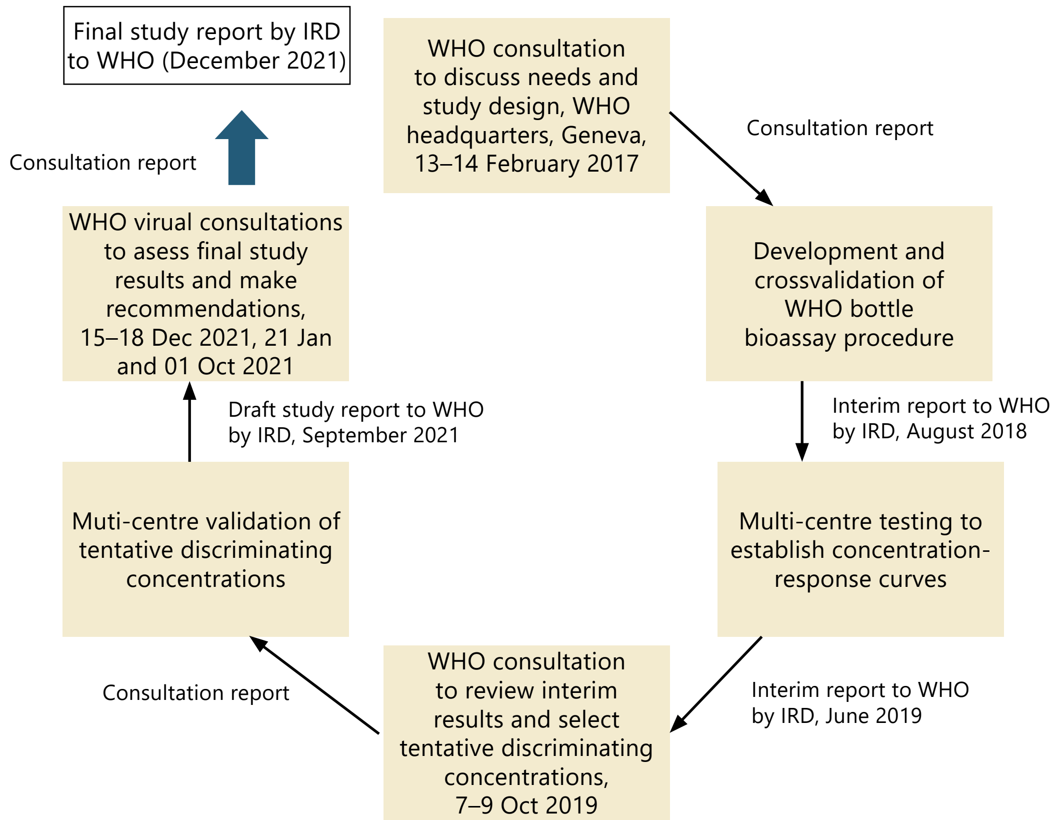
6 General framework of the study

The components of the study are described below and summarized in Fig. 2.

- WHO informal consultation with participating institutions, industry and other stakeholders in 2017 to identify mosquito species, insecticides, test procedures and participating institutions;
- development and discussion of test protocols and initial SOPs, end-points and potential limitations related to specific test compounds;
- signing of WHO technical service agreements with participating institutions and laboratories;
- delivery of test compounds and other materials by WHO to the 23 participating laboratories;
- development and cross-validation of the bottle bioassay method for determining the bio-efficacy of insecticides in group B (Table 5). Six laboratories with extensive expertise in testing and evaluating pesticide products were asked to develop bottle assay protocols and to select appropriate end-points for testing chemicals with distinct properties and modes of action to ensure that the WHO bottle bioassay provided consistent, reproducible results;
- multi-centre testing of serial concentrations of test compounds to establish concentration–response curves against selected test species; preliminary bioassays to assess the range of concentrations that kill 0–100% of tested mosquitoes (step 1) and a complete set of bioassays in triplicate to estimate goodness of fit (slope, *P*) and LC_{99} and LC_{100} with their 95% confidence intervals (step 2);
- review of bioassay results in a WHO consultation, in which the investigators were asked to select a TDC for each compound for testing in step 3. On the basis of data from step 2 (LC_{99} and LC_{100}), filter papers impregnated with TDCs (twice the LC_{99} or sometimes LC_{100}) at the Universiti Sains Malaysia were tested in at least three independent laboratories to evaluate mosquito mortality, with the expectation of killing 100% of each selected species. The quality of the impregnated papers was checked by the Universiti before they were dispatched to the study sites for testing;

- WHO consultations with participating laboratories, industry and other stakeholders to review the results of steps 1–3 and validate the final DCs of all the insecticides and PBO for individual mosquito species;
- data analysis and reporting (steps 1–3), which comprised regular follow-up with participating laboratories to collect, analyse and validate their bioassay results after completion of each testing step and report back to the site before proceeding with the next step or repeating or revalidating certain tests;
- regular technical reports from the participating laboratories to WHO and IRD to ensure that results were made available to WHO and partners in a timely manner;
- preparation of final study report and SOPs and sharing with participating institutions, industry and other stakeholders for final review and comments;
- finalization of recommendations in a WHO consultation restricted to the investigators and invited experts; and
- finalization and dissemination of the report; WHO policy decision to adopt the new DCs.

Fig. 2. General framework of the multi-centre study, with milestones



6.1 Development of bottle bioassay method for group B compounds

6.1.1 Method development

In consultation with study collaborators, six laboratories with extensive expertise in testing and evaluation of new public health pesticides were asked to develop glass bottle bioassay protocols for the 10 test compounds allotted to them (Table 8). Each compound was then allotted to three of the laboratories, one laboratory developing the method and the two others cross-validating the method.

Table 8. Insecticides (group B) and mosquito species allocated to institutions for developing and validating the WHO bottle bioassay

Insecticide	Insecticide class	Institution[a]			Test species
		Method development	Cross-validation of method		
		A	B	C	
Transfluthrin	Pyrethroids	IRD	NIMR-D	NEA	*Ae. aegypti*
Flupyradifurone	Butenolides	IRD	LSHTM	NIMR-D	*Ae. aegypti*
Prallethrin	Pyrethroids	LSHTM	NEA	IRD	*Ae. aegypti*
Clothianidin	Neonicotinoids	LSHTM	IRD	CDC	*An. gambiae*
Metofluthrin	Pyrethroids	NIMR-D	IRD	CDC	*Ae. aegypti*
Imidacloprid	Neonicotinoids	NIMR-D	CDC	LSTM	*Ae. aegypti*
Dinotefuran	Neonicotinoids	LSTM	CDC	IRD	*An. gambiae*
Chlorfenapyr	Pyrroles	LSTM	LSHTM	CDC	*An. gambiae*
Indoxacarb	Oxadiazine	CDC	LSTM	LSHTM	*An. gambiae*
Pyriproxyfen	Insect growth regulator	CDC	LSTM	LSHTM	*An. gambiae*
S-Bioallethrin[b]	Pyrethroids	NEA	NIMR	LSTM	*Ae. aegypti*

[a] The acronyms of the names of the institutions are defined above and in the Annex.

[b] Compound excluded from the study because it was not available.

6.1.2 Cross-validation of bottle bioassay method

The lead laboratories shared their method for testing a particular compound with two other selected centres (columns B and C in Table 8) for cross-validation under the same test conditions and with the same protocol and mosquito species. The results of bottle bioassay development were then shared with IRD for analysis.

Each laboratory was responsible for adapting the bottle bioassay and proposing end-points for the assigned compounds according to the generic study design, in collaboration with WHO and IRD. They were asked to determine and advise on the following technical criteria:

- bottle drying time (1, 2 or 24 h);
- mosquito exposure time (1 or 2 h);
- post-exposure holding period (24, 48 or 72 h); and
- appropriate test conditions (i.e. temperature and relative humidity).

The participating laboratories were also asked to report to IRD and WHO any limitations of the test, such as inconsistent results or changes in testing conditions or concentration of the surfactant for coating bottles. For most compounds, mosquito mortality was recorded at 24, 48 and 72 h after a 1-h exposure to the test insecticide or control. For pyriproxyfen, an insect growth regulator, the holding period was 7 days; mortality was recorded up to 72 h of the holding period after a 2-h drying and a 1-h exposure time; thereafter, the surviving females were chambered individually in paper cups for an additional 96 h to record oviposition. At the end of the 7-day holding period, the sterilizing effects of pyriproxyfen (e.g. reduction in numbers of eggs laid and offspring) were assessed.

The test protocols developed were validated at WHO consultations when consistent, repeatable results had been achieved in the three selected centres.

6.2 Multi-centre testing of serial concentrations of test compounds (groups A and B) to establish concentration–response curves (steps 1 and 2)

At least three laboratories were assigned to establish concentration–response curves for a given compound and mosquito species to cross-validate the bioassay results before proceeding to the next step of testing. Before starting the work, the test conditions (e.g. temperature, relative humidity) and protocols (e.g. bioassay method and sample size, age and physiological status of mosquitoes) were standardized to ensure the comparability of data from different testing laboratories. SOPs for filter paper impregnation, WHO filter paper testing, treatment of bottles and WHO bottle bioassays developed collaboratively were used *(14)*.

The compounds were tested in three sequential steps. The first two steps are described below, and step 3 is described in section 6.3. The requirements for conducting bottle assays and filter paper tests are summarized in Table 9.

Table 9. Scheme of test requirements for conducting bottle bioassays and filter paper tests in steps 1–3

Testing step	Number of test concentrations and controls	Minimum no. of mosquitoes per test concentration or control	No. of replicates (test batches) per concentration or control	Total no. of mosquitoes tested	Expected outcome
Step 1: Screening	Tests: 10–12 Control: 1	50 (25 x 2 bottles or tubes)	1	Tests: 500–600 Control: 50	A range of concentrations that cause 0–100% mortality[a]
Step 2: Determination	Tests: 6 Control: 1	Tests: 100 (25 x 4 bottles or tubes) + Control: 50 (25 x 2 bottles/tubes), except for pyriproxyfen[a], which also required 100 mosquitoes in control (25 x 4 bottles)	3 (3 different batches of mosquitoes)	Test: 1800 + Control: 150 (300 for pyriproxyfen)	Determine LC_{99} (with 95% CI) and LC_{100} (observed) For pyriproxyfen[b] determine OI_{99} with (95% CI) and OI_{100} (observed)
Step 3: Validation	Tests: 1 Control: 1	Tests: 100 (25 x 4 bottles or tubes) + Control: 50 (25 x 2 bottles/tubes), except for pyriproxyfen[a], which also required 100 mosquitoes in control (25 x 4 bottles)	1	Tests: 100 Control: 50 (for pyriproxyfen, also 100 mosquitoes in control[a])	Select a TDC that kills (or sterilizes) 100% of susceptible mosquitoes

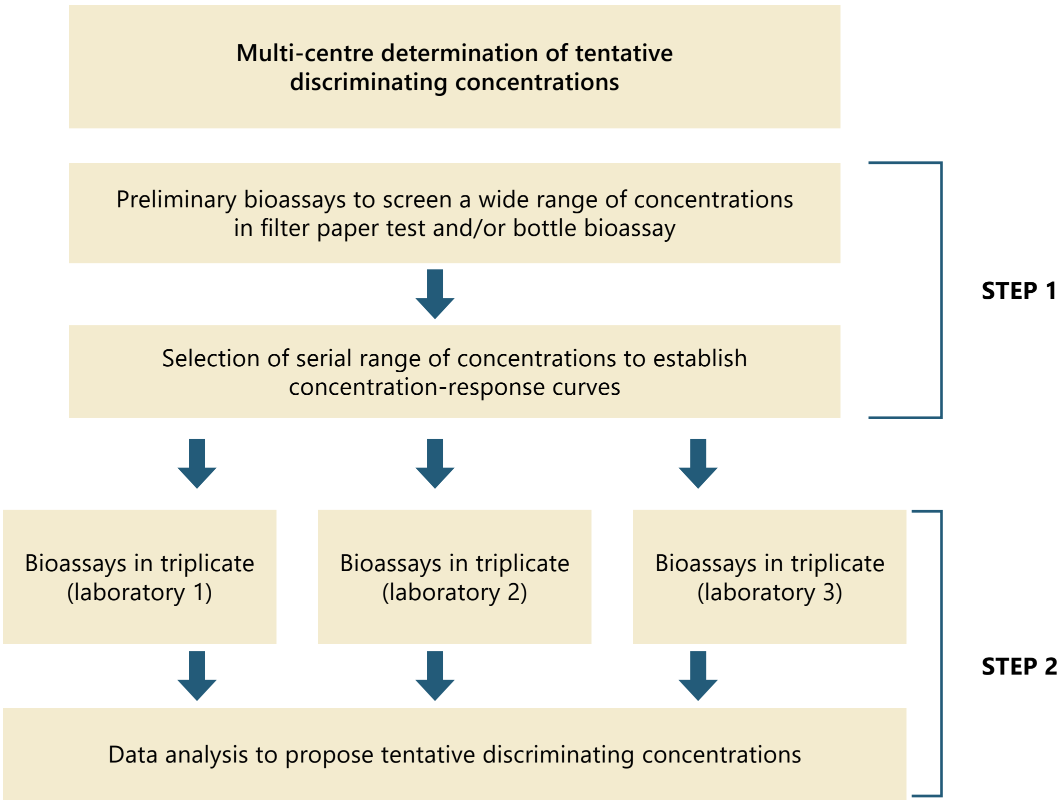
OI: oviposition inhibition.

[a] For specific test requirements for assessing the sterilizing properties of pyriproxyfen in bottle bioassay, refer to the relevant SOP *(14)*.

Step 1. Preliminary bottle and filter paper bioassays for screening serial concentrations of insecticides to obtain 0–100% mortality of mosquitoes

The participating laboratories conducted initial exploratory bioassays to select a broad range of serial concentrations for each compound and mosquito species (Fig. 3). About 10–12 serial concentrations were required to provide a range of responses (from 0 to 100% mortality) for each mosquito species and compound. Well-characterized susceptible mosquitoes were exposed to serial concentrations of each insecticide for 1 h, and appropriate susceptibility end-points were recorded, e.g. mortality at 24 h (or up to 72 h, if relevant, for slow-acting compounds) after 1 h of exposure. During step 1, 50 mosquitoes were tested per concentration with only one replicate (see details in Table 9). Each laboratory impregnated the filter papers and/or coated glass bottles with serial concentrations of the allocated test compounds according to the WHO SOP. Technical assistance was given in the preparation of stock solutions, impregnation of papers, coating of bottles and conducting bioassays by IRD and other collaborating laboratories when requested.

Fig. 3. Scheme for multi-centre determination of tentative discriminating concentrations in steps 1 and 2



Step 2. Bottle and filter paper bioassays to establish LC_{99} (or OI_{99}) and LC_{100} (or OI_{100}) and propose tentative discriminating concentrations

In this step, laboratories were asked to treat bottles and/or impregnate filter papers with a range of serial concentrations selected by IRD and WHO according to the results of testing in step 1. Bioassays were conducted with well-characterized susceptible mosquito species to estimate lethal concentrations (LC_{100} and LC_{99} with 95% CI) in a log-probit statistical model. At least six test concentrations with 100 mosquitoes each and control with 50 mosquitoes each were generally tested in step 2 to generate concentration–response curves; goodness of fit and mortality in controls were also reported. In order for a step-2 test to be considered valid, at least two concentrations that had to kill < 50% of mosquitoes, one concentration that killed about 50%, two concentrations that killed > 50% of mosquitoes and one concentration that killed about 100% mosquitoes were chosen. Each bioassay was performed three times for a given species and mosquito strain and different batches of mosquitoes on different days (Table 9). Impregnated papers were used no more than three times.

In testing of pyriproxyfen, only blood-fed female mosquitoes were exposed for 1 h to the insecticide. They were kept under observation in the laboratory for up to 11 days[1] to record the number of female mosquitoes that laid eggs, the number of eggs laid and the number of offspring emerging as compared with the unexposed (control) mosquito batches. Bioassays were conducted with well-characterized susceptible mosquito strains to estimate concentrations that inhibited 100% (OI_{100}) and 99% (OI_{99}) with 95% CI of the oviposition in a log-probit statistical model. In this case, the TDC proposed was the lowest concentration that induced full inhibition of egg-laying.

For the bioassays with PBO, three laboratories participated in determining the optimum percentage concentration that synergized the effects of pyrethroids against susceptible *Aedes* spp. mosquitoes after a 1-h exposure to filter papers impregnated with serial concentrations of PBO and a 24-h observation time.

6.3 Multi-centre validation of tentative discriminating concentrations against various mosquito species (step 3)

In this final technical step of the study (Fig. 4), TDCs were agreed in WHO consultations on the results of step 2 testing. The consultations recommended that, whenever possible, a **single TDC** be adopted for a given compound and mosquito species tested in order to minimize the number of unique discriminating concentrations for production of impregnated papers at the Universiti Sains Malaysia for routine monitoring of resistance

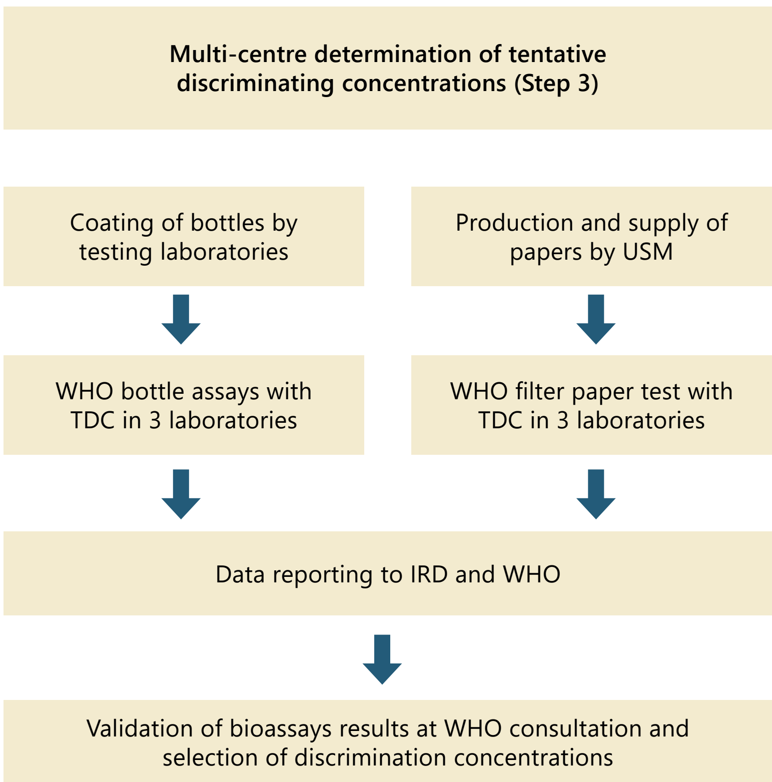
1 Initially, the test lasted up to 11 days to measure egg-laying and hatching (larvae); however, according to the advice of a WHO consultation, the time was reduced to 7 days: 3 days of holding to record mortality and an additional 96 h to record oviposition and emergence of offspring.

by national disease control programmes. A decision to choose a single DC for all species when the differences in the TDCs were narrow (about twofold), was made by consensus during the consultation, acknowledging the risk of either under- or over-estimating resistance in a given species.

WHO provided a list of the TDCs for individual insecticides and species (or group of species if relevant) to the participating centres. Impregnated papers at tentative DCs were prepared by the Universiti Sains Malaysia and supplied directly to the relevant participating laboratories for testing in step 3. Bottles were coated with the TDC for WHO bottle bioassays directly at the testing laboratories.

The TDC of a given insecticide was tested by at least three laboratories against well-characterized susceptible strains in either filter paper or bottle bioassays, with recording of appropriate end-points (e.g. mortality, oviposition inhibition). Bioassays comprised one test with four replicates of a given compound and mosquito species, with at least 100 mosquitoes tested per TDC (see Table 9). The data were reported to WHO for final recommendations.

Fig. 4. Framework for multi-centre validation of tentative discriminating concentrations (TDCs) in step 3



USM, Universiti Sains Malaysia

6.4 Data analysis and reporting

6.4.1 Data analysis, validation and interpretation

The participating laboratories were responsible for collecting, checking quality, collating and reporting data on an Excel® template developed by IRD. Test reports were sent regularly to IRD, where the data were analysed and validated, and real-time feedback was given to the laboratories. The concentration–response data were analysed with SPSS software (v 25). The concentration–response parameters were used to estimate the goodness of fit (with the *P* value) and lethal concentrations (i.e. LC_{100} and LC_{99} with 95% CIs) for each insecticide and species.

The main criteria for validating bioassays results were as follows:

- mortality of controls below the cut-off point of 20% (mortality between 5% and 20%, was corrected with Abbott's formula);
- a minimum of six concentrations to generate concentration–response curves and estimate lethal concentrations;
- goodness of fit ($P > 0.05$); and
- the lowest concentration that killed 100% of mosquitoes (LC_{100}), when available.

After validation of bioassay results by IRD, the concentration–response data were used to propose TDCs for each insecticide and mosquito species, with the following considerations.

- For each testing laboratory, data from three replicates were analysed separately to estimate intra-laboratory variation; when no major difference was found between replicates, the data were pooled to estimate lethal concentrations for the laboratory.
- Datasets from three laboratories were then used, when possible, to cross-validate the results and estimate a TDC.
- The TDC was defined as twice the estimated LC_{99} for a susceptible mosquito strain; when differences in LC_{99} were reported between laboratories, the highest value was selected. When the LC_{99} could not be accurately estimated (e.g. due to a lack of data points or replicates), the LC_{100} was used to establish a TDC.

As all tests were completed and full data sets from steps 1–3 were submitted to IRD for all species and insecticides, a simple performance monitoring score was used to rank the strength of the evidence for establishing TDCs (Table 10) to guide decisions on final DCs in WHO consultations.

Table 10. Scoring system used to rank the strength of the evidence for establishing tentative discriminating concentrations (TDCs)

Criterion	Strength of the evidence
No TDC established because of an insufficient number of validated tests and/or conflicting results between laboratories	No evidence
TDC based on step-1 data validated by at least two laboratories	Weak
TDC based on step-2 data validated by one laboratory	Moderately strong
TDC based on step-2 data validated by two laboratories	Strong
TDC based on step-2 data validated by three laboratories	Very strong

All raw data and identification cards containing key information for each insecticide and species were prepared and archived to facilitate review and traceability of the results.

6.4.2 Data reporting and monitoring of progress

Throughout the study, the participating institutions (including IRD) sent interim reports of bioassays results to WHO headquarters for monitoring of progress. A monitoring system based on the completion rates was established to guide further action (Table 11). Completion rates were calculated as follows:

$$\text{Completion rate (\%)} = \frac{\text{Number of validated tests}}{\text{Total number of test to be performed}} \times 100$$

The rates were estimated for each laboratory, testing step, insecticide and mosquito species and shared with WHO to track the progress of the study, take any corrective measures, identify and resolve any technical difficulties and propose solutions.

Table 11. Classification used to monitor progress and guide further action

Test completion rate (%)	Progress	Actions	Communication frequency
0–35	Low	Strong follow-up, communication by e-mail, phone calls, video calls	Every 2–3 weeks or on WHO request
36–75	Moderate	Strengthen follow-up with regular exchange of e-mails, phone calls, video calls	Every 1–2 months or on WHO request
76–100	Good	Normal follow-up and exchange by e-mail and video calls	About every 3 months or on WHO request

7 Results

The raw data on all the tested compounds with relevant details (e.g. test conditions, concentration–response statistics, sample size) from all three steps of the study are archived at IRD.

7.1 WHO tube tests

7.1.1 Test completion rates

In all, 214 774 mosquitoes belonging to the *Aedes* and *Anopheles* genera were tested in WHO filter paper bioassays (Fig. 5). The sample size was large enough to provide high confidence in the results.

Overall, the completion rates (CRs) were 94%, 87% and 98% in steps 1, 2 and 3, respectively (Table 12). CRs of 100% (all steps) were achieved with deltamethrin, lambda-cyhalothrin, permethrin, bendiocarb and chlorpyrifos-ethyl. The lower rates for alpha-cypermethrin and pirimiphos-methyl were due to the large number of species, especially *Anopheles* spp., to be tested (n = 7) and the lower-than-expected number of bioassays conducted on *An. funestus* and *An. minimus* (Fig. 6).

Fig. 5. Total numbers of *Aedes* and *Anopheles* spp. used for testing Group A insecticides in WHO tube tests

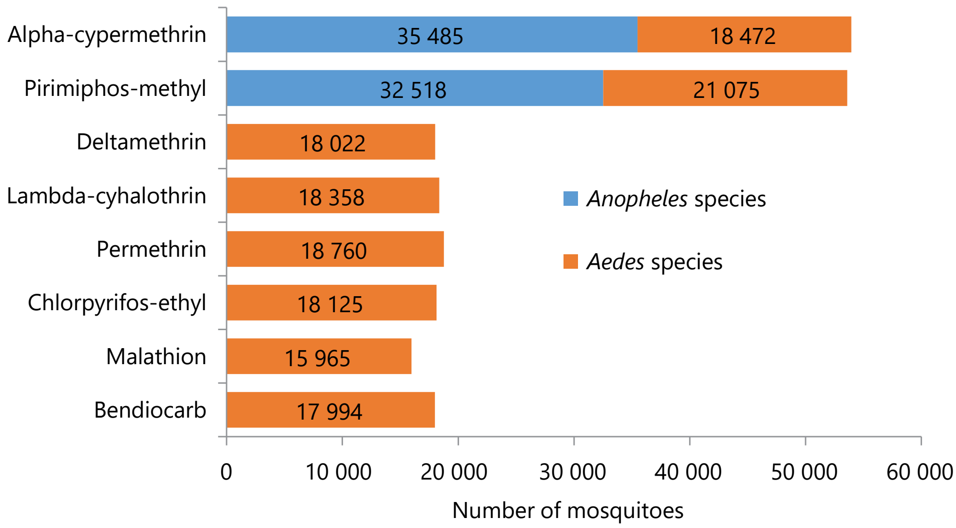
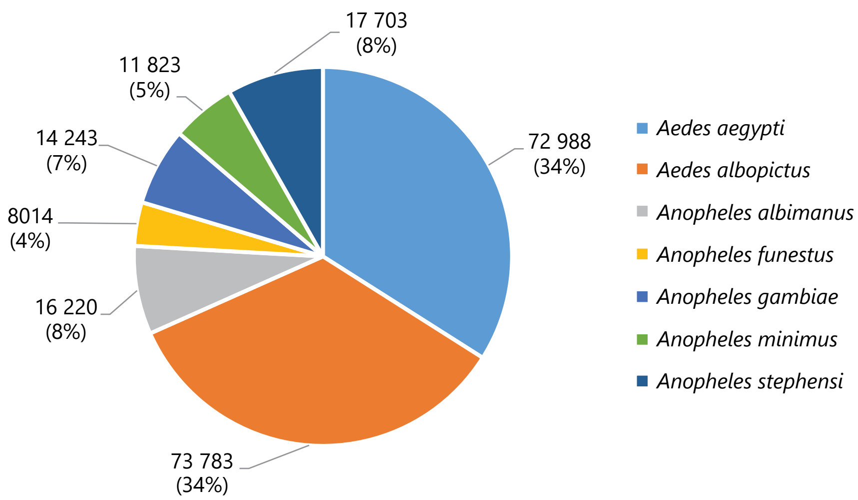


Fig. 6. Total numbers and percentages of mosquito species tested in WHO tube tests



7.1.2 Concentration–response curves and estimated LC_{99} and LC_{100}

The concentration–response curves (step 2), summary tables of LC_{99} and LC_{100} and TDCs for each insecticide and species are discussed below.

Alpha-cypermethrin

Fig. 7 shows the concentration–response curves of alpha-cypermethrin against various *Aedes* and *Anopheles* species.

Reliable, consistent data were provided by the participating laboratories for both *Ae. aegypti* and *Ae. albopictus*. The highest LC_{99} values were 0.019% (95% CI, 0.008 ; 1.847) and 0.038% (0.027 ; 0.075) against *Ae. aegypti* and *Ae. albopictus*, respectively (Table 13). In view of the strength of the evidence (triplicate tests in all centres), the TDCs selected to undergo step-3 testing against *Ae. aegypti* and *Ae. albopictus* were 0.04% and 0.08%, respectively.

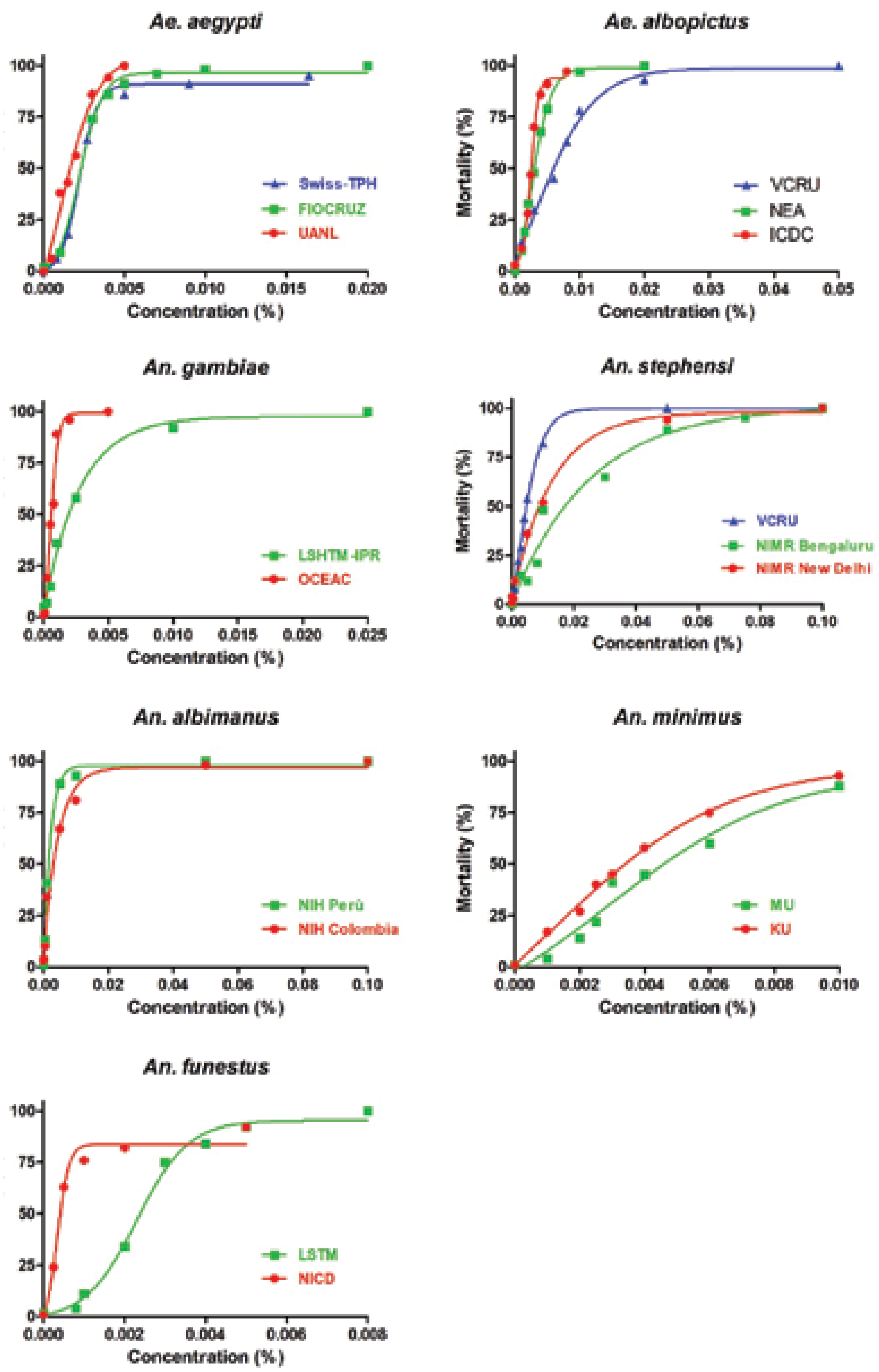
Greater variation in test results was reported for *Anopheles* species (Table 14). Three independent datasets were obtained only for *An. stephensi* (very high evidence), while two datasets were obtained for *An. gambiae, An. minimus, An. funestus* and *An. albimanus* (high evidence). For *An. gambiae*, a significant difference in LC_{99} was detected between OCEAC (0.003% [0.002–0.010]) and LSHTM-IPR (0.026% [0.015; 0.076]) despite the fact that they used the same strain (Kisumu) and that no discrepancies in test conditions (e.g. temperature, age of females) were reported. No LC_{100} was reached for *An. minimus*, but the LC_{99} was 0.027% (0.017; 0.081). The LC_{100} and the LC_{99} for *An. funestus* were both 0.008%. The LC_{99} for *An. stephensi* (0.15% [0.08; 0.81]) and *An. albimanus* (0.072% [0.037; 0.212]) was much higher than that for other *Anopheles* species tested, suggesting higher tolerance of these species to the toxic effects of alpha-cypermethrin..

Table 12. Completion rates (CRs) of WHO tube tests in steps 1–3 for the insecticides tested

Step		Pyrethroids				Organophosphates			Carbamate	Total
		Alpha-cypermethrin	Deltamethrin	Lambda-cyhalothrin	Permethrin	Malathion	Pirimiphos-methyl	Chlorpyrifos-ethyl	Bendiocarb	
	Targeted species	*Ae. aegypti* *Ae. albopictus* *An. albimanus* *An. funestus* *An. gambiae* *An. minimus* *An. stephensi*	*Ae. aegypti* *Ae. albopictus*	*Ae. aegypti* *Ae. albopictus*	*Ae. aegypti* *Ae. albopictus*	*Ae. aegypti* *Ae. albopictus*	*Ae. aegypti* *Ae. albopictus* *An. albimanus* *An. funestus* *An. gambiae* *An. minimus* *An. stephensi*	*Ae. aegypti* *Ae. albopictus*	*Ae. aegypti* *Ae. albopictus*	
	No. of species	7	2	2	2	2	7	2	2	
Step 1	No. of tests performed	21	6	6	6	6	21	6	6	78
	No. of validated tests	19	6	6	6	6	18	6	6	73
	CR (%)	90	100	100	100	100	86	100	100	94%
Step 2	No. of tests performed	63	18	18	18	19	64	18	18	236
	No. of validated tests	51	18	18	18	16	48	18	18	205
	CR (%)	81	100	100	100	84	75	100	100	87%
Step 3	No. of tests performed	24	8	8	8	8	23	12	8	99
	No. of validated tests	24	8	8	8	7	22	12	8	97
	CR (%)	100	100	100	100	88	96	100	100	97%

CR < 35% (little progress); 35–75% (moderate progress); > 75% (good progress).

Fig. 7. Concentration–response curves of alpha-cypermethrin against various *Aedes* and *Anopheles* species in step 2. Each curve represents data pooled from three replicates.



According to the results, the TDCs selected for step-3 validation were:

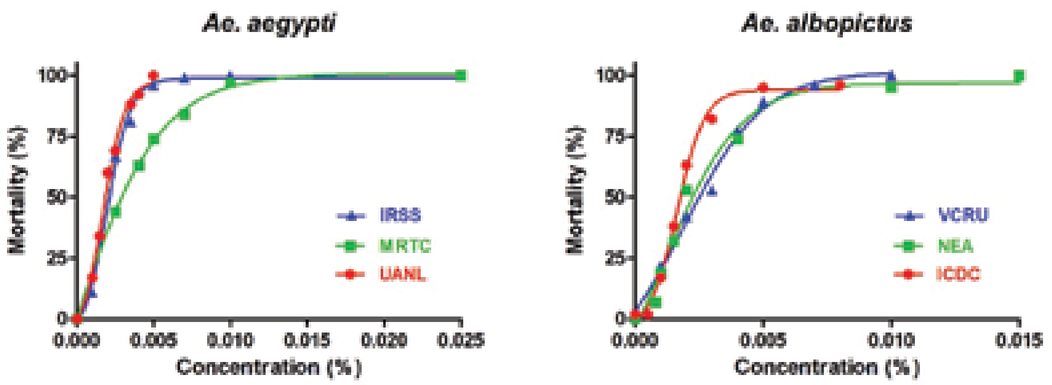
- 0.05% for both *An. gambiae* and *An. minimus*;
- 0.02% for *An. funestus*; and
- 0.3% for both *An. stephensi* and *An. albimanus*

Deltamethrin

Fig. 8 shows the concentration–response curves of deltamethrin against Ae. aegypti and Ae. albopictus in step 2. For both species, reliable, consistent data were found between and within the participating laboratories. In three validated tests, the highest LC_{99} was 0.015% for both species (Table 15).

In view of the very strong evidence, the TDC selected for step-3 evaluation was 0.03% for both *Ae. aegypti* and *Ae. albopictus*.

Fig. 8. Concentration–response curves of deltamethrin against *Ae. aegypti* (left) and *Ae. albopictus* (right) in step 2. Each curve represents data pooled from three replicates.

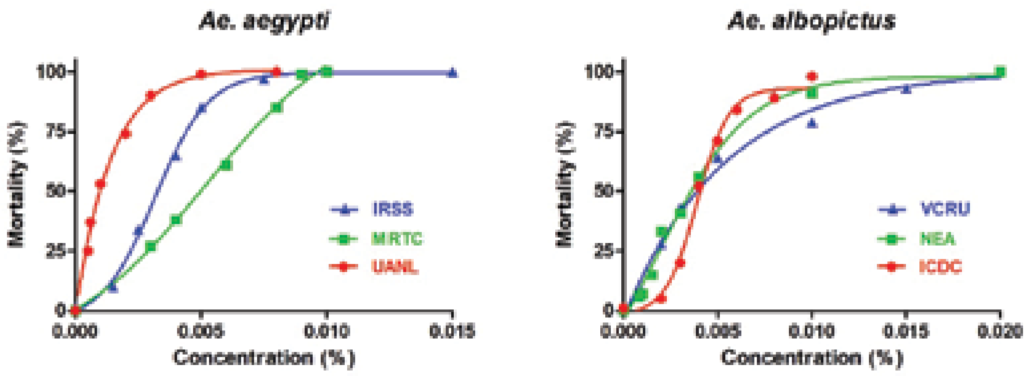


Lambda-cyhalothrin

Fig. 9 shows the concentration–response curves of lambda-cyhalothrin against Ae. aegypti and Ae. albopictus in step 2. Consistent test results were obtained from participating centres for both species. In three validated tests, the highest LC_{99} was 0.010% (0.009 ; 0.243) for *Ae. aegypti* and 0.033% (0.02 ; 0.097) for *Ae. albopictus* (Table 16).

In view of the very strong evidence, the TDCs selected for step-3 evaluation were 0.02% and 0.07% for *Ae. aegypti* and *Ae. albopictus*, respectively.

Fig. 9. Concentration–response curves of lambda-cyhalothrin against *Ae. aegypti* (left) and *Ae. albopictus* (right) in step 2. Each curve represents data pooled from three replicates.

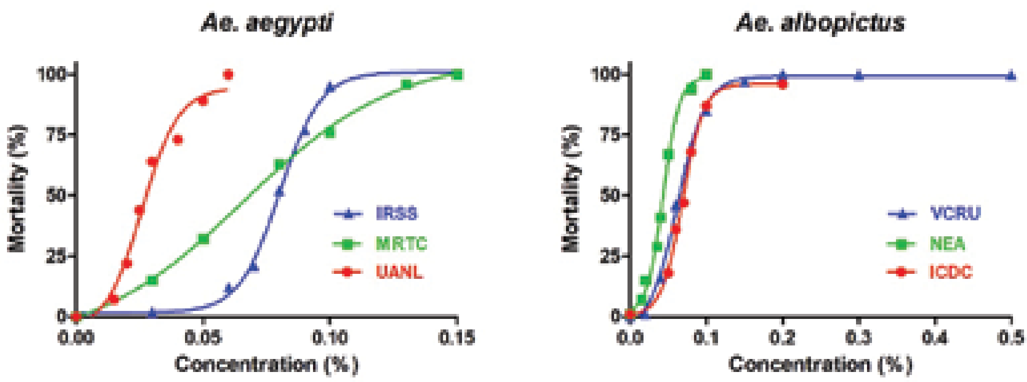


Permethrin

Fig. 10 shows the concentration–response curves of permethrin (cis:trans isomer ratio 40:60) against Ae. aegypti and Ae. albopictus in step 2. With *Ae. aegypti*, the LC_{99} ranged from 0.075% (0.059 ; 0.143) at UANL to 0.17% (0.13 ; 0.47) at MRTC (Table 17). Data were more consistent for *Ae. albopictus*, the LC_{99} ranging from 0.10% at NEA to 0.20% at ICDC (Table 17).

According to the highest LC_{99} values obtained by three centres, the TDC selected for step-3 evaluation was 0.4% for both *Ae. aegypti* and *Ae. albopictus*.

Fig. 10. Concentration–response curves of permethrin 40:60 against *Ae. aegypti* (left) and *Ae. albopictus* (right) in step 2. Each curve represents data pooled from three replicates.

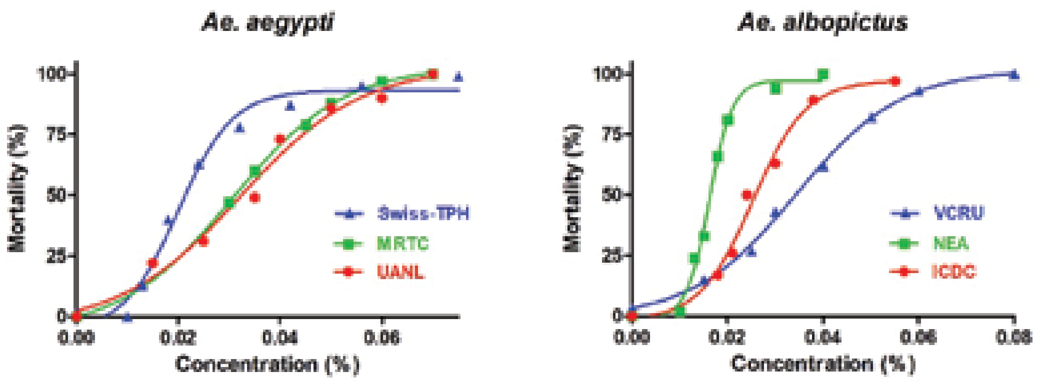


Bendiocarb

Fig. 11 shows the concentration–response curves of bendiocarb against Ae. aegypti and Ae. albopictus in step 2. The data obtained for *Ae. aegypti* were consistent among the centres, the LC_{99} ranging from 0.068% (0.061 ; 0.095) at MRTC to 0.08% (0.066 ; 0.128) at UANL (Table 18). With *Ae. albopictus*, a twofold difference was reported between testing laboratories, the LC_{99} ranging from 0.033% (0.027 ; 0.051) at NEA to 0.083% (0.073 ; 0.101) at VCRU.

According to the highest LC_{99} obtained by three centres, the TDC selected for step 3 evaluation was 0.2% for both *Ae. aegypti* and *Ae. albopictus*.

Fig. 11. Concentration–response curves of bendiocarb against *Ae. aegypti* (left) and *Ae. albopictus* (right) in step 2. Each curve represents data pooled from three replicates.

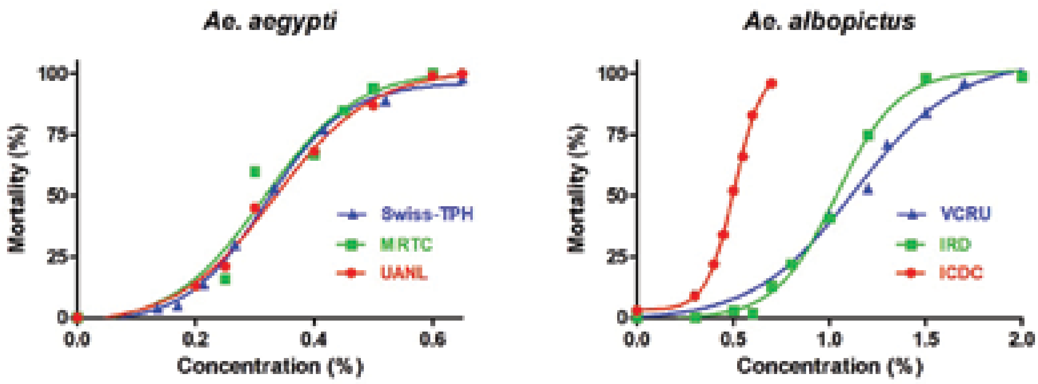


Malathion

Fig. 12 shows the concentration–response curves of malathion against Ae. aegypti and Ae. albopictus in step 2. The data for *Ae. aegypti* were highly reproducible among testing centres, the LC_{99} ranging from 0.66 at MRTC to 0.75% (0.70 ; 0.81) at Swiss TPH (Table 19). With *Ae. albopictus*, significant differences in LC_{99} were reported between ICDC (0.8% [0.78 ; 0.86]) and the two other centres, with LC_{99} 1.9 [1.6 ; 2.3] at IRD and 2% [1.8 ; 2.5] at VCRU. LC_{100} was reported by only one centre (VCRU), at 2%. As a very high LC_{99} (up to 10%) was reported for a laboratory strain of *Ae. aegypti* colonized at NEA (Singapore), it was decided to exclude these data sets from the analysis.

The TDCs selected for step-3 evaluation were 1.5% for *Ae. aegypti* and 4% for *Ae. albopictus*.

Fig. 12. Concentration–response curves of malathion against *Ae. aegypti* (left) and *Ae. albopictus* (right) in step 2. Each curve represents data pooled from three replicates.

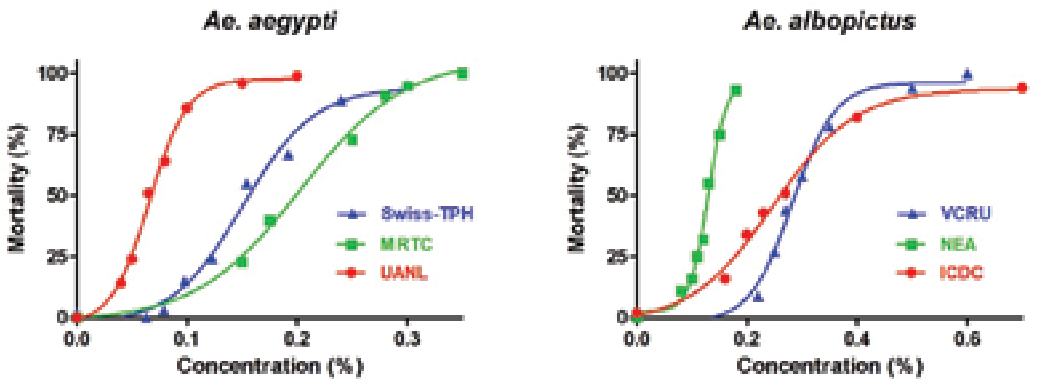


Chlorpyrifos-ethyl

Fig. 13 shows the concentration–mortality curves of chlorpyrifos-ethyl against Ae. aegypti and Ae. albopictus in step 2. Relatively wide variation in the test results (two to five times) was reported with both species. With *Ae. aegypti,* the LC_{99} ranged from 0.18% (0.17 ; 0.20) at UANL to 0.40% (0.34 ; 0.47) at Swiss TPH, and the LC_{100} reported by one centre (VCRU) was 0.35% (Table 20). With *Ae. albopictus*, the LC_{99} ranged from 0.22% (0.20 ; 0.23) at NEA to 1% (0.72 ; 1.8) at ICDC. LC_{100} was reported by only one centre (VCRU) and was 0.6%.

In view of the wide variation, the TDCs selected for step-3 evaluation were 0.8% for *Ae. aegypti* and 1% and 2% for *Ae. albopictus.*

Fig. 13. Concentration–response curves for chlorpyrifos-ethyl against *Ae. aegypti* (left) and *Ae. albopictus* (right) in step 2. Each curve represents data pooled from three replicates.



Pirimiphos-methyl

Fig. 14 shows the concentration–response curves of pirimiphos-methyl against Aedes and Anopheles species. With *Ae. aegypti*, the LC_{99} ranged from 9 mg/m^2 (95% CI, 8.0; 9.8) at UANL to 31 mg/m^2 (19 ; 54) at IRSS (Table 21). The LC_{100} was 40 mg/m^2. Once again, a significant difference in test results was observed between UANL and the two other centres. The data were more consistent for *Ae. albopictus*, the LD_{99} ranging from 18 (15; 24) at ICDC to 23 mg/m^2 at both VCRU and FIOCRUZ (Table 21). The LC_{100} was 25 mg/m^2.

According to the highest LC_{99} obtained in the three laboratories, the TDC selected for step 3 was 60 mg/m^2 for both *Ae. aegypti* and *Ae. albopictus*.

Variation among testing laboratories was also reported with *Anopheles* spp., especially *An. funestus* and *An. minimus* (Table 22, Fig. 14). Three independent datasets were reported only for *An. stephensi* (very strong evidence), while two independent datasets were obtained for *An. gambiae, An. minimus* and *An. albimanus* (strong evidence). With *An. gambiae*, the LC_{99} ranged from 77 to 84 mg/m^2 (95% CI, 69 ; 160), with an LC_{100} of 70 mg/m^2. For *An. stephensi* and *An. albimanus,* the LC_{99} ranged from 29 to 46 mg/m^2, with an $LC_{100\ max}$[1] of 45 mg/m^2. For *An. minimus*, a twofold difference in test results was observed between participating laboratories, LC_{99} ranging from 20 (17 ; 24) at KU to 52 mg/m^2 (43 ; 68) at MU. Much less information was available for *An. funestus* (moderate evidence). Step-2 data reported by LSTM showed some variation among the test replicates (Fig. 14). The overall LC_{99} and $LC_{100\ max}$ were 20 and 28 mg/m^2, respectively.

The TDCs selected for step-3 evaluation were 170 mg/m^2 for *An. gambiae*, 100 mg/m^2 for *An. minimus* and 90 mg/m^2 for *An. stephensi*, *An. albimanus* and *An. funestus.*

1 $LC_{100\ max}$ is the highest LC_{100} obtained at different laboratories for the same insecticide–species combination, e.g. if LC_{100} is found to be 40, 43 and 45 mg/m^2 in three different laboratories, the $LC_{100\ max}$ will be 45.

Fig. 14. Concentration–response curves of pirimiphos-methyl against various *Aedes* and *Anopheles* species in step 2. Each curve represents data pooled from three replicates, except for *An. funestus* at LSTM.

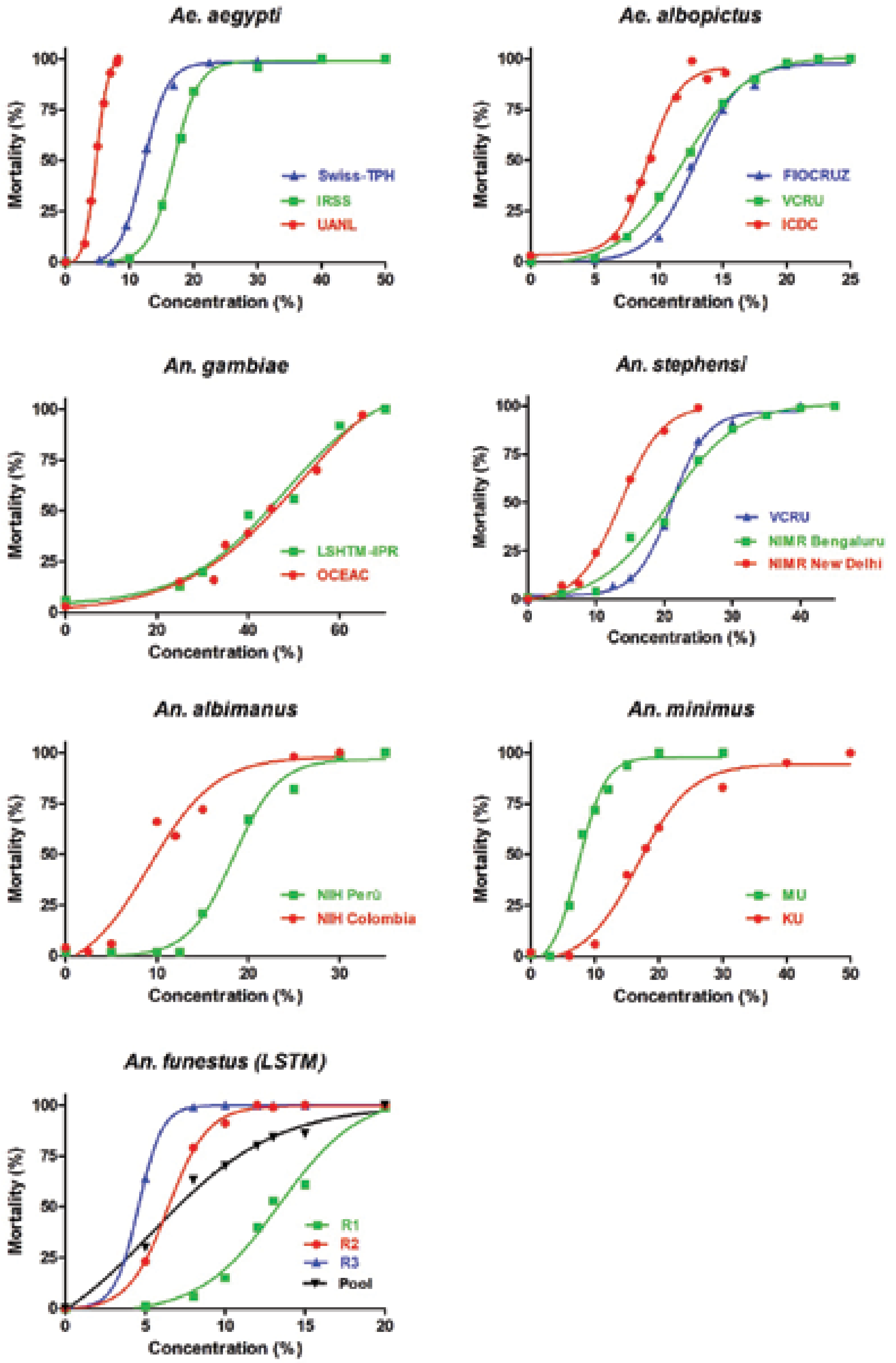


Table 13. LC_{100}, LC_{99} (and 95% CI) and tentative discriminating concentrations of alpha-cypermethrin against *Aedes* species from results in step 2 of the study

Species	Centre (country)	Sample size (no. of test concentrations)	Observed LC_{100} (%)	Twice the LC_{100} (%)	Estimated LC_{99} (%) (95% CI)	Twice the LC_{99} (%)	TDC selected for step 3	Strength of evidence
Ae. aegypti	UANL (Mexico)	2250 (7)	0.005	0.01	0.007 (0.004–0.029)	0.014	**0.04%**	Very strong
	FIOCRUZ (Brazil)	2219 (7)	0.02	0.04	0.009 (0.008–0.012)	0.018		
	Swiss TPH (Switzerland)	2073 (6)	NA	NA	0.019 (0.008–1.847)	0.038		
Ae. albopictus	ICDC (China)	2223 (7)	NA	NA	0.008 (0.006–0.017)	0.016	**0.08%**	Very strong
	NEA (Singapore)	2546 (8)	0.02	0.04	0.013 (0.012–0.016)	0.026		
	VCRU (Malaysia)	2206 (7)	0.05	0.10	0.038 (0.027–0.075)	0.076		

The highest LC_{99} used to establish the TDCs for a given species are shown in blue; the TDCs selected to undergo step 3 (validation) are given in **bold**.

NA: not available.

Table 14. LC_{100}, LC_{99} (and 95% CI) and tentative discriminating concentrations of alpha-cypermethrin selected against *Anopheles* species from results in step 2 of the study

Species	Centre (country)	Sample size (no. of concentrations)	Observed LC_{100} (%)	Twice the LC_{100} (%)	Estimated LC_{99} (%) (95% CI)	Twice the LC_{99} (%)	TDC selected for step 3	Strength of evidence
An. gambiae	OCEAC (Cameroon)	2168 (7)	0.005	0.01	0.003 (0.002–0.010)	0.006	**0.05%**	High
	LSHTM-CREC (Benin)[a]	647 (7)	0.005	0.01	NA	NA		
	LSHTM-IPR (Côte d'Ivoire)	1994 (6)	0.025	0.05	0.026 (0.015–0.076)	0.052		
An. minimus	KU (Thailand)	2395 (7)	NA	NA	0.020 (0.017–0.025)	0.04	**0.05%**	High
	MU (Thailand)	2392 (7)	NA	NA	0.027 (0.017–0.081)	0.055		
An. funestus	LSTM (UK)	1521 (7)	0.008	0.02	0.008 (0.006–0.013)	0.016	**0.02%**	High
	NICD (Soth Africa)[a]	1717 (8)	0.008	0.02	NA	NA		
An. stephensi	NIMR-D (India)	1814 (7)	0.1	0.2	0.13 (0.07–0.54)	0.26	**0.30%**	Very High
	NIMR-B (India)	2329 (8)	0.1	0.2	0.15 (0.08–0.81)	0.3		
	VCRC (India)	2850 (9)	0.05	0.1	0.034 (0.027–0.044)	0.07		
An. albimanus	NIH (Colombia)	2365 (7)	0.1	0.2	0.072 (0.037–0.212)	0.15	**0.30%**	High
	NIH (Peru)	2304 (7)	0.1	0.2	0.02 (0.01-0.05)	0.04		

The highest LC_{99} used to establish the TDCs for a given species are shown in blue; the TDCs selected to undergo step 3 (validation) are given in **bold**.

NA: not available.

[a] Only LC_{100} available.

Table 15. LC_{100}, LC_{99} (and 95% CI) and tentative discriminating concentrations of deltamethrin selected against *Aedes* species from results in step 2 of the study

Species	Centre (country)	Sample size (no. of concentrations)	Observed LC_{100} (%)	Twice the LC_{100} (%)	Estimated LC_{99} (%) (95% CI)	Twice the LC_{99} (%)	TDC selected for step 3	Strength of evidence
Ae. aegypti	UANL (Mexico)	2250 (7)	0.005	0.01	0.006 (0.005–0.009)	0.012	0.03%	Very strong
	MRTC (Mali)	1950 (6)	0.025	0.05	0.015 (0.013–0.019)	0.030		
	IRSS (Burkina Faso)	2304 (7)	0.01	0.02	0.007 (0.006–0.008)	0.015		
Ae. albopictus	ICDC (China)	2219 (7)	NA	NA	0.008 (0.006–0.016)	0.016	0.03%	Very strong
	NEA (Singapore)	2267 (7)	0.015	0.03	0.015 (0.010–0.032)	0.030		
	VCRU (Malaysia)	2230 (7)	0.01	0.02	0.009 (0.008–0.014)	0.018		

The highest LC_{99} used to establish the TDCs for a given species are shown in blue; the TDCs selected to undergo step 3 (validation) are given in **bold**.

NA: not available.

Table 16. LC_{100}, LC_{99} (and 95% CI) and tentative discriminating concentrations of lambda-cyhalothrin for selected against *Aedes* species from results in step 2 of the study

Species	Centre (country)	Sample size (no. of concentrations)	Observed LC_{100} (%)	Twice the LC_{100} (%)	Estimated LC_{99} (%) (95% CI)	Twice the LC_{99} (%)	TDC selected for step 3	Strength of evidence
Ae. aegypti	UANL (Mexico)	2250 (7)	0.008	0.016	0.007 (0.005–0.015)	0.014	**0.02%**	Very strong
	MRTC (Mali)	1950 (6)	0.01	0.020	0.010 (0.009–0.243)	0.020		
	IRSS (Burkina Faso)	2356 (7)	0.01	0.020	0.009 (0.008–0.010)	0.018		
Ae. albopictus	ICDC (China)	2361 (7)	NA	NA	0.012 (0.009–0.017)	0.024	**0.07%**	Very strong
	NEA (Singapore)	2518 (8)	0.02	0.04	0.020 (0.015–0.035)	0.040		
	VCRU (Malaysia)	2241 (7)	0.02	0.04	0.033 (0.020–0.097)	0.066		

The highest LC_{99} used to establish the TDCs for a given species are shown in blue; the TDCs selected to undergo step 3 (validation) are given in **bold**.

NA: not available.

Table 17. LC_{100}, LC_{99} (and 95% CI) and tentative discriminating concentrations of permethrin (40:60) selected against *Aedes* species from results in step 2 of the study

Species	Centre (country)	Sample size (no. of concentrations)	Observed LC_{100} (%)	Twice the LC_{100} (%)	Estimated LC_{99} (%) (95% CI)	Twice the LC_{99} (%)	TDC selected for step 3	Strength of evidence
Ae. aegypti	UANL (Mexico)	2250 (7)	0.06	0.012	0.075 (0.059–0.143)	0.15	**0.4%**	Very strong
	MRTC (Mali)	1950 (6)	0.15	0.30	0.17 (0.13–0.47)	0.34		
	IRSS (Burkina Faso)	2312 (7)	0.15	0.30	0.12 (0.103–0.19)	0.24		
Ae. albopictus	ICDC (China)	1914 (6)	NA	NA	0.20 (0.12–7680743[a])	0.40	**0.4%**	Very strong
	NEA (Singapore)	2252 (7)	0.1	0.2	0.10 (0.08–0.14)	0.20		
	VCRU (Malaysia)	2850 (9)	0.2	0.4	0.18 (0.17–0.20)	0.36		

The highest LC_{99} used to establish the TDCs for a given species are shown in blue; the TDCs selected to undergo step 3 (validation) are given in **bold**.

NA: not available.

[a] Very high upper confidence limit due to poor goodness of the fit (high chi square value with low P value).

Table 18. LC_{100}, LC_{99} (and 95% CI) and tentative discriminating concentrations of bendiocarb selected against *Aedes* species from results in step 2 of the study

Species	Centre (country)	Sample size (no. of concentrations)	Observed LC_{100} (%)	Twice the LC_{100} (%)	Estimated LC_{99} (%) (95% CI)	Twice the LC_{99} (%)	TDC selected for step 3	Strength of evidence
Ae. aegypti	UANL (Mexico)	2250 (7)	0.07	0.14	0.080 (0.066–0.128)	0.16	**0.2%**	Very strong
	MRTC (Mali)	1950 (6)	0.065	0.13	0.068 (0.061–0.095)	0.14		
	Swiss TPH (Switzerland)	2279 (8)	NA	NA	0.078 (0.071–0.088)	0.16		
Ae. albopictus	ICDC (China)	1988 (6)	NA	NA	0.060 (0.048–0.127)	0.12	**0.2%**	Very strong
	NEA (Singapore)	2581 (7)	0.04	0.08	0.033 (0.027–0.051)	0.07		
	VCRU (Malaysia)	2231 (7)	0.08	0.16	0.083 (0.073–0.101)	0.17		

The highest LC_{99} used to establish the TDCs for a given species are shown in blue; the TDCs selected to undergo step 3 (validation) are given in **bold**.

NA: not available.

Table 19. LC_{100}, LC_{99} (and 95% CI) and tentative discriminating concentrations of malathion selected against *Aedes* species from results in step 2 of the study

Species	Centre (country)	Sample size (no. of concentrations)	Observed LC_{100} (%)	Twice the LC_{100} (%)	Estimated LC99 (%) (95% CI)	Twice the LC_{99} (%)	TDC selected for step 3	Strength of evidence
Ae. aegypti	UANL (Mexico)	2250 (7)	0.7	1.4	0.69 (0.595–0.91)	1.38	**1.5%**	Very strong
	MRTC (Mali)	1950 (6)	0.6	1.2	0.66 (NA)	1.32		
	Swiss TPH (Switzerland)	2276 (8)	NA	NA	0.75 (0.70–0.81)	1.50		
Ae. albopictus	ICDC (China)	1957 (7)	NA	NA	0.81 (0.78-0.86)	1.62	**4%**	Very strong
	VCRU (Malaysia)	2231 (7)	2	4	2 (1.8–2.5)	4		
	IRD (France)	1055 (9)	NA	NA	1.86 (1.62-2.34)	3.72		

The highest LC_{99} used to establish the TDCs for a given species are shown in blue; the TDCs selected to undergo step 3 (validation) are given in **bold**.

NA: not available.

Table 20. LC_{100}, LC_{99} (and 95% CI) and tentative discriminating concentrations of chlorpyrifos-ethyl selected against *Aedes* species from results in step 2 of the study

Species	Centre (country)	Sample size (no. of concentrations)	Observed LC_{100} (%)	Twice the LC_{100} (%)	Estimated LC_{99} (%) (95% CI)	Twice the LC_{99} (%)	TDC selected for step 3	Strength of evidence
Ae. aegypti	UANL (Mexico)	2250 (7)	NA	NA	0.18 (0.17–0.20)	0.36	**0.8%**	Very strong
	MRTC (Mali)	1950 (6)	0.35	0.7	0.35 (NA)	0.70		
	Swiss TPH (Switzerland)	2285 (8)	NA	NA	0.39 (0.34–0.47)	0.78		
Ae. albopictus	ICDC (China)	1943 (6)	NA	NA	0.96 (0.72–1.80)	1.9	**1%** and **2%**[a]	Very strong
	NEA (Singapore)	2157 (7)	NA	NA	0.22 (0.203–0.23)	0.44		
	VCRU (Malaysia)	2243 (7)	0.6	1.2	0.54 (0.45–0.80)	1.0		

The highest LC_{99} used to establish the TDCs for a given species are shown in blue; the TDCs selected to undergo step 3 (validation) are given in **bold**.

NA: not available.

[a] Two concentrations were selected for testing in step 3 due to high variation in step-2 data.

Table 21. LC_{100}, LC_{99} (and 95% CI) and tentative discriminating concentrations of pirimiphos-methyl selected against *Aedes* species from results in step 2 of the study

Species	Centre (country)	Sample size (no. of concentrations)	Observed LD100 (%)	Twice the LD_{100} (%)	Estimated LD_{99} (%) (95% CI)	Twice the LD_{99} (%)	TDC selected for step 3	Strength of evidence
Ae. aegypti	UANL (Mexico)	2250 (7)	8.2	16.4	9 (8.0–9.8)	18	**60 mg/m²**	Very strong
	IRSS (Burkina Faso)	2322 (7)	40	80	31 (25.5–57.5)	62		
	Swiss TPH (Switzerland)	2283 (8)	40	80	25 (19.03–54.4)	50		
Ae. albopictus	ICDC (China)	2308 (8)	NA	NA	18 (15.5–24.0)	36	**60 mg/m²**	Very strong
	FIOCRUZ (Brazil)	2222 (7)	25	50	23 (21.6–23.6)	47		
	VCRU (Malaysia)	950 (9)	22.5	45	23 (21.5–25.7)	47		

The highest LC_{99} used to establish the TDCs for a given species are shown in blue; the TDCs selected to undergo step 3 (validation) are given in **bold**.

NA: not available.

Table 22. LC_{100}, LC_{99} (and 95% CI) and tentative discriminating concentrations of pirimiphos-methyl selected against *Anopheles* species from results in step 2 of the study

Species	Centre (country)	Sample size (no. of concentrations)	Observed LC_{100} (%)	Twice the LC_{100} (%)	Estimated LC_{99} (%) (95% CI)	Twice the LC99 (%)	TDC selected for step 3	Strength of evidence
An. gambiae	OCEAC (Cameroon)	2127 (7)	NA	NA	84 (69.5-160)	169	**170 mg/m²**	Strong
	IPR (Côte d'Ivoire)	1996 (6)	70	140	77 (NA)	155		
An. stephensi	NIMR-D (India)	1694 (6)	NA	NA	29 (26.6-31.01)	57	**90 mg/m²**	Very strong
	NIMR-B (India)	2196 (9)	45	90	46 (39.3-61.69	93		
	VCRC (India)	2250 (7)	40	80	39 (33.8-49.8)	77		
An. albimanus	NIH (Colombia)	2617 (7)	30	60	30 (21.5-75.4)	60	**90 mg/m²**	Strong
	NIH (Peru)	3424 (8)	*35*	*70*	33 (29.3-39.1)	66		
An. minimus	KU (Thailand)	1929 (8)	50	100	52 (43.2-68.4)	100	**100 mg/m²**	Strong
	MU (Thailand)	2021 (8)	20	40	20 (17.2-24.3)	40		
An. funestus	LSTM (UK)	2127 (7)	20	40	28 (21.6-220)	60	**90 mg/m²**	Moderately strong

The highest LC_{99} used to establish the TDCs for a given species are shown in blue; the TDCs selected to undergo step 3 (validation) are given in **bold**.

NA: not available.

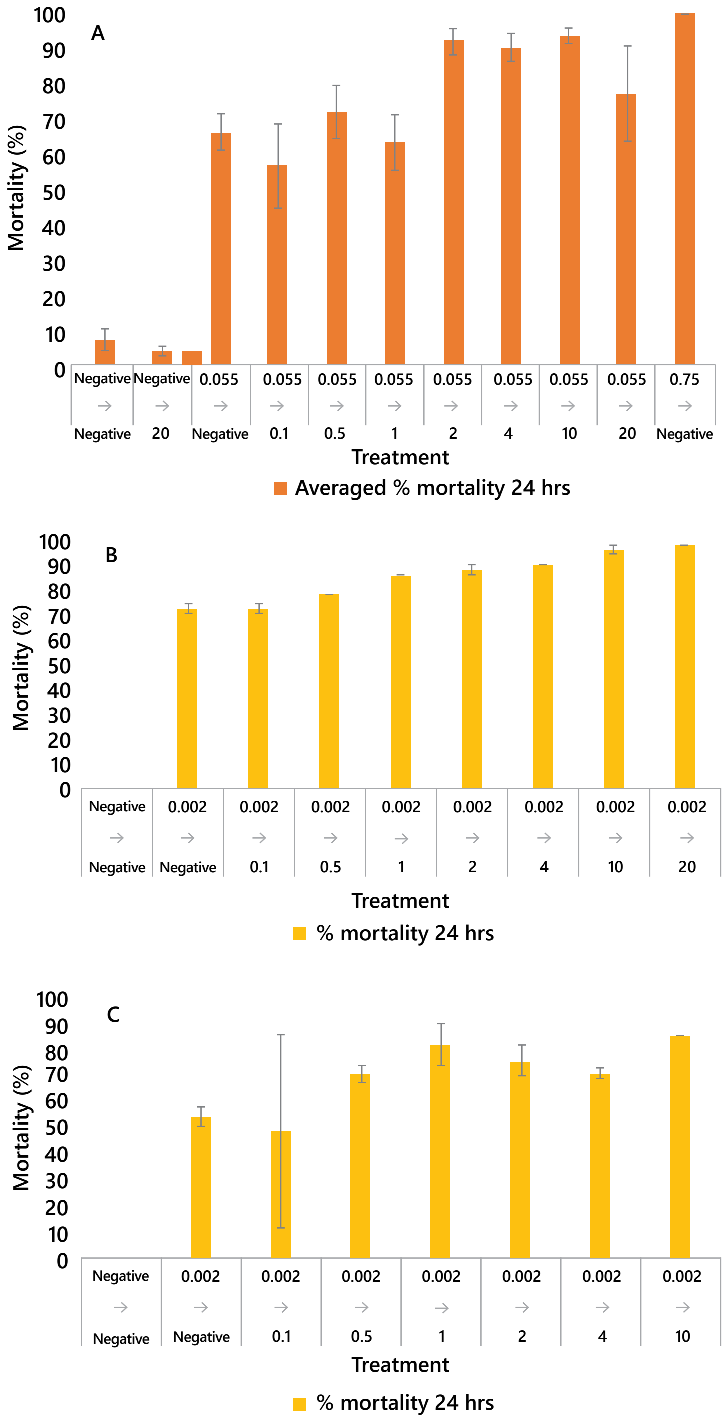
Piperonyl butoxide

The aim of testing the synergist PBO was not to determine a DC as for other compounds but to find the optimum percentage concentration to be used for synergist-insecticide bioassays against *Aedes* mosquitoes. It is commonly accepted that the optimum concentration of a synergist corresponds to the highest concentration that does not kill the targeted mosquito species (the sublethal concentration). Unexpectedly, preliminary test results showed that PBO alone did not kill susceptible *Aedes* strains (New Orleans and Rockefeller strains) at concentrations of 0.1–20%. Consequently, a new provisional method was developed to determine the optimum percentage concentration of PBO that synergizes the effects of pyrethroids against susceptible *Aedes* spp. mosquitoes *(14)*.

Briefly, the method consisted of exposing susceptible female mosquitoes for 1 h to PBO at a concentration of 0.1–20% before exposing them for 1 h to test papers impregnated at the LC_{50} of alpha-cypermethrin and to determine the first concentration at which mortality reached a plateau.

PBO increased the toxicity of alpha-cypermethrin from a concentration of 1% (Fig. 15), although a plateau of mortality rates was rapidly reached for concentrations from 2% to 20%. Only LSTM reported 100% mosquito mortality at the highest concentration of 20%; however, the mosquito colonies used in this study did not show any pyrethroid resistance genes, which may explain the outcome. The same protocol might also be used to test mixed function oxidase-based resistant strains to establish an optimum concentration of PBO for synergist-insecticide bioassays with *Aedes* species.

Fig. 15. Potentiation by PBO of the effect of alpha-cypermethrin (at LC_{50}) on susceptible strains of *Ae. aegypti* (A: LSTM, New Orleans; B: UANL, New Orleans; C: FIOCRUZ, Rockefeller)



The X-axis of each graph indicates the concentration of alpha-cypermethrin in the first row and the concentration of PBO in the second.

7.1.3 Validation of tentative discriminating concentrations in filter paper tests (step 3)

According to the results of step 2, 19 TDCs were selected for further testing in step 3 against seven mosquito species. In all, 96 bioassays (30 bioassays with *Anopheles* spp., 66 with *Aedes* spp.) with 9924 mosquitoes were conducted. At all the TDCs tested, mosquito mortality was ≥ 98%, the WHO susceptibility threshold (see tables 23 and 24 for *Aedes* and *Anopheles*, respectively).

Table 23. Mortality of *Aedes* spp. exposed to tentative discriminating concentrations in step-3 filter paper tests (1 h exposure; 24 h recording time)

Insecticide	Species	Centre	TDC	Mortality (%)	N
Permethrin	*Aedes aegypti*	UANL	0.40%	100%	100
		MRTC	0.40%	100%	100
		IRSS	0.40%	100%	102
		IRD	0.40%	100%	117
	Aedes albopictus	ICDC	0.40%	100%	110
		NEA	0.40%	98%	84
		VCRU	0.40%	100%	100
		IRD	0.40%	100%	116
Deltamethrin	*Aedes aegypti*	UANL	0.03%	100%	100
		MRTC	0.03%	100%	100
		IRSS	0.03%	100%	102
		IRD	0.03%	100%	111
	Aedes albopictus	ICDC	0.03%	100%	103
		NEA	0.03%	99%	91
		VCRU	0.03%	100%	100
		IRD	0.03%	99%	105
Lambda-cyhalothrin	*Aedes aegypti*	UANL	0.02%	100%	100
		MRTC	0.02%	100%	100
		IRSS	0.02%	100%	102
		IRD	0.02%	100%	113
	Aedes albopictus	ICDC	0.07%	100%	98
		NEA	0.07%	100%	95
		VCRU	0.07%	100%	100
		IRD	0.07%	100%	114

Table 23 (Cont'd). Mortality of *Aedes* spp. exposed to tentative discriminating concentrations in step-3 filter paper tests (1 h exposure; 24 h recording time)

Insecticide	Species	Centre	TDC	Mortality (%)	N
Alpha-cypermethrin	*Aedes aegypti*	UANL	0.04%	100%	100
		FIOCRUZ	0.04%	100%	100
		Swiss TPH	0.04%	100%	108
		IRD	0.04%	99%	107
	Aedes albopictus	ICDC	0.08%	100%	110
		NEA	0.08%	100%	96
		VCRU	0.08%	100%	100
		IRD	0.08%	98%	114
Bendiocarb	*Aedes aegypti*	UANL	0.20%	100%	100
		MRTC	0.20%	100%	100
		Swiss TPH	0.20%	100%	105
		IRD	0.20%	99%	111
	Aedes albopictus	ICDC	0.20%	100%	101
		NEA	0.20%	100%	93
		VCRU	0.20%	100%	100
		IRD	0.20%	100%	95
Chlorpyrifos-ethyl	*Aedes aegypti*	UANL	0.80%	100%	100
		MRTC	0.80%	100%	100
		Swiss TPH	0.80%	100%	87
		IRD	0.80%	100%	108
	Aedes albopictus	ICDC	1%	100%	96
		NEA	1%	99%	91
		VCRU	1%	100%	100
		IRD	1%	100%	110
		ICDC	2%	100%	106
		NEA	2%	100%	93
		VCRU	2%	100%	100
		IRD	2%	100%	109
Malathion	*Aedes aegypti*	UANL	1.50%	100%	100
		MRTC	1.50%	100%	100
		Swiss TPH	1.50%	100%	108
		IRD	1.50%	100%	111
	Aedes albopictus	ICDC	4%	100%	100
		VCRU	4%	100%	100
		IRD	4%	98%	112

Table 23 (Cont'd). Mortality of *Aedes* spp. exposed to tentative discriminating concentrations in step-3 filter paper tests (1 h exposure; 24 h recording time)

Insecticide	Species	Centre	TDC	Mortality (%)	N
Pirimiphos-methyl	*Aedes aegypti*	UANL	60 mg/m²	100%	100
		IRSS	60 mg/m²	100%	103
		Swiss TPH	60 mg/m²	100%	120
		IRD	60 mg/m²	99%	110
	Aedes albopictus	ICDC	60 mg/m²	100%	104
		IRD	60 mg/m²	100%	106
		FIOCRUZ	60 mg/m²	100%	101
		VCRU	60 mg/m²	100%	100

Table 24. Mortality of *Anopheles* spp. mosquitoes exposed to tentative discriminating concentrations in step 3 of filter paper tests (1 h exposure; 24 h recording time)

Insecticide	Species	Centre	TDC	Mortality (%)	N
Alpha-cypermethrin	*Anopheles funestus*	LSTM	0.02%	100%	99
		CDC	0.02%	100%	63
		NICD	0.02%	98%	100
	Anopheles gambiae	OCEAC	0.05%	100%	92
		LSHTM-CREC	0.05%	99%	99
		LSHTM-IPR	0.05%	100%	111
		IRD	0.05%	100%	112
	Anopheles minimus	KU	0.05%	100%	101
		MU	0.05%	100%	102
		CDC	0.05%	100%	120
	Anopheles albimanus	NIH Colombia	0.30%	100%	95
		NIH Perù	0.30%	100%	101
		CDC	0.30%	100%	117
	Anopheles stephensi	NIMR ND	0.30%	100%	100
		NIMR B	0.30%	100%	99
		VCRC	0.30%	100%	100

Table 24 (Cont'd). Mortality of *Anopheles* spp. mosquitoes exposed to tentative discriminating concentrations in step 3 of filter paper tests (1 h exposure; 24 h recording time)

Insecticide	Species	Centre	TDC	Mortality (%)	N
Pirimiphos-methyl	*Anopheles albimanus*	NIH Colombia	90 mg/m²	100%	103
		NIH Perù	90 mg/m²	100%	100
		CDC	90 mg/m²	100%	114
	Anopheles funestus	LSTM	90 mg/m²	100%	100
		NICD	90 mg/m²	100%	105
	Anopheles stephensi	NIMR ND	90 mg/m²	99%	100
		NIMR B	90 mg/m²	100%	102
		VCRC	90 mg/m²	100%	100
	Anopheles minimus	CDC	100 mg/m²	100%	100
		KU	100 mg/m²	100%	102
		MU	100 mg/m²	100%	106
	Anopheles gambiae	OCEAC	170 mg/m²	100%	96
		LSHTM-KCMC	170 mg/m²	100%	99
		LSHTM-IPR	170 mg/m²	100%	313

7.1.4 Main constraints encountered

While no major problems were reported in conducting the filter paper tests, certain constraints were reported by the laboratories:

- initial delays in the procurement and supply of insecticides, carrier oils and other materials at the test sites due to logistical problems;
- a technical challenge to proposing common DCs that applied to all the mosquito species because of substantial differences in the LC_{99} values among the testing laboratories and use of different strains;
- some difficulty in regular production of sufficient numbers of mosquitoes for step 2 (bioassays in triplicate), especially of *An. minimus* and *An. funestus*, which are particularly difficult to breed;
- lack of reproducibility of test results for some insecticides (e.g. organophosphates), which required selection of two TDCs to undergo step 3;
- suspected resistance in some mosquito colonies, which delayed testing, as mosquitoes from susceptible colonies of other participating laboratories had to be sent to such a laboratory to ensure the continuity of testing; and
- restrictions in accessing facilities at almost all sites due to the COVID-19 pandemic, which affected testing and the organization of meetings and consultations.

7.2 WHO bottle bioassays

7.2.1 Development and validation of the WHO bottle bioassay protocol

As indicated in section 6.1, six laboratories with a high level of expertise in insecticide testing were asked to advise on adequate test conditions for bottle assays for group B compounds (e.g. drying time, exposure time, holding period, temperature, relative humidity) and to report any limitations of the tests, such as inconsistent results, abnormal mortality among controls, inadequate dose of surfactant.

Overall, the WHO bottle assay gave consistent, reproducible results for seven of the nine insecticides tested (metofluthrin, prallethrin, transfluthrin, pyriproxyfen, flupyradifurone, clothianidin and chlofenapyr). The number of tests performed by each centre and the test conditions used for these insecticides are summarized in Table 25.

Briefly, the number of tests required to validate a test protocol ranged from three for pyrethroids and flupyradifurone to 10 for clothianidin and 12 for chlorfenapyr because of greater variation in the test results. We noted a strong influence of temperature and to a lesser extend humidity on the test results; therefore, the centres were asked to strictly maintain the testing and holding temperature and relative humidity at 27° ± 2 °C and 75 ± 10%, respectively.

A 1-h exposure time was found suitable for evaluating the efficacy of all the insecticides; therefore, no other exposure times were investigated. A 24-h bottle-drying time (the interval between coating the bottles and testing) was found adequate for all the insecticides, including the volatile pyrethroids metofluthrin, prallethrin and transfluthrin. The bottles were dried horizontally with their lids open for 24 h before the test started (Fig. 16). A 1-h exposure time was found suitable for evaluating the efficacy of insecticides having a killing action, i.e. all insecticides except pyriproxyfen and no other exposure times were therefore investigated.

A 24-h holding period (time to record end-point after exposure of mosquitoes to insecticides in bottles) was found to be adequate for most of the insecticides to induce mortality, including clothianidin (Fig. 17). More consistent, repeatable results were reported for chlorfenapyr, however, when the holding period was extended from 24 h to 72 h (Fig. 18). Interestingly, no abnormal mortality was reported in the control up to 72 h of holding (Fig. 19). Two insecticides, flupyradifurone and clothianidin, required addition of a surfactant, 81% rapeseed oil methyl ester (MERO), as per the manufacturers' instructions, to prevent crystallization of the active ingredients and allow for adequate coating of bottles. The manufacturer-recommended concentration of 1500 ppm per bottle was found suitable with *Aedes* spp. Certain laboratories, however, reported high mortality (above the WHO threshold of 20%) in control bottles coated with 1500 ppm MERO against *Anopheles*

Table 25. Compounds for which the WHO bottle assay method was validated for establishing discriminating concentrations

Insecticide	Testing steps				Species	Solvent/ surfactant	Drying time (h)	Exposure time (h)	Holding time (h)	Status
	Development	Validation		Confirmation						
Metofluthrin	NIMR-D (1 test)	IRD (1 test)	CDC (1 test)	None	*Ae. aegypti*	Acetone	24	1	24	Validated[a]
Prallethrin	LSHTM-CREC (1 test)	NEA (1 test)	IRD (1 test)	None	*Ae. aegypti*	Acetone	24	1	24	Validated[a]
Transfluthrin	IRD (1 test)	NIMR-D (1 test)	NEA (1 test)	None	*Ae. aegypti*	Acetone	24	1	24	Validated[a]
Pyriproxyfen	CDC (1 test)	LSTM (2 test)	LSHTM-CREC (1 test)	IRD (1 test)	*An. gambiae*	Acetone	**2**	1	**72**	Validated[a]
Flupyradifurone	IRD (1 test)	LSHTM-KCMC (1 test)	NIMR-D (1 test)	None	*Ae. aegypti*	Acetone + MERO	24	1	24	Validated[a]
Clothianidin	LSHTM-KCMC (4 tests)	IRD (4 tests)		NIH + NIMR-D (1 test + 1 test)	*An. gambiae*	Acetone + MERO	24	1	24	Validated[a]
Chlorfenapyr	LSTM (1 test)	LSHTM (2 tests)	CDC (3 tests)	IRD + NIMR-D (2 tests + 4 tests)	*An. gambiae*	Acetone	24	1	**72**	Validated[b]

[a] Test protocol validated at WHO consultation in October 2019.

[b] Test protocol validated at WHO consultations on 15–18 December 2020 and 21 January 2021.

spp. (Fig. 20). As shown in Fig. 21, some mosquitoes were found sticking to the walls of the bottles, possibly due to the presence of MERO and/or water droplets or humidity, causing their death. Consequently, the concentration of MERO was reduced to ensure the feasibility of conducting bottle assays with *Anopheles* species. After calibration, the concentration of MERO adopted for the bioassays was 800 ppm for *Anopheles* spp., except for *An. albimanus*, which required 200 ppm of the surfactant (Fig. 20).

Fig. 16. Coated bottles being dried overnight in the dark with their lids open

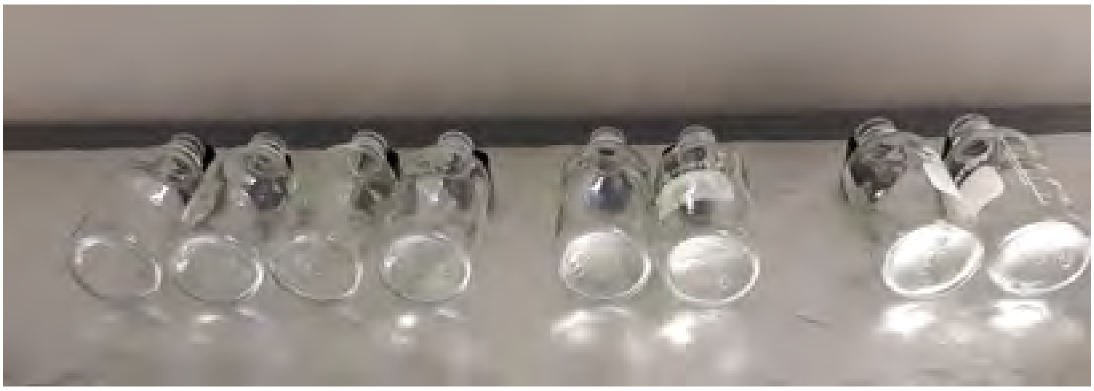


Photo courtesy of Rosemary Lees, LSTM, United Kingdom.

Fig. 17. Mortality of mosquitoes in bioassays in clothianidin-coated bottles after 24-, 48- and 72-h holding periods (test conditions: 1 h exposure, 24 h bottle drying). Changing the holding period (24, 48 or 72 h) did not affect mortality rates.

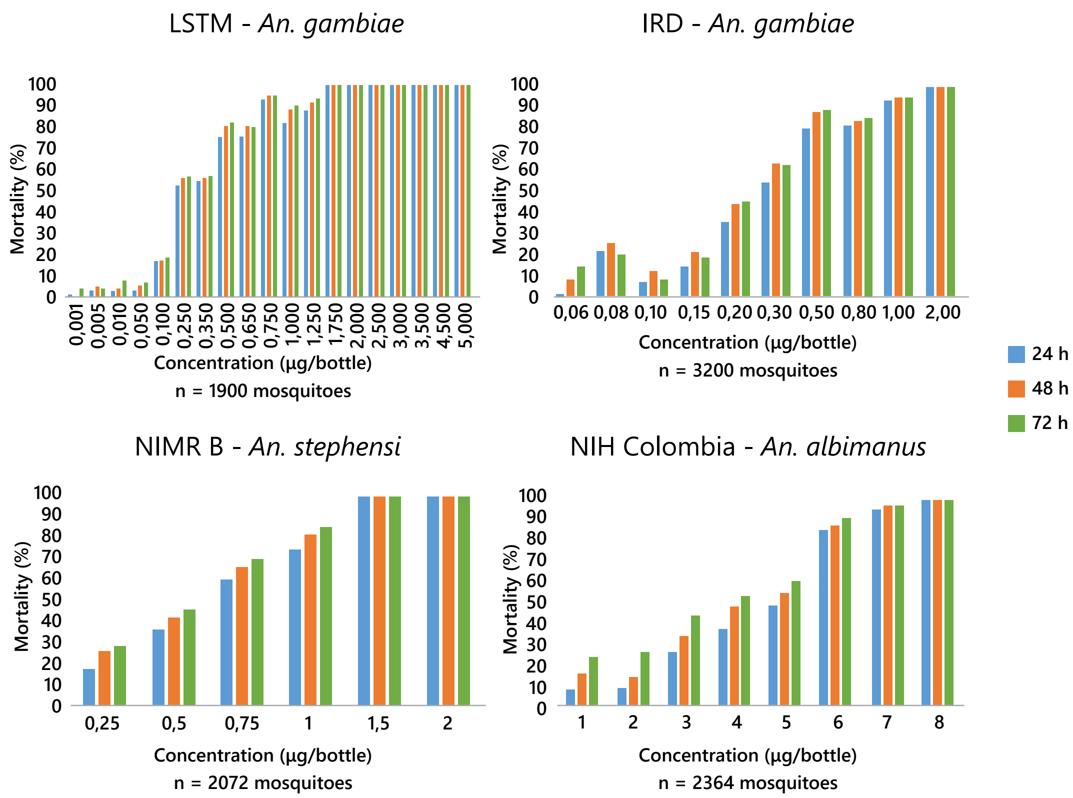


Fig. 18. Mosquito mortality in bioassays in chlorfenapyr-coated bottles after 24-, 48- and 72-h holding periods (test conditions: 1 h exposure, 24 h bottle drying). More consistent results were seen after a 72-h holding period than a 24- or 48-h period (n ≈ 4700 mosquitoes)

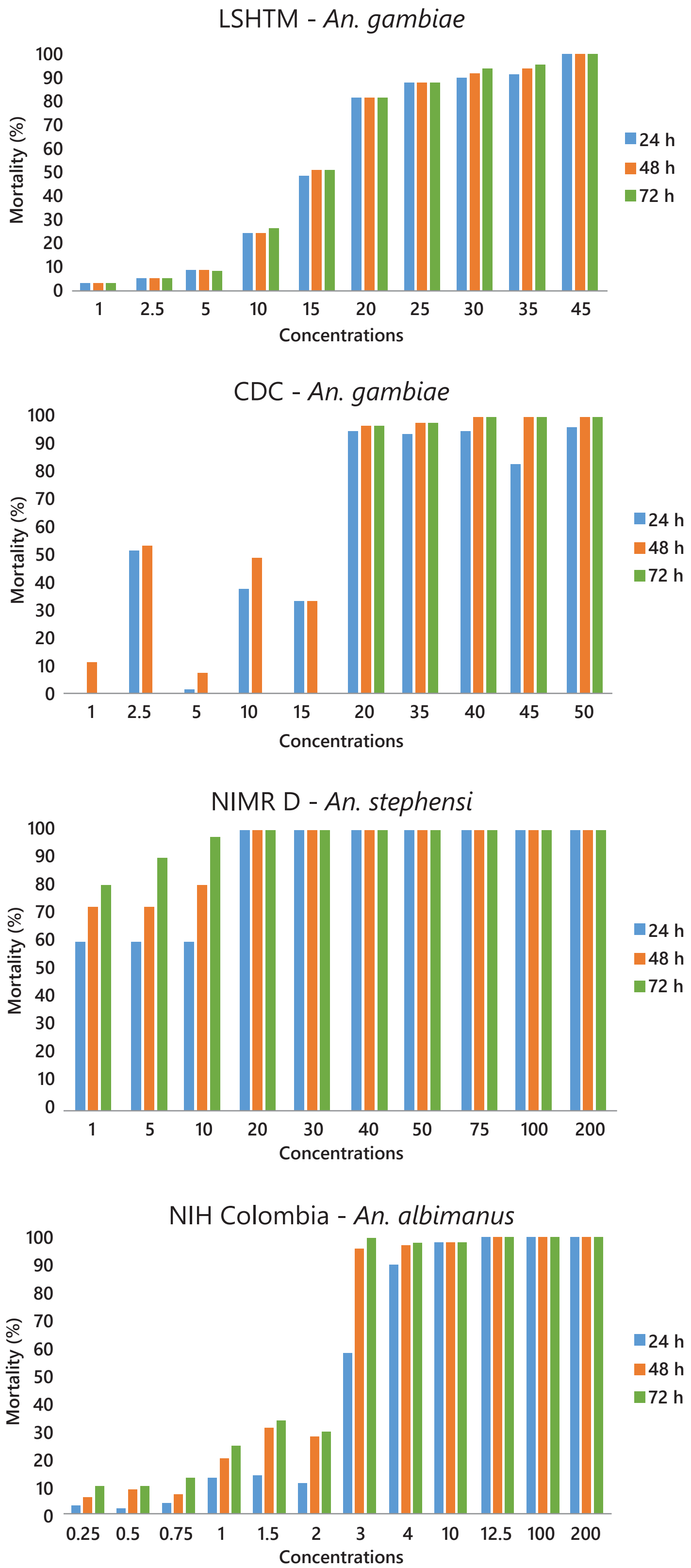


Fig. 18 (Cont'd). Mosquito mortality in bioassays in chlorfenapyr-coated bottles after 24-, 48- and 72-h holding periods (test conditions: 1 h exposure, 24 h bottle drying). More consistent results were seen after a 72-h holding period than a 24- or 48-h period (n ≈ 4700 mosquitoes)

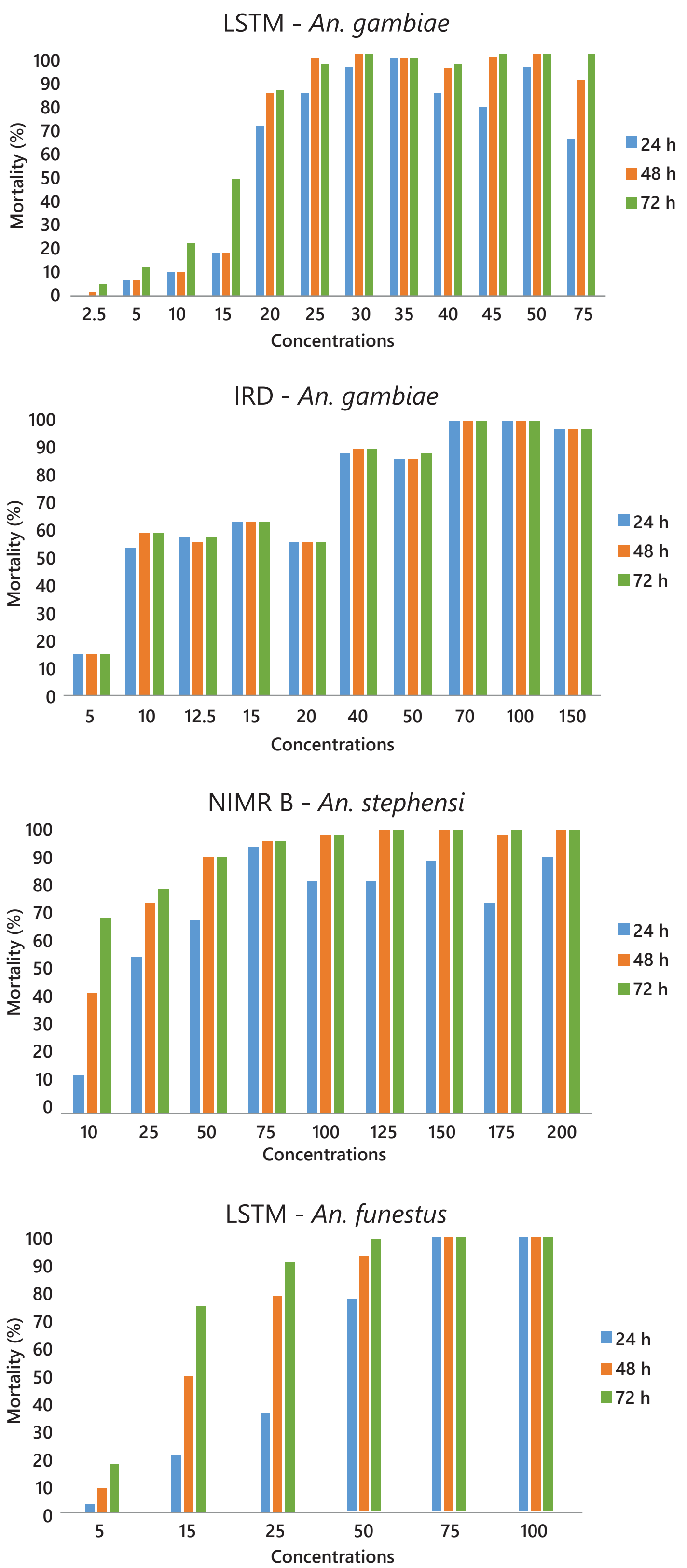


Fig. 19. Mortality in control mosquitoes in bottle bioassays treated with acetone after 24-, 48- and 72-h holding periods (used as control in chlorfenapyr bioassays). The test conditions were 1 h exposure and 24 h bottle drying time. Approximately 1000 mosquitoes of five species were tested in 10 laboratories. Each dot represents 50 mosquitoes tested.

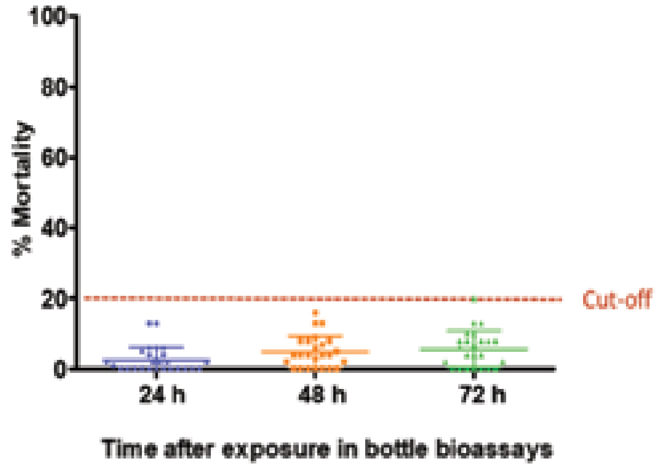


Fig. 20. Mortality of control *Anopheles* species in bottle assays after exposure to 1500, 800 and 200 ppm of MERO (test conditions: 1 h exposure; 24 h bottle drying). Each dot represents ≈ 25 mosquitoes.

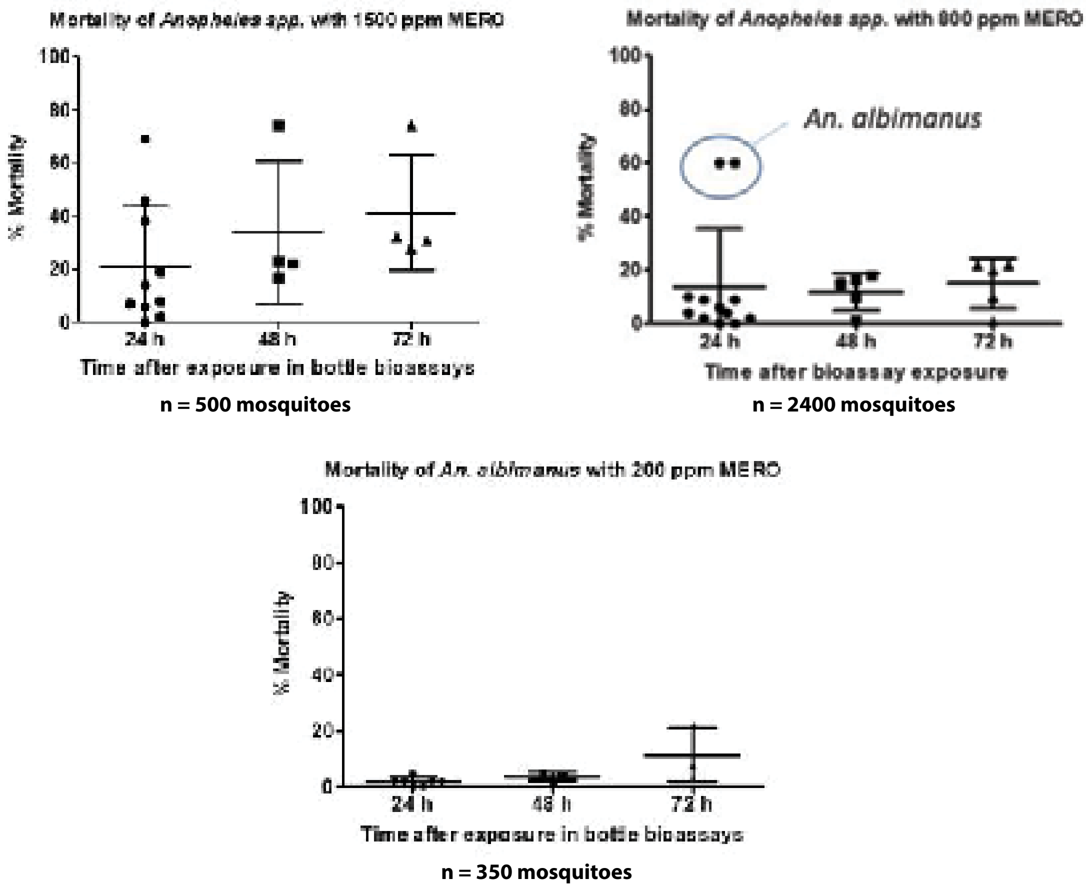


Fig. 21. *Anopheles albimanus* mosquitoes sticking to the walls of bottles coated with a mixture of acetone and 1500 ppm MERO

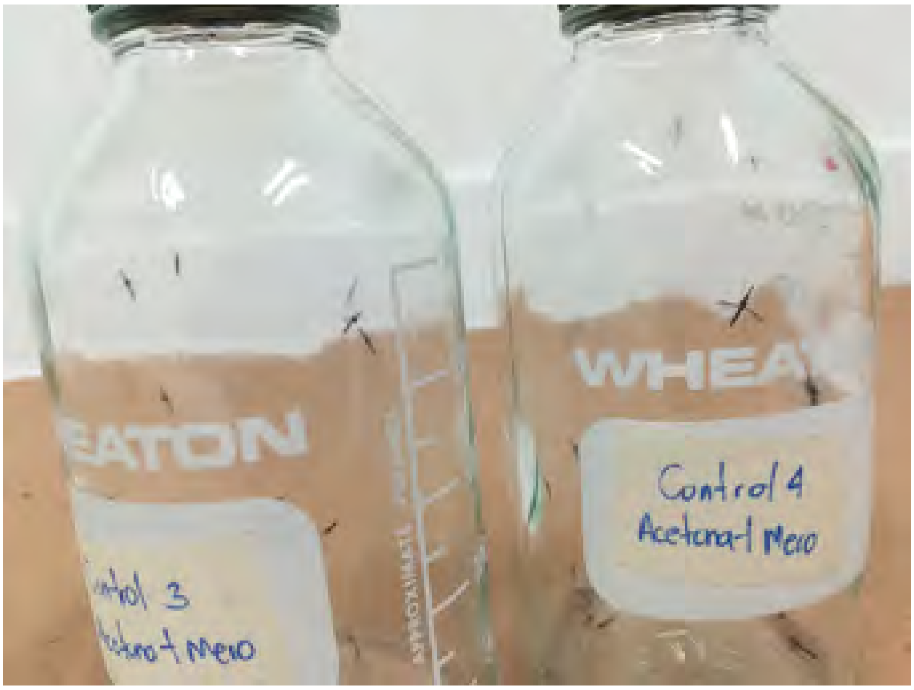


Photo courtesy of Martha Ahumada, NIH-Colombia.

The results of the bioassays indicated the following test conditions for all the insecticides that induced mortality:

- 24 h bottle drying time;
- 1 h exposure time; and
- 24 h holding time, except for chlorfenapyr, for which a 72-h holding period was used for recording adult mortality.

For pyriproxyfen, our study showed that the bottle bioassay method is suitable for assessing the sterilizing properties of this growth regulator against susceptible adult mosquitoes. The initial scope of the bioassay was to assess the capacity of pyriproxyfen to inhibit or reduce the fertility and fecundity of female mosquitoes (i.e. oviposition, fecundity, hatching and number of offspring) in order to establish a suitable DC for monitoring resistance. The SOP was finalized after discussion in WHO consultations *(14)*.

Briefly, glass bottles were treated 2 h before starting the test and dried with open lids. Mosquitoes were then introduced into pyriproxyfen-coated bottles and exposed for 1 h. Mortality was measured for up to 72 h after exposure. After that period, both control and treated mosquitoes were chambered individually for an additional 96 h to assess the individual oviposition rates. Initially, 11 days were required to count the number of eggs laid and larvae hatched, which required suitable testing and holding conditions (27 °C ± 2 °C and 75% relative humidity ± 10%) to be maintained throughout. Preliminary assays conducted with *An. gambiae* and *An. stephensi* showed that all the measured indicators (oviposition, fecundity, offspring) except hatching rates followed a concentration–response trend and hence were considered suitable for detecting mosquito resistance to sterilization by pyriproxyfen (Fig. 22). The rate of oviposition inhibition (OI) was selected as the end-point for establishing the DC because it (i) provided reliable, consistent results, (ii) allowed shortening of the testing time from 11 to 7 days,[1] and (iii) significantly reduced the amount of work required of the technical staff to complete the tests.

The test conditions suitable for pyriproxyfen are thus as follows:

- Allow healthy males to mate with female mosquitoes in the days before selection for bioassays.
- Dry coat the bottles for 2 h before exposure and testing.
- Blood-feed the 5–7-day old females within 1 h before exposure to pyriproxyfen.
- Expose blood-fed females to pyriproxyfen or control in bottles for 1 h.
- Hold exposed females in paper cups for 72 h to record mortality.
- 72 h after the exposure, chamber individual females for a subsequent 96 h to assess oviposition and mortality in both controls and treated mosquitoes.
- Validate the test by ensuring that mortality of control mosquitoes is < 20% after the initial 72-h holding period and the oviposition rate is > 30% at the end of chambering for 96 h.
- Perform the test with a susceptible laboratory mosquito strain (e.g. Kisumu or other local species) in parallel to confirm that any signs of resistance in wild mosquito population are not due to the test conditions.

Lack of consistency and repeatability was observed within and among laboratories that tested imidacloprid, indoxacarb and dinotefuran in bottle bioassays. For dinotefuran (10 tests) and imidacloprid (5 tests), strong intra- and inter-laboratory variation was reported, with no plateau of mortality with increasing concentrations (Figs. 23 and 24). For indoxacarb, no clear concentration–response relation was seen for *An. gambiae* at concentrations of 1–1700 μg/bottle (7 tests performed, Fig. 25). Changing the bottle drying time did not improve the outcome. We assumed that the drying process might be critical for these three compounds and that bottle bioassays might also require addition of a suitable surfactant to coat bottles adequately. We could not, however, invest more time and resources in developing a suitable bioassay method for these compounds, and no DCs were established (Table 26). A decision to stop testing the compounds was made after a recommendation by the WHO consultation in October 2019. Further work is necessary to develop test protocols for these compounds, which may be used increasingly in formulating vector control products.

1 First 72 h of initial holding of mosquitoes after 1 h exposure to record mortality; thereafter, an additional 96 h to record oviposition and emergence of offspring.

Fig. 22. Assessment of sterilizing properties of pyriproxyfen against susceptible *Anopheles* spp. measured by reduction of oviposition, fecundity, hatching and offspring

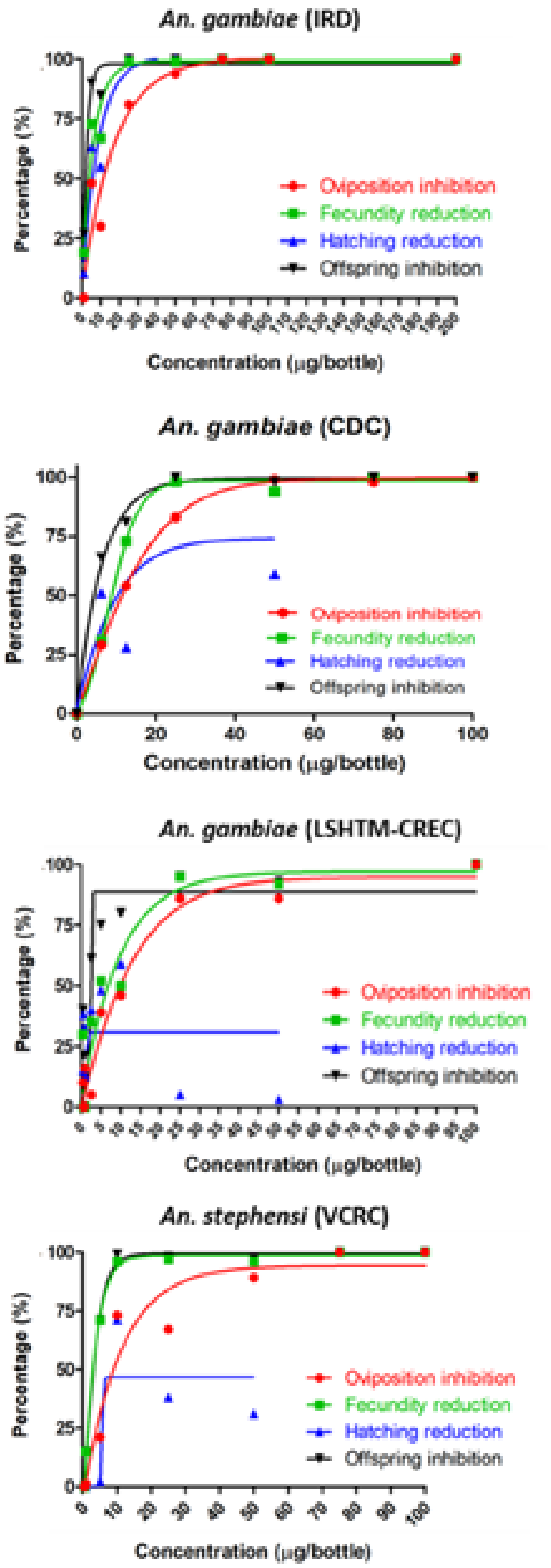
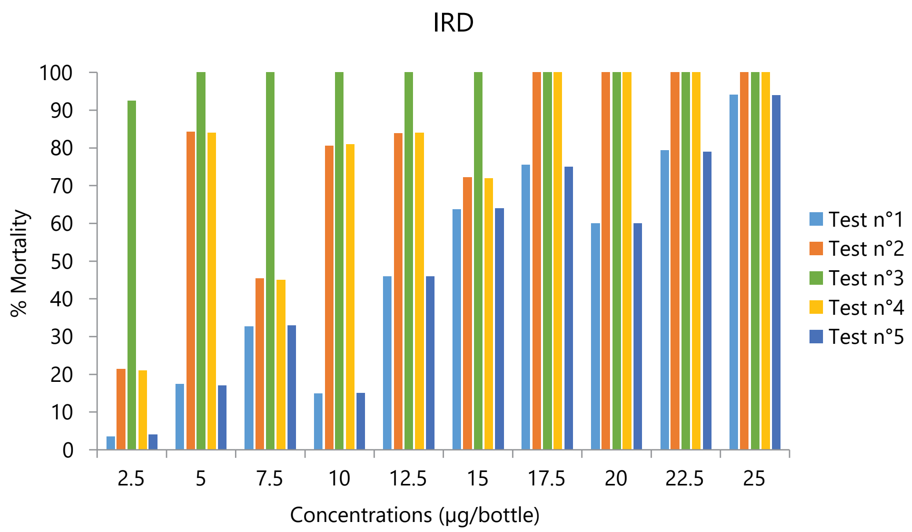


Fig. 23. Concentration–mortality response in bottle bioassays of dinotefuran against the susceptible Kisumu strain of *An. gambiae* (test conditions: 1 h exposure; 24 h bottle drying). No surfactant was used.



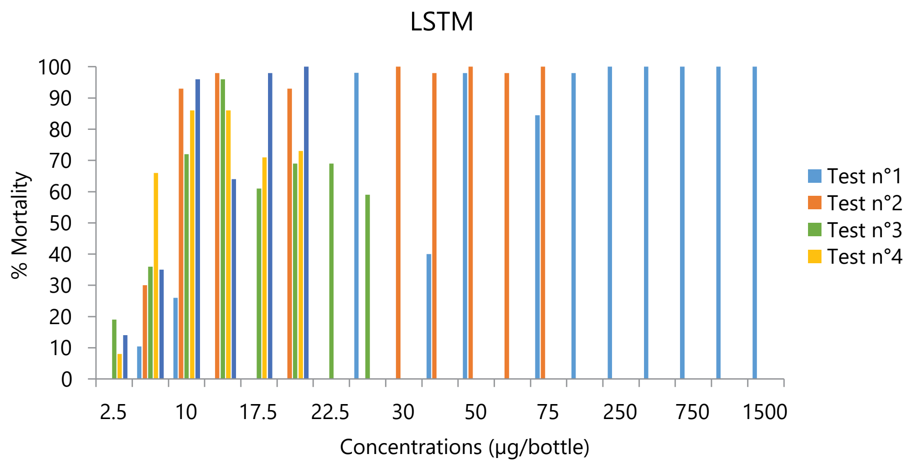
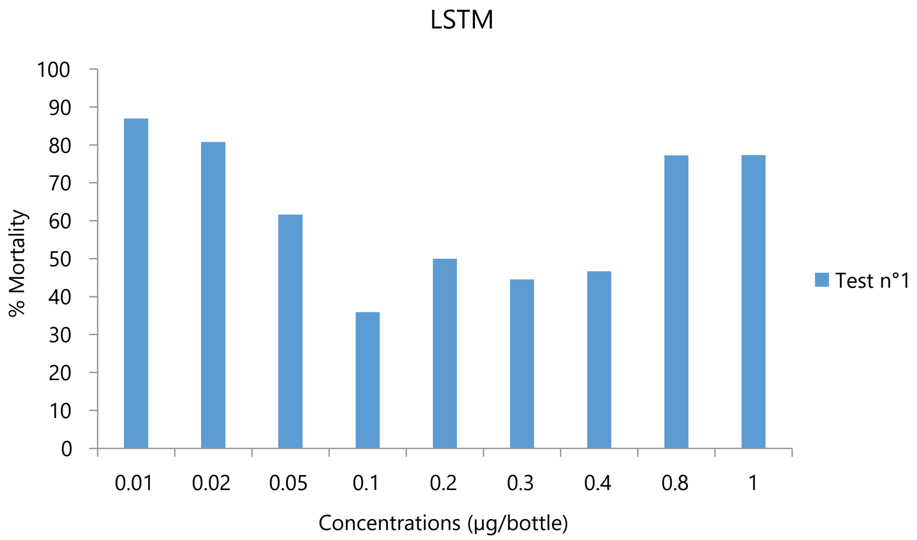


Fig. 24. Concentration–mortality response in bottle bioassays of imidacloprid against susceptible *An. gambiae* (test conditions: 1 h exposure; 24 h bottle drying). No surfactant was used.



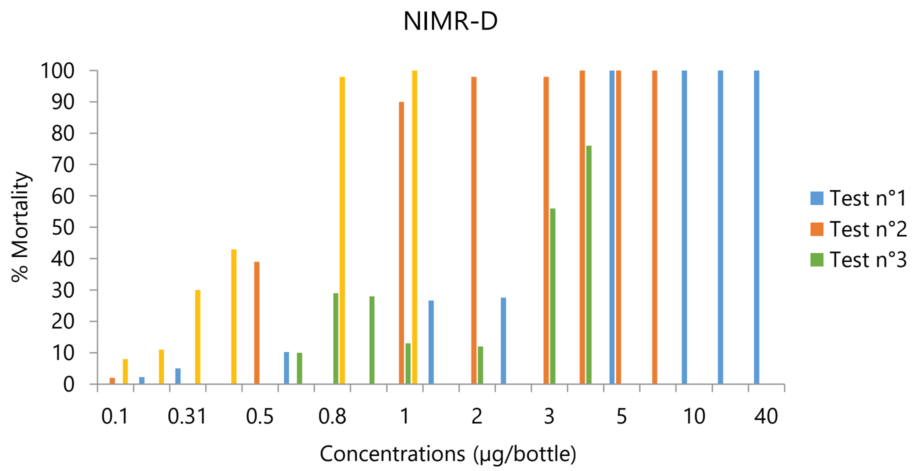


Fig. 25. Concentration–mortality response in bottle bioassays with indoxacarb against susceptible *An. gambiae* (test conditions: 1 h exposure; 24 h bottle drying time (DT) at CDC and LSTM and 1 h and 24 h DT at IRD). No surfactant was used.

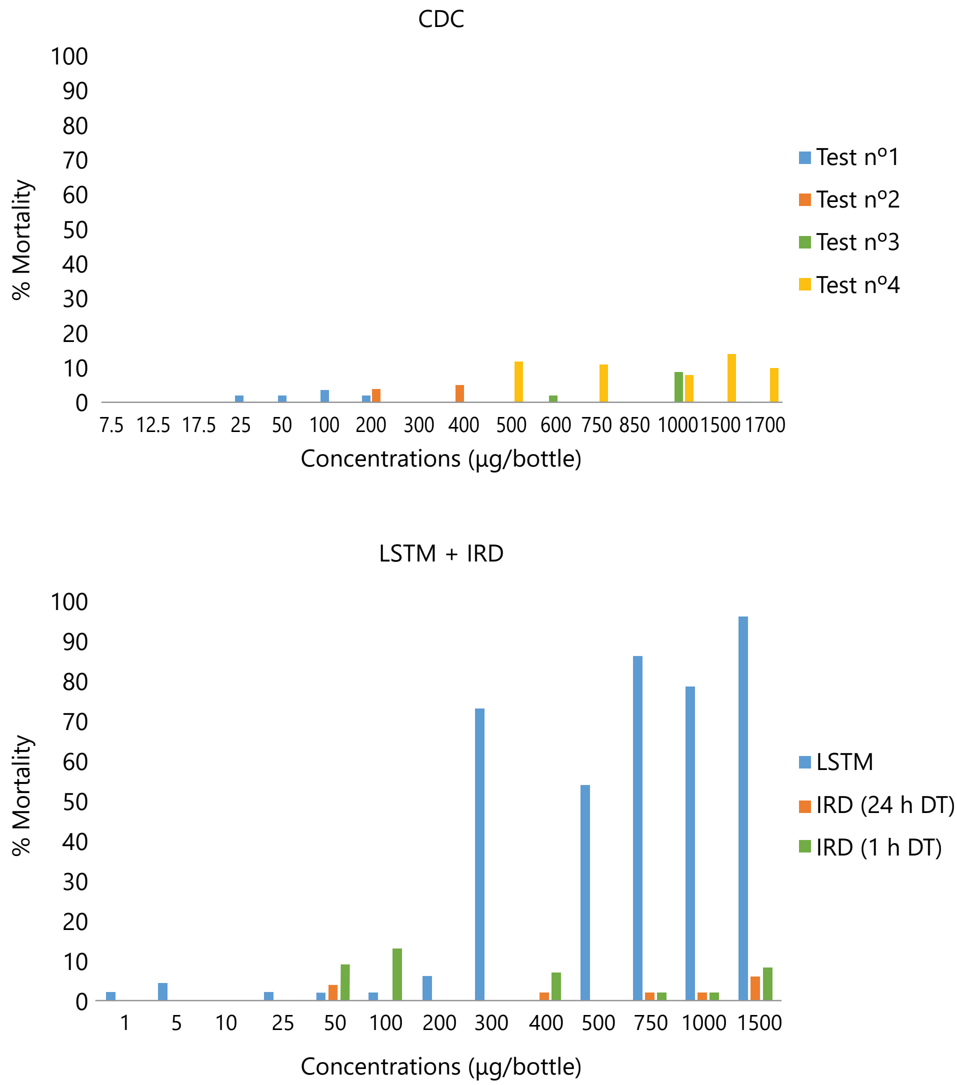


Table 26. Compounds for which bottle assays could not be validated for establishing discriminating concentrations (testing conditions: 24 h bottle drying time, 1 h exposure time, 24 h holding time)

Insecticide	Testing steps			Species	Solvent
	Development	Validation	Confirmation		
Dinotefuran	LSTM (5 tests)	IRD (5 tests)	None	*An. gambiae*	Acetone
Imidacloprid	NIMR-D (4 tests)	LSTM (1 test)	None	*Ae. aegypti*	Acetone
Indoxacarb	US CDC (4 tests)	LSTM (1 test)	IRD (2 tests)	*An. gambiae*	Acetone

7.2.2 Bioassay completion rates

In all, 203 104 *Aedes* and *Anopheles* mosquitoes were tested in WHO bottle assays (Fig. 26). *Ae. albopictus* (24%; n = 48 382), *Ae. aegypti* (20%; n = 41 108) and *An. gambiae* (20%; n = 39 627) together accounted for 64% of the total number of mosquitoes tested in bottle assays (Fig. 27). The five other species tested (*An. funestus*, *An. stephensi, An. minimus*, *An. albimanus* and *An. arabiensis*) constituted the remaining 36% of the total. As for the filter paper bioassays, the sample size was large enough to give strong confidence in the results.

Fig. 26. Numbers of *Aedes* and *Anopheles* spp. mosquitoes used for testing group B insecticides in WHO bottle assays

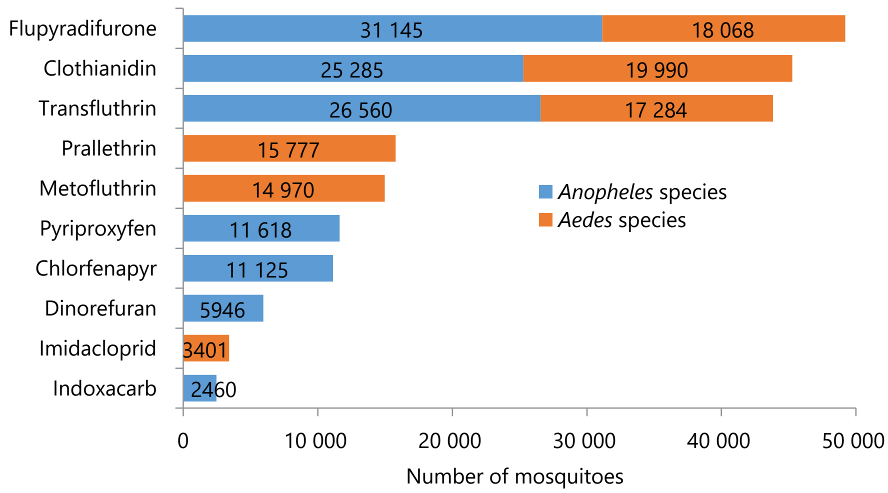


Fig. 27. Numbers and percentages of *Aedes* and *Anopheles* spp. mosquitoes used for testing group B compounds in WHO bottle assays

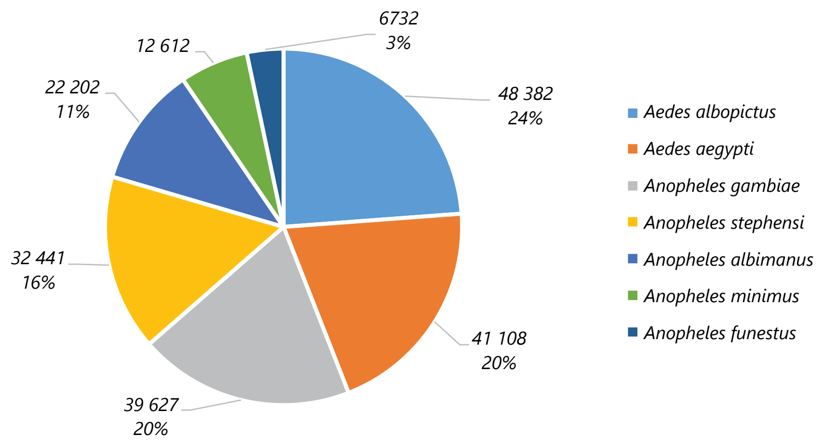


Table 27. Test completion rates (CR) in steps 1–3 of WHO bottle bioassays and insecticides tested

Step	Insecticide class	Pyrethroids			Butenolides	Insect growth regulator	Neonicotinoids	Overall
	Insecticide	**Transfluthrin**	**Prallethrin**	**Metofluthrin**	**Flupyradifurone**	**Pyriproxyfen**	**Clothianidin**	
	Targeted species	*Ae. aegypti*	*Ae. aegypti*	*Ae. aegypti*	*Ae. aegypti*	*An. albimanus*	*Ae. aegypti*	
		Ae. albopictus	*Ae. albopictus*	*Ae. albopictus*	*Ae. albopictus*	*An. funestus*	*Ae. albopictus*	
		An. albimanus			*An. albimanus*	*An. gambiae*	*An. albimanus*	
		An. funestus			*An. funestus*	*An. minimus*	*An. funestus*	
		An. gambiae			*An. gambiae*	*An. stephensi*	*An. gambiae*	
		An. minimus			*An. minimus*		*An. minimus*	
		An. stephensi			*An. stephensi*		*An. stephensi*	
Step 1	No. of tests performed	21	6	6	21	4	21	89
	No. of validated tests	19	6	6	17	4	17	79
	CR (%)	90	100	100	81	100	81	89%
Step 2	No. of tests performed	63	18	18	63	–	66	228
	No. of validated tests	39	12	12	43	–	43	149
	CR (%)	62	67	67	68	–	65	65%
Step 3	No. of tests performed	21	6	7	21	32	26	129
	No. of validated tests	21	6	7	12	27	24	112
	CR (%)	100	100	100	57	84	92	87%

CR < 35% (little progress); 35–75% (moderate progress); > 75% (good progress)

Test completion rates were estimated only for the group B compounds for which bottle bioassay protocols were developed and validated at the second WHO consultation (metofluthrin, prallethrin, transfluthrin, flupyradifurone, clothianidin and pyriproxyfen). For chlorfenapyr, the test protocol was validated at the third WHO consultation on 15–18 December 2020 and 21 January 2021 after review of evidence from WHO-supervised and non-WHO-supervised studies; the completion rates were therefore not estimated.

The test completion rates were 89%, 65% and 87% in steps 1, 2 and 3, respectively (Table 27). Overall, more variation in test results was reported in bottle bioassays than in filter paper tests, and it was difficult to repeat results for some compounds (see details in section 7.2.3). For flupyradifurone, the lower completion rates were due to a lower number of test results obtained with *An. funestus* and *An. albimanus* than expected. For pyriproxyfen, the completion rates were not estimated in step 2 because concentration–response bioassays could not be conducted in all the laboratories and against all the target species.

7.2.3 Concentration–response curves and estimate of LC_{99} and LC_{100}

The concentration–response curves (step 2) and summaries of LC_{99} and LC_{100} with TDCs for each insecticide and species are discussed below and shown in Tables 28 and 29.

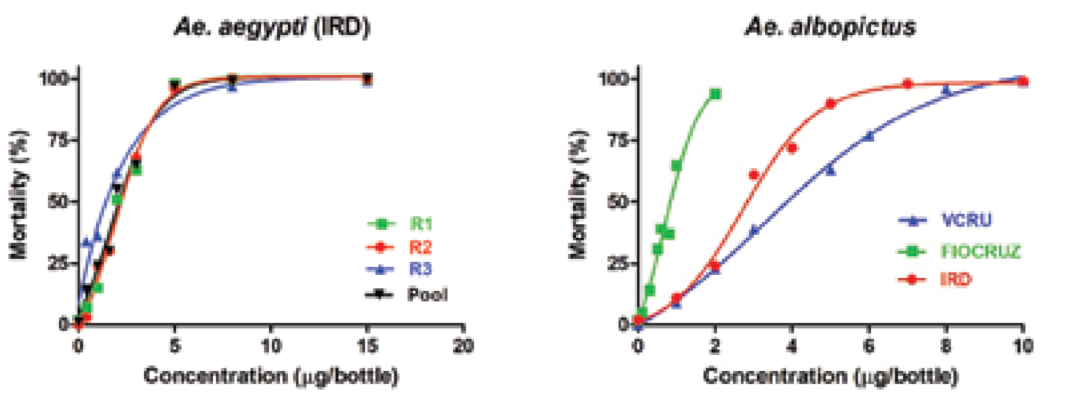
Prallethrin

Fig. 28 shows the concentration–mortality curves for prallethrin against *Ae. aegypti* and *Ae. albopictus*.

For *Ae. aegypti*, data were obtained from only one centre (IRD). Moderately strong evidence indicated LD_{99} and LD_{100} of 8 (95% CI 5.8–19) and 15 µg per bottle, respectively (Table 28). For *Ae. albopictus*, three independent datasets were obtained and analysed, providing very strong evidence. The LD_{99} ranged from 4 (2–19) at FIOCRUZ to 13 µg/bottle (9–31) at VCRU. The $LC_{100\ max}$ was 10 µg/bottle.

The TDC selected for step 3 evaluation was 30 µg active ingredient (AI) per bottle for both *Ae. aegypti* and *Ae. albopictus*.

Fig. 28. Concentration–response curves for prallethrin against *Ae. aegypti* (left) and *Ae. albopictus* (right) in step 2



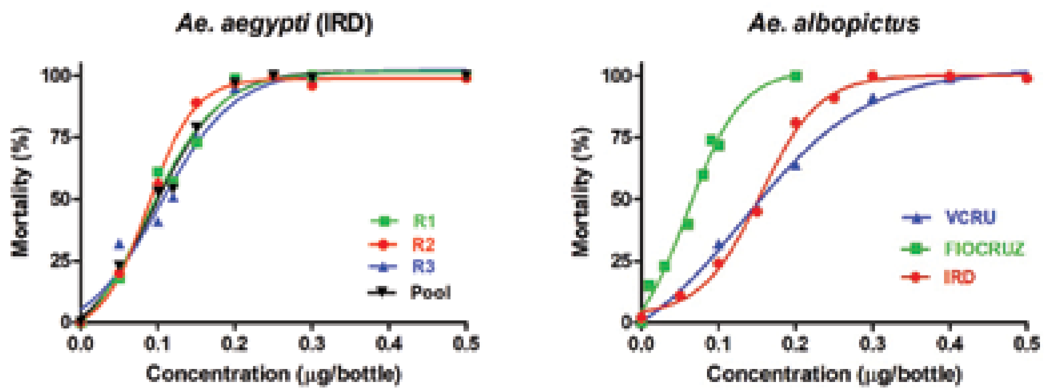
Metofluthrin

Fig. 29 shows the concentration–mortality curves for metofluthrin against *Ae. aegypti* and *Ae. albopictus*.

As for prallethrin, only one centre (IRD) submitted step-2 data for *Ae. Aegypti*, which provided moderately strong evidence. Data for three replicates indicated that the LD_{99} was 0.3 (0.21–1.65) and the LC_{100} was 0.5 µg per bottle (Table 29). Three independent datasets were obtained and analysed for *Ae. albopictus*, providing very strong evidence. The LD_{99} ranged from 0.21 (0.16–0.42) at FIOCRUZ to 0.5 µg/bottle (0.37–0.81) at VCRU, and the $LC_{100\ max}$ was 0.5 µg per bottle.

The TDC selected for step 3 was 1 µg per bottle for both *Ae. aegypti* and *Ae. albopictus.*

Fig. 29. Concentration–response curves for metofluthrin against *Ae. aegypti* (left) and *Ae. albopictus* (right) in step-2 evaluation



Transfluthrin

Fig. 30 shows the concentration–mortality curves for transfluthrin against *Aedes* and *Anopheles* spp. For *Ae. aegypti*, the LC_{99} ranged from 0.70 (0.60–0.75) at NIMR to 1.5 (1.2–2.3) µg/bottle at IRD (Table 30). For *Ae. albopictus*, the LC_{99} ranged from 0.2 (0.15–0.22) at FIOCRUZ to 1.5 (1.12–12.2) at VCRU. The LC_{100} was 0.15 at FIOCRUZ and 1.2 at both VCRU and IRD, confirming the lower tolerance of *Ae. albopictus* colonized strain at the test site in Brazil to insecticides.

The TDC selected for step 3 evaluation was 3 µg per bottle for both *Ae. aegypti* and *Ae. albopictus*.

Some variation in laboratory test results was reported for *Anopheles* mosquitoes (Table 31). Three independent datasets could be obtained only for *An. stephensi* (very strong evidence), while two independent datasets were obtained for *An. gambiae* and *An. albimanus* (strong evidence). Overall, the LC_{99} ranged from 0.05 (95% CI, 0.03 ; 0.19) for *An. albimanus*

(NIH-Colombia) to 1.12 µg/bottle (0.67 ; 3.67) for *An. stephensi* (NIMR), while the LC_{100} ranged from 0.1 to 0.8 regardless of the species. Much less information was available for *An. funestus* and *An. minimus*, with only two validated tests obtained in step 1 (weak evidence). For these two species, the LC_{99} could not be estimated, but the LC_{100} ranged from 0.2 to 1.5 µg/bottle. Despite differences in test results (mainly due to lack of replicates for some species), similar concentration–response values for transfluthrin were observed.

The TDC selected for step 3 was 2 µg per bottle for all the mosquito species tested.

Fig. 30. Concentration–response curves for transfluthrin against *Aedes* and *Anopheles* species evaluated in step 2

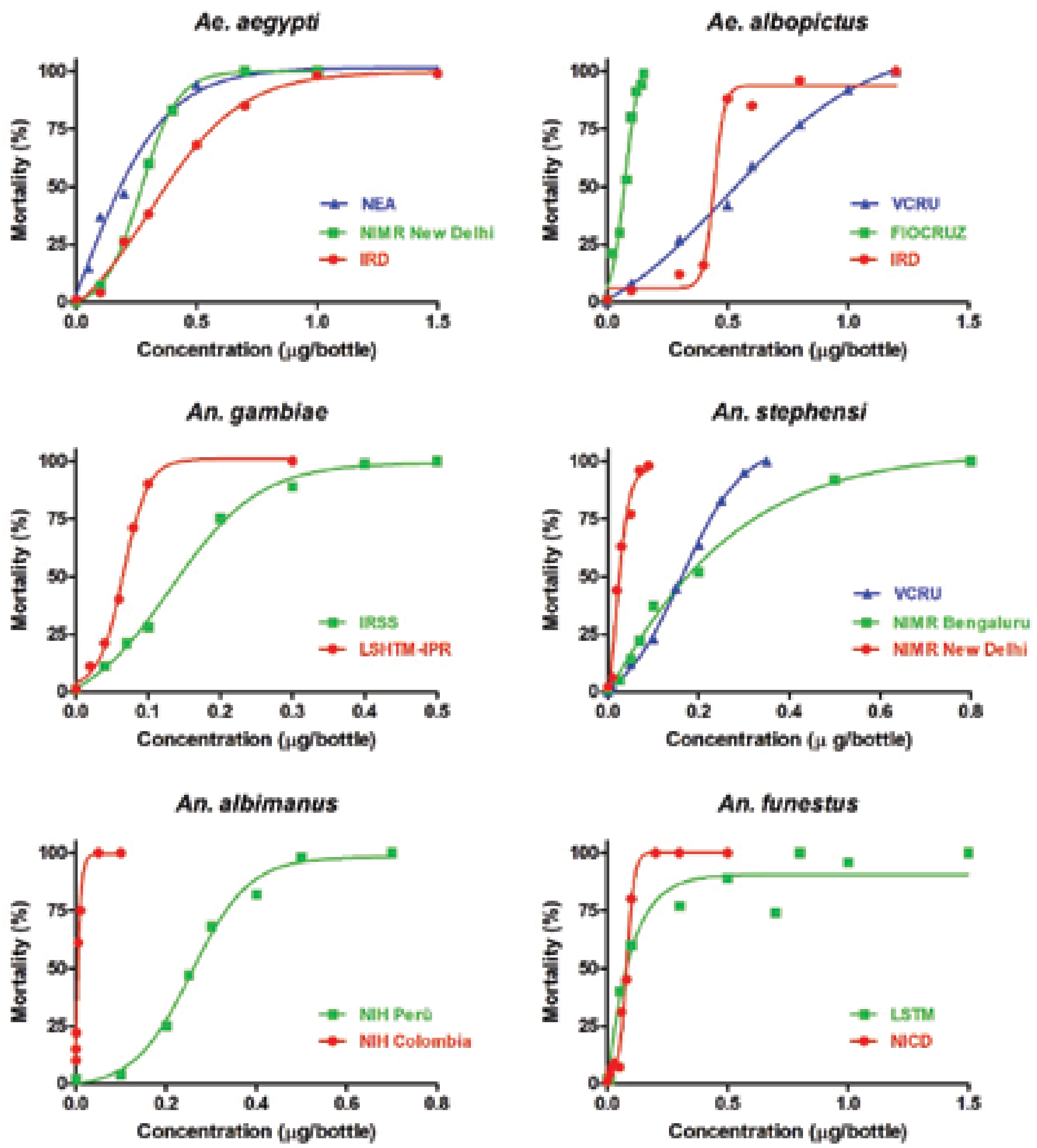
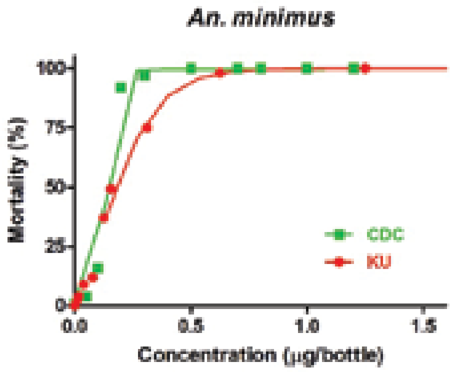


Fig. 30 (Cont'd). Concentration–response curves for transfluthrin against *Aedes* and *Anopheles* species evaluated in step 2



Clothianidin

Fig. 31 shows the concentration–mortality curves for clothianidin against *Aedes* and *Anopheles* species.

For *Ae. aegypti,* significant differences in LC_{99} were observed among the testing centres, ranging from 2 µg bottle at IRSS to 13 (95% CI, 11 ; 16) µg/bottle at KCMC. Unexpectedly higher values were reported by the latter centre than by the three others (see Table 32); for example, $LC_{100\ max}$ (20 µg/bottle) was four times higher than that obtained at IRD (5 µg/bottle). It was therefore decided to exclude those data in selecting the TDC. The LC_{99} for *Ae. albopictus* ranged from 3 (95% CI, 2 ; 12) to 4 (95% CI, 2 ; 37) µg/bottle.

The TDCs selected for step-3 evaluation were:

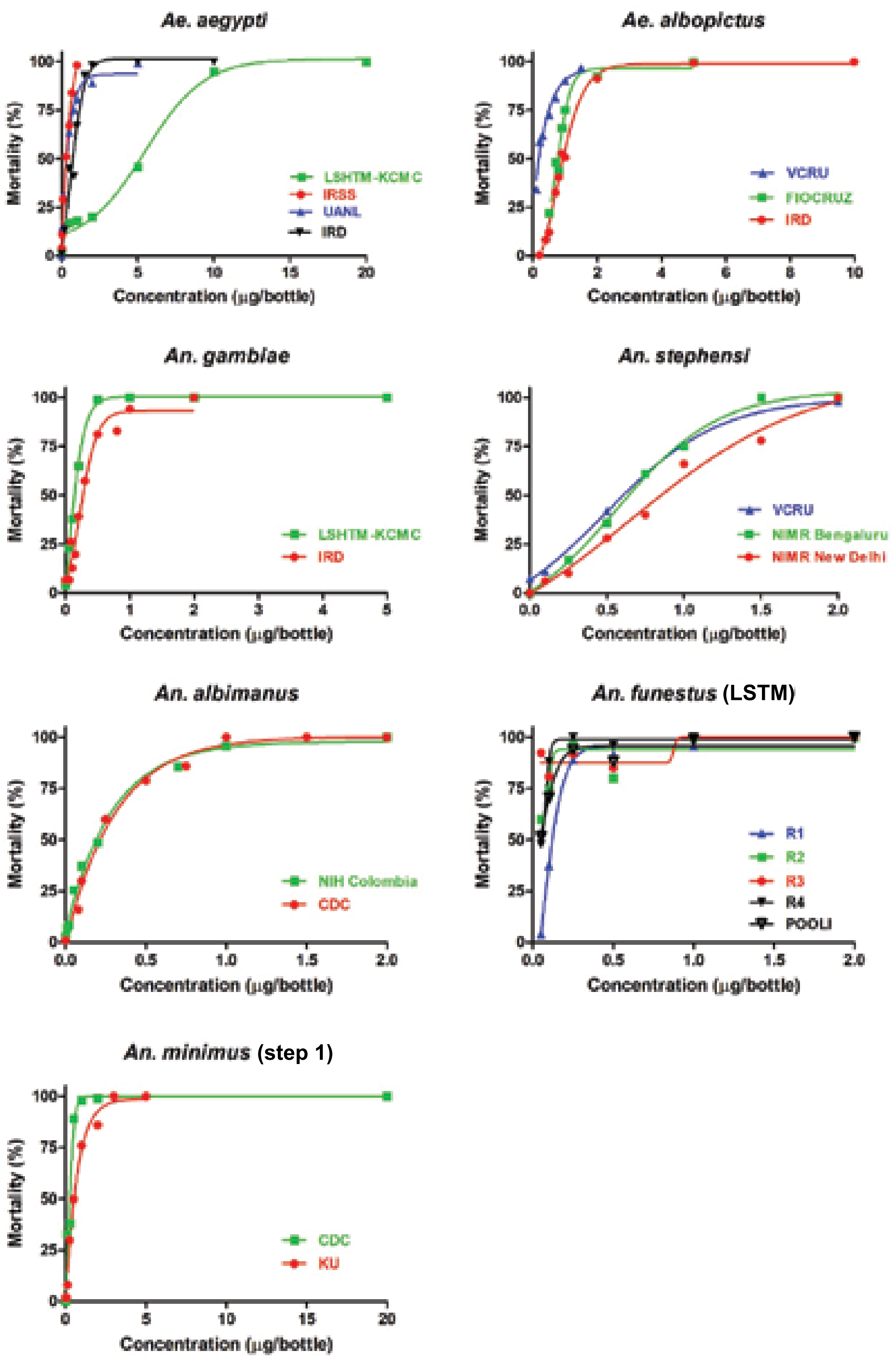
- 10 and 20 µg per bottle for *Ae. aegypti* and
- 10 µg per bottle for *Ae. albopictus.*

For *Anopheles* mosquitoes, the data were relatively consistent within and among laboratories (Table 33; Fig. 31). Three independent datasets could be obtained for *An. stephensi* (very strong evidence), while two datasets were obtained for *An. gambiae* and *An. albimanus* (strong evidence). Only one step-2 (moderately strong evidence) and two step-1 (weak evidence) data sets were used to estimate the end-points and establish the TDC for *An. funestus* and *An. minimus*, respectively. Overall, the LC_{99} ranged from 0.6 (95% CI, 0.4; 1) µg/bottle for *An. gambiae* (KCMC) to 4.9 (95% CI, 3.4 ; 8.5) µg/bottle for *An. stephensi* (NIMR). The $LC_{100\ max}$ ranged from 1 to 3 µg/bottle for all *Anopheles* species.

The TDCs selected for step-3 evaluation were:

- 10 µg/bottle for *An. stephensi*
- 6 µg/bottle for *An. albimanus* and *An. minimus*
- 4 µg/bottle for *An. gambiae*
- 3 µg/bottle for *An. funestus.*

Fig. 31. Concentration–response curves for clothianidin against *Aedes* and *Anopheles* in step-2 evaluation (except for *An. minimus*)



Flupyradifurone

Fig. 32 shows the concentration–mortality curves for flupyradifurone with *Aedes* and *Anopheles* species.

For both *Ae. aegypti* and *Ae. albopictus*, the results of the bioassays were consistent between and among the testing laboratories. The highest LC_{99} and $LC_{100\ max}$ in three validated tests (very strong evidence) were 40 and 30 µg/bottle, respectively (Table 34).

The TDC selected for step-3 testing was 80 µg/bottle for both *Ae. aegypti* and *Ae. albopictus*.

The concentration–response relation of flupyradifurone for *Anopheles* species are summarized in Fig. 32 and Table 35. Overall, the concentrations of flupyradifurone required to kill mosquitoes were higher than those for other insecticides (LC_{100}, 12–150 µg/bottle according to species). Significant inter-laboratory variation was reported in the results of tests with *An. minimus*, with an eight times difference in the LC_{99}. To avoid overestimation of the DC, which could obviate detection of resistance in field populations, it was decided to exclude the highest value (reported by KU) in estimating the TDC for *An. minimus*.

For *An. gambiae* and *An. stephensi*, the LC_{99} ranged from 8.5 (95% CI, 5 ; 42) to 27 (95% CI, 20 ; 41) µg/bottle, and the $LC_{100\ max}$ was 30 µg/bottle for both species. Despite weak evidence (step-1 data from two independent centres), the results showed that the tolerance of *An. funestus* to flupyradifurone was similar to that of *An. minimus*, with an $LC_{100\ max}$ of 50 µg/bottle for both species. *An. albimanus* showed the highest tolerance to flupyradifurone, with LC_{100} and LC_{99} values of 150 and 240 µg/bottle, respectively. A low concentration of surfactant (200 ppm MERO) used in the bottle assays might explain these outcomes.

The TDC selected for step-3 testing were:

- 60 µg/bottle for both *An. gambiae* and *An. stephensi*
- 60 and 100 µg/bottle for *An. minimus*
- 100 µg/bottle for *An. funestus*
- 300 and 500 µg/bottle for *An. albimanus*.

Fig. 32. Concentration–response curves for flupyradifurone against *Aedes* and *Anopheles* in step-2 evaluations (except for *An. funestus*)

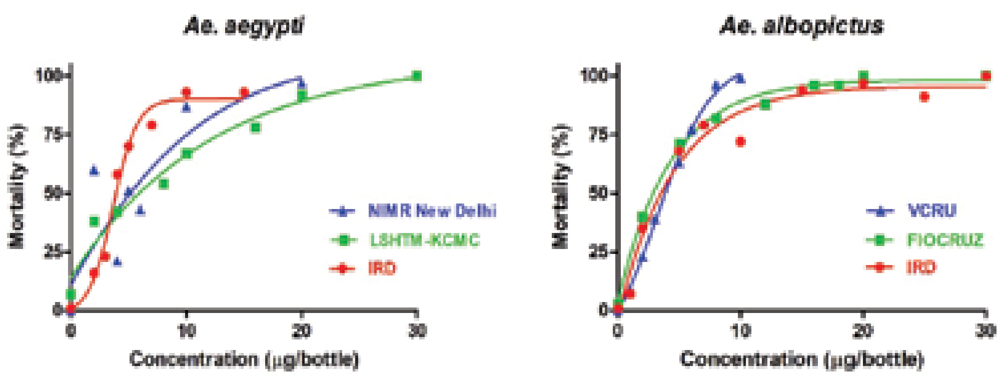
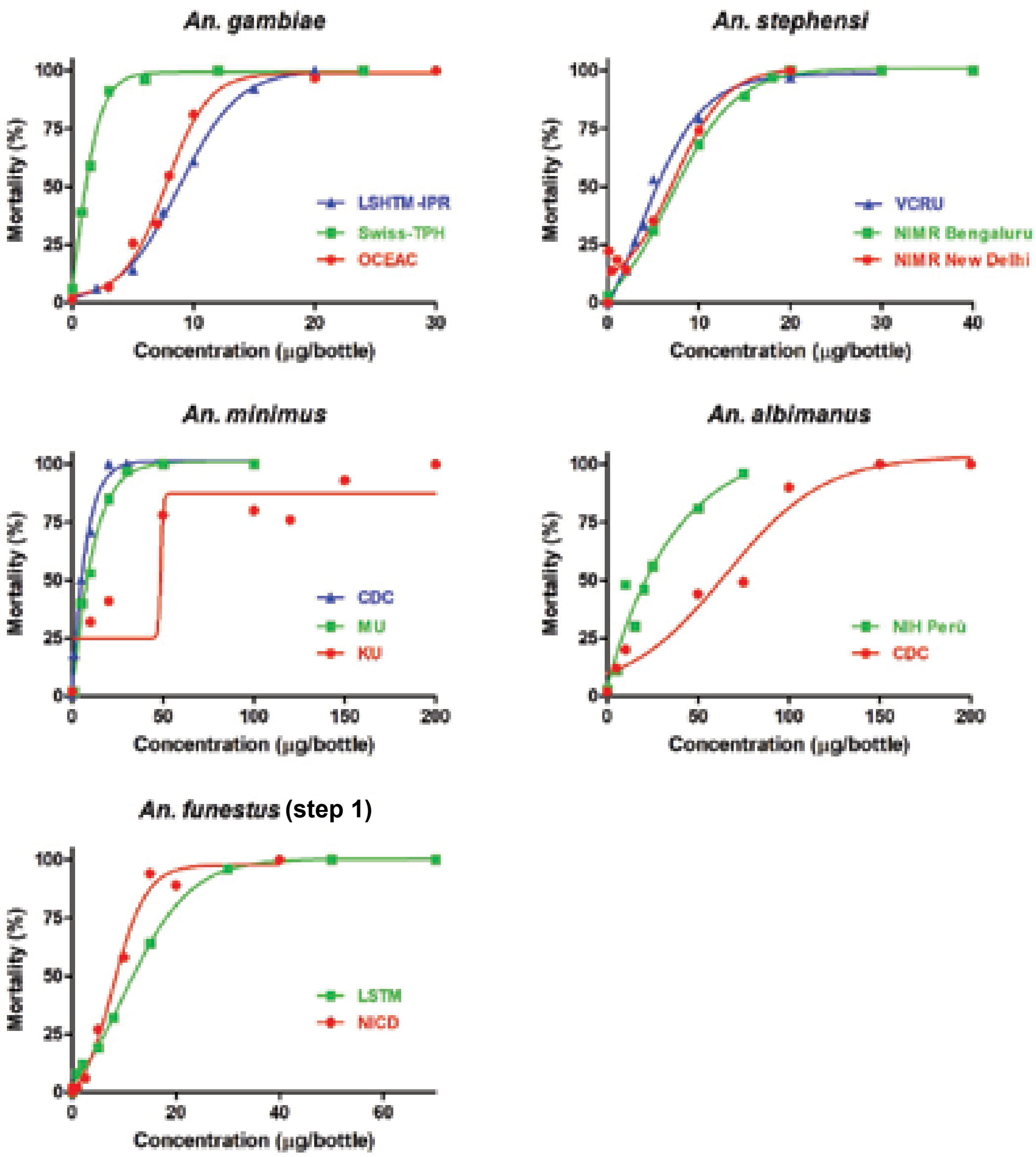


Fig. 32 (Cont'd). Concentration–response curves for flupyradifurone against *Aedes* and *Anopheles* in step-2 evaluations (except for *An. funestus*)

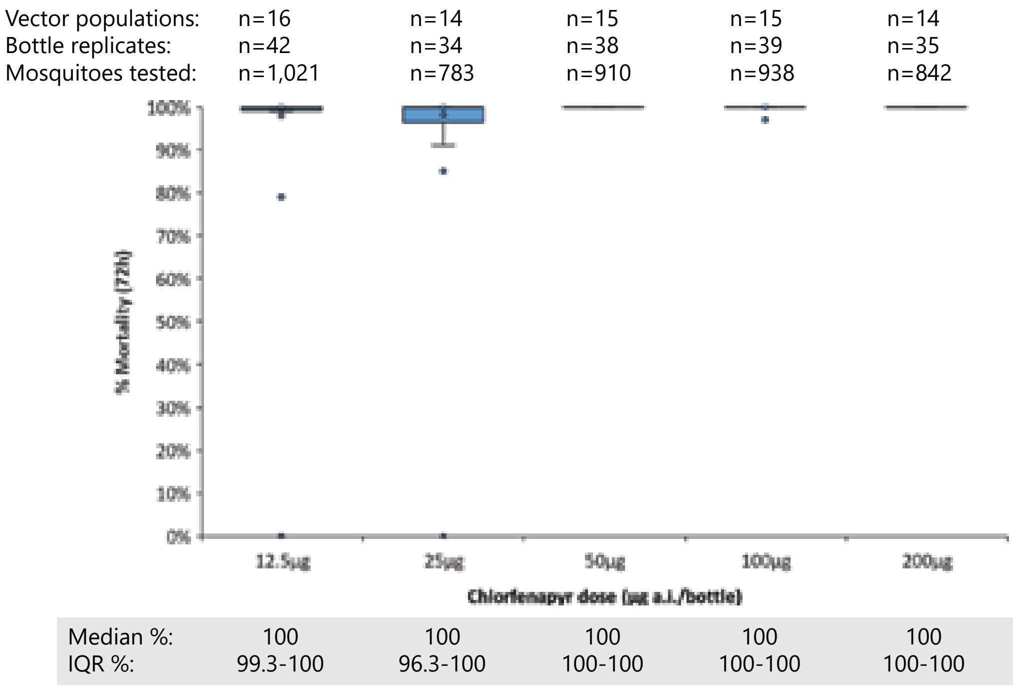


Chlorfenapyr

Chlorfenapyr was tested somewhat differently from the other compounds, as a strict "step-by-step" protocol could not be used for technical reasons. The test protocol was not validated at the second WHO consultation because of inconsistent results and difficulty in achieving a plateau of mortality at some participating centres (see section 7.2.1). New datasets from WHO- and non-WHO-supervised studies were subsequently sent to WHO, which were reviewed at the third consultation in December 2020 to decide whether to revise the recommendations for this compound. With the new evidence, a standardized test protocol was validated and shared with participating laboratories, and two TDCs (100 and 200 µg/m^2) were selected for step-3 testing against various *Anopheles* species.

Data from the Vector Link project of the US President's Malaria Initiative on chlorfenapyr resistance in Africa *(14)* indicated a concentration–response relation for chlorfenapyr of 200 µg AI/bottle, which was the only concentration to cause > 98% mortality at 72 h (Fig. 33). In the WHO study, 100% mortality was almost achieved for concentrations ranging from 12.5 for *An. albimanus* to 125 µg/bottle for *An. stephensi* (Fig. 34; Table 36).

Fig. 33. Mortality rates with chlorfenapyr in bottle assays with multiple insectary-reared susceptible colonies and wild populations of *An. gambiae* s.l. in eight countries in sub-Saharan Africa



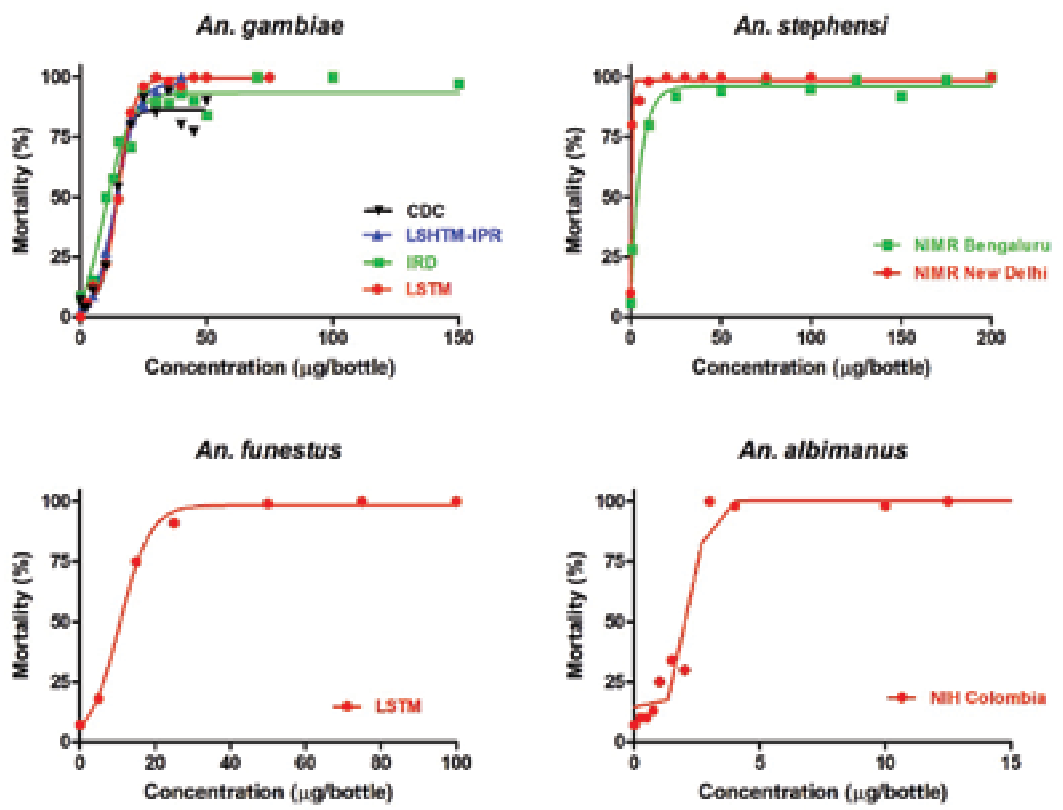
Source: reproduced with permission from reference *15.*

An. gambiae Kisumu – Ghana, Kenya, Madagascar, Nigeria, Uganda, Zambia; *An. coluzzii* Ngousso – Mali; *An. arabiensis* KGB – Ethiopia. The test conditions were 1-h exposure time, 72-h holding period; the target temperature was 27–29 °C and relative humidity 70–90%.

The LC_{99} with both An. gambiae and An. funestus ranged from 36 (95% CI, 29 ; 55) to 128 (95% CI, 76 ; 461) µg/bottle, whereas the LC_{100} ranged from 30 to 75 µg/bottle. The wide 95% CI of the estimated value for LC_{99} is due to the difficulty in achieving a plateau of mortality at high concentrations. The differences were even more pronounced with An. stephensi, for which the LC_{99} ranged from 18 (95% CI, 9 ; 80) at NIMR-D to 234 (95% CI, 104 ; 961) µg/bottle at NIMR-B. *An.* albimanus showed the greatest susceptibility to chlorfenapyr, with LC_{100} and LC_{99} values of 3 and 6 (95% CI, 4 ; 61) µg/bottle, respectively. As observed during the development phase (see section 7.2.1), relatively high inter-laboratory variation in test results was reported, which was due to the lower number of tests with chlorfenapyr than with other insecticides, which represented only 5% of the total number of mosquitoes tested.

On the basis of the results and data from both WHO-supervised and non-WHO-supervised tests, the TDCs for step-3 testing were 100 and 200 µg/bottle for all *Anopheles* species.

Fig. 34. Concentration–response curves for chlorfenapyr against various *Anopheles* species (WHO-supervised study)



For *An. gambiae,* the numbers of replicates tested were 3 by CDC, 2 by IRD, 1 by LSHTM and 1 by LSTM. For *An. stephensi*, the numbers of replicates were 3 by NIMR-B and 1 by NIMR-D. For *An. funestus* and *An. albimanus*, only one replicate each was tested.

Pyriproxyfen

Fig. 35 shows the percentage mortality of susceptible adult *Anopheles* mosquitoes exposed to serial concentrations of pyriproxyfen after a 72-h holding period. Bioassays were conducted only with *An. gambiae* (IRD, LSTM, CDC, LSHTM) and *An. stephensi* (NIMR) because of technical difficulties in conducting bottle bioassays with pyriproxyfen at all sites and against all the targeted species.

Overall, 6480 mosquitoes were tested in WHO bottle bioassays (including controls). Mosquito mortality varied by laboratory and no clear concentration–response relation was seen for the acute toxicity of pyriproxyfen. At the end of the holding period (72 h), all surviving females at each concentration and control were chambered individually in paper cups for measurement of oviposition rates.

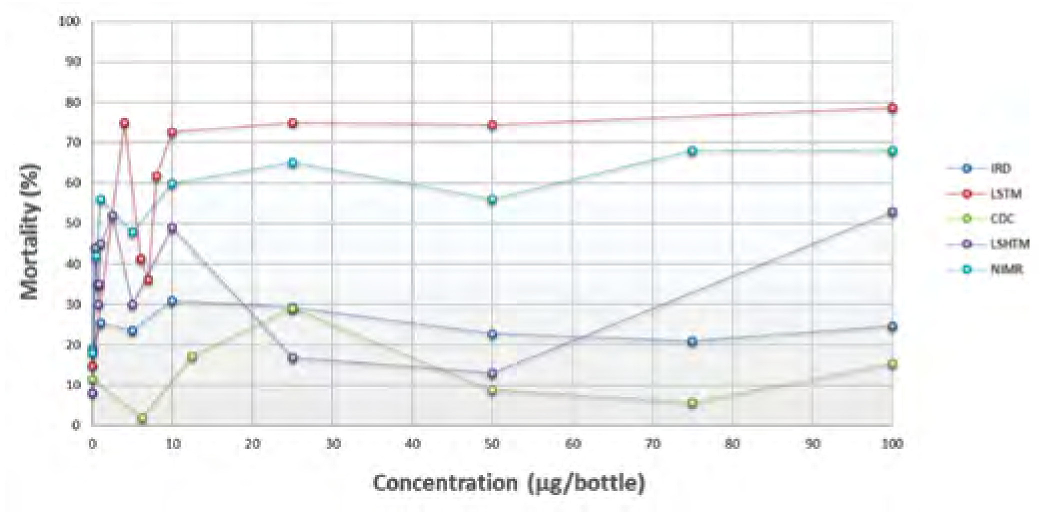
Fig. 36 shows the OI rates for *An. gambiae* and *An. stephensi* after the chambering period (see details in section 7.2.1 and in the SOP *(14)*). Because of time constraints, only one or two replicates were tested at each centre. Bioassay results with *An. gambiae* were consistent among laboratories, except at LSTM, where the range of concentration required to inhibit

100% of oviposition was 10 times lower (0.8–4 µg/bottle) than at the other centres (75–100 µg/bottle at IRD, CDC and LSHTM). The small number of live females chambered because of relatively high mortality 72 h after exposure may partly explain the difference.

The highest OI_{100} and OI_{99} (estimated by log-probit analysis) values were 100 and 220 (95% CI, 73 ; 2000) µg AI/bottle, respectively (Table 37). A similar trend was seen for *An. stephensi*, with OI_{100} and OI_{99} of 75 and 120 (95% CI, 60 ; 592) µg AI/bottle, respectively.

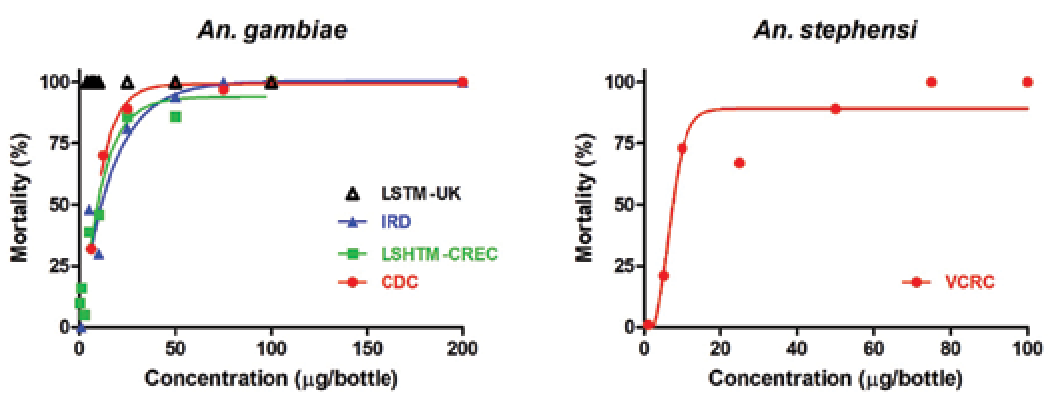
In view of the wide confidence intervals of the estimates, the TDCs were based on the observed values for OI_{100}, which were 100 and 200 µg AI/bottle for all *Anopheles* species tested.

Fig. 35. Mortality of susceptible *Anopheles* strains exposed to pyriproxyfen-coated bottles after a 72-h holding period



An. gambiae Kisumu strain was used at IRD, LSHTM, LSTM and CDC; *An. stephensi* was used at VCRC; total sample size, 6480 mosquitoes.

Fig. 36. Oviposition inhibition rates with pyriproxyfen tested in bottle bioassays against susceptible adult *An. gambiae* and *An. stephensi*



One replicate was tested at each centre, except for CDC and LSTM, where two replicates were tested.

Table 28. LC_{100}, LC_{99} (and 95% CI) and tentative discriminating concentrations of prallethrin against *Aedes* species

Species	Centre (country)	Sample size (no. of test concentrations)	Observed LC_{100} (µg/ bottle)	Twice the LC_{100} (µg/ bottle)	Estimated LC_{99} (95% CI) (µg/ bottle)	Twice the LC_{99} (µg/ bottle)	TDC selected for step 3	Strength of evidence
Ae. aegypti	IRD (France)	2879 (8)	15	30	8 (5.8–19)	16	**30 µg/bottle**	Moderately strong
Ae. albopictus	IRD (France)	2627 (7)	10	20	8.5 (7.007–12.04)	17	**30 µg/bottle**	Very strong
	FIOCRUZ (Brazil)	2211 (7)	NA	NA	4 (2.3–19.4)	8		
	VCRU (Malaysia)	2261 (7)	10	20	13 (9.6–31.03)	26		

The highest LC_{99} used to establish the TDCs for a given species are shown in blue; the TDCs selected to undergo step 3 (validation) are given in **bold**.

NA: not available.

Table 29. LC_{100}, LC_{99} (and 95% CI) and tentative discriminating concentrations of metofluthrin against *Aedes* species

Species	Centre (country)	Sample size (no. of test concentrations)	Observed LC_{100} (µg/ bottle)	Twice the LC_{100} (µg/ bottle)	Estimated LC_{99} (95% CI) (µg/ bottle)	Twice the LC_{99} (µg/ bottle)	TDC selected for step 3	Strength of evidence
Ae. aegypti	IRD (France)	2922 (8)	0.5	1	0.3 (0.21–1.65)	0.6	**1 µg/bottle**	Moderately strong
Ae. albopictus	IRD (France)	2556 (8)	0.4	0.8	0.35 (0.29–0.54)	0.7	**1 µg/bottle**	Very strong
	FIOCRUZ (Brazil)	2216 (7)	0.2	0.4	0.21 (0.16–0.42)	0.4		
	VCRU (Malaysia)	2250 (7)	0.5	1	0.50 (0.37–0.81)	1		

The highest LC_{99} used to establish the TDCs for a given species are shown in blue; the TDCs selected to undergo step 3 (validation) are given in **bold**.

NA: not available.

Table 30. LC_{100}, and LC_{99} (and 95% CI) and tentative discriminating concentrations of transfluthrin against *Aedes* species

Species	Centre (country)	Sample size (no. of test concentrations)	Observed LC_{100} (µg/bottle)	Twice the LC_{100} (µg/bottle)	Estimated LC_{99} (95% CI) (µg/bottle)	Twice the LC_{99} (µg/bottle)	TDC selected for step 3	Strength of evidence
Ae. aegypti	IRD (France)	2541 (7)	NA	NA	1.5 (1.16–2.35)	3.0	**3 µg/bottle**	Very strong
	NIMR-D (India)	1594 (6)	0.7	1.4	0.7 (0.60–0.75)	1.3		
	NEA (Singapore)	1795 (6)	1.5	3	1.0 (NA)	2.0		
Ae. albopictus	IRD (France)	2510 (7)	1.2	2.4	0.8 (0.62–77.1)	1.6	**3 µg/bottle**	Very strong
	VCRU (Malaysia)	2257 (7)	1.2	2.4	1.5 (1.12–12.2)	3.0		
	FIOCRUZ (Brazil)	2188 (7)	0.15	0.3	0.2 (0.15–0.22)	0.4		

The highest LC_{99} used to establish the TDCs for a given species are shown in blue; the TDCs selected to undergo step 3 (validation) are given in **bold**.

NA: not available.

Table 31. LC_{100}, LC_{99} (and 95% CI) and tentative discriminating concentrations of transfluthrin against *Anopheles* species

Species	Centre (country)	Sample size (no. of test concentrations)	Observed LC_{100} (µg/bottle)	Twice the LC_{100} (µg/bottle)	Estimated LC99 (95% CI) (µg/bottle)	Twice the LC_{99} (µg/bottle)	TDC selected for step 3	Strength of evidence
An. gambiae	IRSS (Burkina Faso)	2317 (7)	0.5	1	0.48 (0.38–0.72)	0.95	**2 µg/bottle**	Strong
	IPR (Côte d'Ivoire)	1963 (6)	0.3	0.6	0.15 (0.12–0.22)	0.3		
An. stephensi	NIMR-D (India)	1674 (6)	NA	NA	0.12 (0.08–0.42)	0.25	**2 µg/bottle**	Very strong
	NIMR-B (India)	2286 (7)	0.8	1.6	1.12 (0.67–3.67)	2.24		
	VCRC (India)	2487 (8)	0.4	0.8	0.43 (0.34–0.77)	0.87		
An. albimanus	NIH (Colombia)	2666 (7)	0.1	0.2	0.05 (0.03–0.19)	0.1	**2 µg/bottle**	Strong
	NIH (Peru)	2215 (7)	0.7	1.4	0.62 (0.53–0.807)	1.2		
An. funestus (step 1 data)	NICD (South Africa)	659 (10)	0.2	0.4	NA	NA	**2 µg/bottle**	Weak
	LSTM (UK)	450 (10)	1.5	3	NA	NA		
An. minimus (step 1 data)	KU (Thailand)	621 (12)	1.25	2.5	NA	NA	**2 µg/bottle**	Weak
	CDC (USA)	794 (9)	0.5	1	NA	NA		

The highest LC_{99} used to establish the TDCs for a given species are shown in blue; the TDCs selected to undergo step 3 (validation) are given in **bold**.

NA: not available.

Table 32. LC_{100}, LC_{99} (with their 95% CI) and tentative discriminating concentrations of clothianidin against *Aedes* species

Species	Centre (country)	Sample size (no. of test concentrations)	Observed LC_{100} (µg/bottle)	Twice the LC_{100} (µg/bottle)	Estimated LC_{99} (95% CI) (µg/bottle)	Twice the LC_{99} (µg/bottle)	TDC selected for step 3	Strength of evidence
Ae. aegypti	IRSS (Burkina Faso)	1952 (6)	NA	NA	2 (NA)	4	**10 and 20 µg/bottle[a]**	Very strong
	KCMC (U.R. Tanzania)	1298 (6)	20	40	13 (11.8–16.05)	26		
	UANL (Mexico)	2250 (7)	NA	NA	9 (5.02–33.4)	18		
	IRD (France)	2445 (8)	5	10	2.5 (NA)	5		
Ae. albopictus	IRD (France)	3583 (10)	10	20	3.6 (3.1–4.5)	7	**10 µg/bottle**	Very strong
	FIOCRUZ (Brazil)	*2224 (7)*	5	10	3.0 (2.03–12.6)	6		
	VCRU (Malaysia)	2250 (7)	NA	NA	4 (2.3–37.3)	8		

The highest LC_{99} used to establish the TDCs for a given species are shown in blue; the TDCs selected to undergo step 3 (validation) are given in **bold**.

NA: not available.

[a] Two concentrations were selected for testing in step 3 because of wide variation in step-2 data.

Table 33. LC_{100}, LC_{99} (and 95% CI) and tentative discriminating concentrations of clothianidin against *Anopheles* species

Species	Centre (country)	Sample size (no. of test concentrations)	Observed LC_{100} (µg/bottle)	Twice the LC_{100} (µg/bottle)	Estimated LC_{99} (95% CI) (µg/bottle)	Twice the LC_{99} (µg/bottle)	TDC selected for step 3	Strength of evidence
An. gambiae	IRD (France)	3204 (10)	2	4	2 (1.3–5.2)	4	**4 µg/bottle**	Strong
	KCMC (U.R. Tanzania)	2252 (7)	1	2	0.6 (0.4–1.03)	1.5		
	LSTM (UK)[a]	NA	1.75	3.5	NA	NA		
An. stephensi	NIMR B (India)	2072 (6)	1.5	3	1.8 (1.3–8.9)	3.6	**10 µg/bottle**	Very strong
	NIMR-D (India)	2095 (7)	2	4	4 (2.6–11.3)	8		
	VCRC (India)	2250 (7)	NA	NA	3.2 (2.5–4.8)	6.5		
An. albimanus	CDC(USA)	2471 (8)	1	2	1.6 (1.1–5.2)	3	**6 µg/bottle**	Strong
	NIH (Colombia)	2364 (8)	2	4	2.8 (1.6–7.5)	6		
An. funestus	LSTM (UK)	599 (6)	2	4	1.5 (NA)	3	**3 µg/bottle**	Moderately strong
	NICD (South Africa)	NA	NA	NA	NA	NA		
	CDC (USA)	NA	NA	NA	NA	NA		
An. minimus (step-1 data)	KU (Thailand)	457 (8)	3	6	4.9 (3.4–8.5)	10	**6 µg/bottle**	Weak
	CDC (USA)	684 (6)	NA	NA	1.15 (NA)	2		

The highest LC_{99} used to establish the TDCs for a given species are shown in blue; the TDCs selected to undergo step 3 (validation) are given in **bold**.

NA: not available.

[a] Non-WHO-supervised trial data (provided as complementary information).

Table 34. LC_{100}, LC_{99} (and 95% CI) and tentative discriminating concentrations of flupyradifurone against *Aedes* species

Species	Centre (country)	Sample size (no. of test concentrations)	Observed LC_{100} (µg/bottle)	Twice the LC_{100} (µg/bottle)	Estimated LC_{99} (95% CI) (µg/bottle)	Twice the LC_{99} (µg/bottle)	TDC selected for step 3	Strength of evidence
Ae. aegypti	IRD (France)	2235 (7)	NA	NA	20 (12.2–161.8)	40	**80 µg/bottle**	Very strong
	KCMC (U.R Tanzania)	2713 (7)	30	60	40 (27.9–112.9)	80		
	NIMR-D (India)	1687 (6)	NA	NA	20 (NA)	40		
Ae. albopictus	FIOCRUZ (Brazil)	2360 (8)	30	60	29 (21.9–51.5)	58	**80 µg/bottle**	Very strong
	VCRU (Malaysia)	2261 (7)	10	20	13 (9.6–31.03)	26		
	IRD (France)	2355 (9)	NA	NA	41 (23–166)	80		

The highest LC_{99} used to establish the TDCs for a given species are shown in blue; the TDCs selected to undergo step 3 (validation) are given in **bold**.

NA: not available.

Table 35. LC_{100}, LC_{99} (and 95% CI) and tentative discriminating concentrations of flupyradifurone against *Anopheles* species

Species	Centre (country)	Sample size (no. of test concentrations)	Observed LC_{100} (µg/bottle)	Twice the LC_{100} (µg/bottle)	Estimated LC_{99} (95% CI) (µg/bottle)	Twice the LC_{99} (µg/bottle)	TDC selected for step 3	Strength of evidence
An. stephensi	VCRC (India)	2270 (7)	30	60	27(20.6–41.2)	54	**60 µg/bottle**	Very strong
	NIMR-B (India)	2349 (7)	20	40	22 (18.9–29.5)	44		
	NIMR-D (India)	1585 (7)	20	40	22.6 (NA)	45		
An. gambiae	OCEAC (Cameroon	2032 (7)	30	60	23 (16.4–54.8)	46	**60 µg/bottle**	Very strong
	Swiss TPH (Switzerland)	1774 (6)	12	24	8.4 (5.5–42.3)	17		
	IPR (Côte d'Ivoire)	2019 (6)	20	40	21 (17.5–29.9	42		
An. minimus	KU (Thailand) [a]	1963 (7)	200	400	440 (NA)	880	**60 and 100 µg/bottleb**	Strong
	MU (Thailand)	1495 (7)	50	100	56 (34.5–164.1)	112		
	CDC (USA)	2179 (7)	20	40	27.3 (17.8–92.3)	56		
An. funestus (step 1)	NICD (South Africa)	437 (8)	40	80	NA	NA	**100 µg/bottle**	Weak
	LSTM (UK)	236 (8)	50	100	NA	NA		
An. albimanus	NIH (Peru)	1607 (7)	NA	NA	239 (98–3195)	480	**300 and 500 µg/bottleb**	Moderately strong
	CDC (USA)	2413 (7)	150	300	170 (120–2158)	340		

The highest LC_{99} used to establish the TDCs for a given species are shown in blue; the TDCs selected to undergo step 3 (validation) are given in **bold**.

NA: not available.

[a] Centre with much higher values than other laboratories; data were excluded in selecting the TDC.

[b] Two concentrations were selected for testing in step 3 because of wide variation in step-2 data.

Table 36. LC_{100}, LC_{99} (and 95% CI) and tentative discriminating concentrations of chlorfenapyr against *Anopheles* species

Species	Centre (country)	Sample size (no. of test concentrations)	Observed LC_{100} (µg/bottle)	Twice the LC_{100} (µg/bottle)	Estimated LC_{99} (95% CI) (µg/bottle)	Twice the LC_{99} (µg/bottle)	TDC selected for step 3[a]
An. gambiae	CDC (USA)	1730 (12)	NA	NA	128 (76–461)	256	**100 and 200 µg/bottle**
	IRD (France)	1370 (14)	70	140	111 (70.5–262)	222	
	LSTM (UK)	681 (12)	30	60	36 (29–55.4)	72	
	LSTHM (Côte d'Ivoire)	579 (10)	45	95	46 (38.8–60.3)	92	
An. stephensi	NIMR-D (India)	440 (10)	20	40	18.6 (9.3–79.8)	36	
	NIMR B (India)	1618 (10)	125	250	234 (105–961)	468	
An. albimanus	NIH (Colombia)	743 (12)	3	6	6 (3.8–61.6)	12	
An. funestus	LSTM (UK)	653 (6)	75	150	46 (36.7–61.2)	92	

The TDCs selected to undergo step 3 (validation) are shown in **bold**.

NA: not available.

[a] The strength of the evidence could not be assessed.

Table 37. OI_{100}, OI_{99} (and 95% CI) and tentative discriminatory concentrations of pyriproxyfen against *Anopheles* species

Species	Centre (country)	Sample size (no. of test concentrations)	Observed LC_{100} (µg/ bottle)	Twice the LC_{100} (µg/ bottle)	Estimated LC_{99} (95% CI) (µg/ bottle)	Twice the LC_{99} (µg/bottle)	TDC selected for step 3
An. gambiae	IRD (France)	513 (8)	75	150	115 (47.6–2275)	230	**100 and 200 µg/bottlea**
	CDC (USA)	1038 (10)	100	200	69 (43.3–168)	140	
	LSHTM (Benin)	1130 (11)	100	200	220 (72.8–2093)	440	
	LSTM (UK)	359 (8)	4	8	NA	NA	
An. stephensi	VCRC (India)	457 (9)	75	150	123 (59-592)	250	

The highest LC_{99} used to establish the TDCs for a given species are shown in blue; the TDCs selected to undergo step 3 (validation) are given in **bold**.

NA: not available; OI: oviposition inhibition.

[a] Two concentrations were selected for testing in step 3 because of wide variation in step-2 data.

7.2.4 Validation of tentative discriminating concentrations in step 3 for WHO bottle bioassays

Twenty-one TDCs were tested against seven mosquito species in a total of 138 WHO bottle bioassays (34 with *Aedes* and 104 with *Anopheles* spp.) and a total of 14 305 mosquitoes (excluding controls) (Tables 38 and 39). When bioassays using a TDC did not provide the expected outcomes (i.e. ≥ 98% mortality or oviposition inhibition for pyriproxyfen), the test centres were asked to replicate the test before validating the results. Overall, mortality was ≥ 98% at all TDCs, except for flupyradifurone and pyriproxyfen, for which substantial inter-laboratory variation was observed with some *Anopheles* species.

Table 38. Mortality (%) of *Aedes* mosquitoes exposed to tentative discriminating concentrations in step 3 of WHO bottle assays (1-h exposure; 24-h recording time)

Insecticide	Species	Centre	TDC (µg/ bottle)	Mortality (%)	N
Metofluthrin	*Ae. aegypti*	IRD	1 µg/bottle	100	89
		CDC		100	121
		FIOCRUZ		100	99
		NIMR-D		100	100
	Ae. albopictus	IRD		100	100
		FIOCRUZ		100	98
		VCRU		100	100
Prallethrin	*Ae. aegypti*	IRD	30 µg/bottle	100	100
		LSHTM-CREC		100	271
		NEA		100	95
	Ae. albopictus	IRD		100	126
		FIOCRUZ		100	102
		VCRU		100	100
Transfluthrin	*Ae. aegypti*	IRD	3 µg/bottle	99	100
		NIMR-D		100	100
		NEA		100	87
	Ae. albopictus	IRD		100	112
		FIOCRUZ		100	99
		VCRU		100	100

Table 38 (Cont'd). Mortality (%) of *Aedes* mosquitoes exposed to tentative discriminating concentrations in step 3 of WHO bottle assays (1-h exposure; 24-h recording time)

Insecticide	Species	Centre	TDC (µg/bottle)	Mortality (%)	N
Clothianidin	*Ae. albopictus*	IRD	10 µg/bottle	100	100
		FIOCRUZ		100	98
		VCRU		100	100
	Ae. aegypti	IRSS	10 µg/bottle	100	105
		IRD		100	105
		UANL		100	100
		IRSS	20 µg/bottle	100	103
		IRD		100	105
		UANL		100	100
Flupyradifurone	*Ae. aegypti*	IRD	80 µg/bottle	100	90
		LSHTM-KCMC		100	101
		NIMR-New Delhi		100	100
	Ae. albopictus	IRD		100	116
		FIOCRUZ		100	97
		VCRU		100	100

For flupyradifurone, all the TDCs caused > 98% mortality of *An. gambiae*, *An. stephensi and An. funestus* (Table 39). With *An. minimus*, a TDC of 60 µg/bottle was insufficient to cause mortality above the WHO threshold, whereas 100 µg/bottle caused > 98% mortality at most sites. With *An. albimanus*, the TDC of 500 µg/bottle caused 100% mortality at CDC and NIH Peru, whereas the rate was < 98% at NIH Colombia regardless of the concentration and the replicate. Technical difficulties in testing flupyradifurone and MERO were reported by the Colombian laboratory; however, changing testing conditions (or even using a fan to accelerate drying of the bottles) did not change the outcomes.

With pyriproxyfen, 100 µg/bottle was sufficient to inhibit > 98% oviposition of *An. gambiae*, *An. stephensi* and *An. funestus* (Table 39). With *An. albimanus*, 100% OI was achieved at NIH Peru and NIH Colombia regardless of the concentration (100 and 200 µg/bottle), whereas OI was always < 98% at CDC. Despite the presence of eggs in oviposition chambers, the CDC reported that a few eggs laid after exposure of females to pyriproxyfen were viable. With *An. minimus*, OI ranged from 70 to 100% with 100 and 200 µg/bottle, but data were inconsistent among the centres. Establishment of TDCs using the data originally generated for *An. gambiae* and *An. stephensi* may explain the variable outcomes for other species. More work is necessary to establish and validate a DC of pyriproxyfen against *An. albimanus* and *An. minimus*.

Table 39. Mortality (%) of *Anopheles* spp. exposed to tentative discriminating concentrations in step 3 of WHO bottle bioassays (1-h exposure; 24-h recording time, except for chlorfenapyr, with which a 72-h holding period was adopted)

Insecticide	Species	Centres	Tentative DC	Mortality (%)	N
Transfluthrin	*An. albimanus*	CDC	2 µg/bottle	100%	116
		NIH Colombia		100%	99
		NIH Peru		100%	101
	An. stephensi	NIMR-B		100%	103
		NIMR-D		100%	100
		VCRC		100%	100
	An. funestus	LSTM		100%	97
		NICD		100%	107
		CDC		100%	111
	An. minimus	CDC		100%	120
		KU		100%	102
		MU		100%	101
	An. gambiae	IRSS		100%	104
		OCEAC		100%	93
		LSHTM-IPR		100%	298
Clothianidin	*An. stephensi*	NIMR-B	10 µg/bottle	100%	100
		VCRC		100%	100
		NIMR-D		100%	97
	An. gambiae	IRD	4 µg/bottle	99%	106
		LSHTM-KCMC		100%	96
		LSTM		100%	101
	An. funestus	LSTM	3 µg/bottle	100%	100
		NICD		100%	115
		CDC		100%	106
	An. albimanus	CDC	6 µg/bottle	100%	104
		NIH Colombia		100%	98
		NIH Peru		100%	97
	An. minimus	CDC		100%	117
		KU		98%	94
		MU		100%	104

Table 39 (Cont'd). Mortality (%) of *Anopheles* spp. exposed to tentative discriminating concentrations in step 3 of WHO bottle bioassays (1-h exposure; 24-h recording time, except for chlorfenapyr, with which a 72-h holding period was adopted)

Insecticide	Species	Centres	Tentative DC	Mortality (%)	N
Flupyradifurone	*An. gambiae*	Swiss TPH	60 µg/bottle	100%	100
		OCEAC		100%	98
		IRD		99%	104
		LSHTM-IPR		100%	302
	An. stephensi	NIMR-B		100%	102
		NIMR-D		100%	100
		VCRC		100%	100
	An. funestus	LSTM	100 µg/bottle	100%	98
		NICD		100%	115
	An. minimus	MU (R1)	60 µg/bottle	88%	48
		MU (R2)		94%	102
		KU (R1)		71%	91
		KU (R2)		82%	95
	An. minimus	CDC	100 µg/bottle	100%	43
		MU (R1)		98%	49
		MU (R2)		98%	102
		KU (R1)		97%	96
		KU (R2)		99%	98
	An. albimanus	CDC	300 µg/bottle	88%	100
		NIH Peru		100%	104
		NIH Colombia (R1)		38%	91
		NIH Colombia (R2)		88%	122
		NIH Colombia (R3)		32%	138
	An. albimanus	CDC	500 µg/bottle	100%	88
		NIH Peru		100%	103
		NIH Colombia(R1)		49%	105
		NIH Colombia (R2)		92%	149
		NIH Colombia (R3)		51%	152

Table 39 (Cont'd). Mortality (%) of *Anopheles* spp. exposed to tentative discriminating concentrations in step 3 of WHO bottle bioassays (1-h exposure; 24-h recording time, except for chlorfenapyr, with which a 72-h holding period was adopted)

Insecticide	Species	Centres	Tentative DC	Mortality (%)	N
Chlorfenapyr	*An. gambiae*	IRD	100 µg/bottle	100%	107
		LSTM		100%	128
	An. albimanus	NIH P		100%	96
		NIH C		100%	108
	An. funestus	NICD		100%	97
	An. stephensi	NIMR-D		100%	100
		NIMR-B		100%	102
		VCRC (R1)		79%	101
		VCRC (R2)		100%	100
	An. gambiae	IRD	200 µg/bottle	100%	116
	An. albimanus	NIH P		100%	98
		NIH C		100%	110
	An. funestus	NICD		100%	97
	An. stephensi	NIMR-D		100%	100
		NIMR-B		98%	98
		VCRC (R1)		89%	100
		VCRC (R2)		100%	100
Pyriproxyfen	*An. gambiae*	IRD	100 µg/bottle	100%	96
		LSHTM-CREC		100%	154
		CDC		100%	100
	An. gambiae	IRD	200 µg/bottle	100%	104
		LSHTM-CREC		100%	181
		CDC		100%	91
	An. stephensi	NIMR-B	100 µg/bottle	100%	81
		NIMR-D		100%	34
		VCRC		100%	49
	An. stephensi	NIMR-B	200 µg/bottle	100%	80
		NIMR-D		100%	25
		VCRC		100%	44
	An. funestus	LSTM	100 µg/bottle	100%	53
		NICD		100%	97
		CDC		94%	104
	An. funestus	LSTM	200 µg/bottle	81%	49
		NICD		98%	100
		CDC		100%	72

Table 39 (Cont'd). Mortality (%) of *Anopheles* spp. exposed to tentative discriminating concentrations in step 3 of WHO bottle bioassays (1-h exposure; 24-h recording time, except for chlorfenapyr, with which a 72-h holding period was adopted)

Insecticide	Species	Centres	Tentative DC	Mortality (%)	N
	An. albimanus	CDC	100 µg/bottle	41%	109
		NIH Colombia		100%	112
		NIH Peru		100%	116
	An. albimanus	CDC	200 µg/bottle	88%	99
		NIH Colombia		100%	84
	An. minimus	CDC	100 µg/bottle	70%	109
		KU		94%	98
		MU		99%	97
	An. minimus	CDC	200 µg/bottle	86%	111
		KU		98%	97
		MU		100%	100

N: number of mosquitoes tested; NA: not available. R1, R2 and R3 are replicates 1–3.
For pyriproxyfen, "N" indicates the total number of females alive at 72 h after exposure and chambered.

7.2.5 Main constraints encountered

The main limitations and constraints reported by the participating laboratories were as follows:

- Difficulty in establishing adequate bottle bioassay protocols for testing dinotefuran, imidacloprid (neonicotinoids) and indoxacarb (oxadiazine) against either *Aedes* or *Anopheles* spp. mosquitoes. The distinct modes of action and/or physical properties of these compounds may require use of specific testing and/or holding conditions (e.g. exposure time, bottle drying time, surfactant, temperature, humidity), and further work is necessary.

- Lack of reproducibility and inconsistent results observed with some compounds, e.g. chlorfenapyr, flupyradifurone, transfluthrin and clothianidin. This may be due to difficulty in ensuring homogeneous coating and drying of glass bottles, especially with volatile compounds and insecticides that require use of a surfactant. For chlorfenapyr, inconsistent results were reported when the temperature during the test was below 25 °C. The testing laboratories were advised to perform tests strictly according to the SOPs, i.e. testing and holding temperature and relative humidity at 27° ± 2 °C and 75 ± 10%, respectively.

- Difficulty in generating large concentration–response data sets for *An. minimus* and *An. funestus*, which resulted in weaker evidence for establishing TDCs than for other species.

- Lack of evidence to propose and validate adequate DCs for pyriproxyfen for all *Anopheles* species because of the difficulty of conducting the test, which requires maintenance of adequate testing and long holding conditions throughout the study.
- Delay in obtaining results in step 3 because of limited access to testing laboratories due to the COVID-19 pandemic.

8 Conclusions

This report provides details of a WHO-led multi-centre study conducted in 2017–2021 to determine DCs of 18 insecticide compounds and one synergist (PBO). The compounds belong to nine classes and were either already used in formulated vector control products or were being evaluated for use in products for indoor residual sprays, insecticide-treated nets, space sprays or spatial repellents for control of vectors of malaria, arboviral diseases and household pests (see Tables 5 and 6 for details). A total of 23 laboratories worldwide participated in the study and used standardized test protocols with laboratory-colonized, well-characterized susceptible *Aedes* and *Anopheles* species.

Overall, 417 878 mosquitoes were tested in 715 validated tests. To our knowledge, this study provides the largest toxicology dataset available on mosquitoes. From the evidence generated, we established and validated 17 new DCs for *Aedes* spp. (Tables 1 and 2) and 13 new DCs for *Anopheles* spp. (Tables 3 and 4) in either WHO tube tests or WHO bottle bioassays. The DCs were established according to a step-by-step design (go/no-go at each of three steps) and validated by independent experts during WHO consultations. When the DCs of a particular compound for different species were within a close range, a common or single DC for the species was adopted by consensus to reduce the number of DCs for routine monitoring of resistance in national disease control programmes.

In the following sections, the results and technical difficulties reported by participating laboratories are discussed and a way forward is proposed to improve laboratory-testing procedures and methods for future selection of DCs.

8.1 WHO tube tests

A total of 215 000 mosquitoes, representing five *Anopheles* spp. and two *Aedes* spp., were used to test the 18 insecticides and PBO. WHO-recommended carrier oils were used for impregnating insecticides onto Whatman no. 1 filter papers, i.e. silicone oil for pyrethroids and olive oil for carbamates and organophosphates, except for pirimiphos-methyl, for which no oil was used as per the manufacturer's instructions. The tests were performed under WHO-recommended conditions of temperature (27 ± 2 °C) and relative humidity (75 ± 10%). The mosquitoes were exposed for 1 h in tubes and held for 24 h as per the WHO guidelines *(10)*.

All the participating institutions had good knowledge and expertise in performing the WHO filter paper test before the study started. Overall, consistent data were obtained for replicates.

For *Anopheles* species, three new DCs were established in addition to the list previously published by WHO *(10)*, which were 0.3% alpha-cypermethrin against both *An. stephensi* and *An. albimanus* (the existing DC of 0.05% remains valid for all other *Anopheles* species) and 100 or 150 mg/m^2 for pirimiphos-methyl according to the species (see Table 3 for details). It should be noted that the unit used for the DC for pirimiphos-methyl is mg AI/m^2 rather than the percentage used previously, as no carrier oil is used for impregnating filter papers. The previous WHO-recommended DC for this insecticide was 0.25% *(10)*, which corresponds to approximately 100 mg AI/m^2. These values will enable entomologists to compare historical data on this compound with new data to detect any change in resistance frequencies in wild populations of mosquitoes. WHO has started a separate study of storage stability to determine the shelf-life of insecticide-treated papers, including those treated with pirimiphos-methyl.

For *Aedes* spp., 11 new DCs were established and validated for pyrethroids (permethrin, lambda-cyhalothrin, deltamethrin and alpha-cypermethrin), organophosphates (chlorpyrifos-ethyl, malathion and pirimiphos-methyl) and a carbamate (bendiocarb) (see Table 1 for details). The results indicate that all the interim DCs published in the WHO interim guidance on Zika virus disease *(11)* were underestimates, except that for deltamethrin (0.03%). For most insecticides (except malathion and alpha-cypermethrin), a single DC was adopted for both *Ae. albopictus* and *Ae. aegypti,* which will facilitate the logistics of treating papers for monitoring resistance.

There was insufficient evidence to propose a suitable concentration of PBO for synergistic bioassays with *Aedes* spp., as no mosquito mortality was observed with PBO-treated papers at concentrations of 0.1–20% and no sublethal concentration could be determined for *Ae. aegypti*. In addition, the results with the provisional test protocol established by the participating laboratories to measure the potentiation effect of PBO on alpha-cypermethrin (used at LC_{50}) were inconclusive, as no plateau of mortality (100%) was reached with susceptible mosquito strain regardless of the concentration (1–20%) of PBO. Recently colonized mosquitoes that express oxidase-based resistance might have to be used in synergistic tests with PBO to determine an optimum concentration of PBO for *Aedes* spp.

8.2 WHO bottle bioassays

During the first WHO consultation in 2017, experts in the public and private sectors reviewed the test protocols for monitoring insecticide resistance in mosquitoes and concluded that the CDC bottle assay *(13)* could allow testing of insecticide compounds that are not technically suitable for impregnation on filter papers because of instability. Consequently, glass bottles were explored for testing susceptibility to some compounds with certain properties and/or modes of action (group B compounds). The bottle bioassay offers greater flexibility and allows use of additives or surfactants that prevent crystallization of insecticides and ensure uniform coating of bottles *(16–18)*. The CDC bottle assay, however, measures the immediate effect (knock down) after a diagnostic period of exposure (typically 30 min) *(13)*. To harmonize the end-points with those measured in WHO filter paper bioassays *(10, 12)*, the bottle assays were adapted by WHO to measure knock down after 1 h of exposure and mortality at 24 h (or up to 72 h for insecticides with slow killing action). This modified assay is referred to as the "WHO bottle bioassay".

Overall, 203 104 mosquitoes were used for testing 11 insecticides in WHO bottle bioassays. Despite technical difficulties reported during the study (see section 7.2.1), the WHO method was found to be adequate for testing mosquito susceptibility to insecticides of various chemical classes, including pyrethroids (metofluthrin, prallethrin and transfluthrin), neonicotinoids (clothianidin), pyrroles (chlorfenapyr), juvenile hormone mimics (pyriproxyfen) and butenolides (flupyradifurone). The evidence from the multi-centre study was used to establish and validate 11 new DCs for *Aedes* spp. (see Table 2) and 6 for *Anopheles* spp. (see Table 4). The new bottle bioassay will be useful for countries and research institutions in determining the baseline susceptibility of wild populations of vectors of malaria and *Aedes*-borne diseases to these new compounds for public health use.

Despite the good results obtained, wide variation was reported among laboratories for some mosquito species–insecticide combinations (especially at the top of concentration–response curves). Lack of reproducibility was frequent with transfluthrin, flupyradifurone and clothianidin against *Anopheles* strains (up to 30% of tests failed). For volatile pyrethroids such as transfluthrin, prallethrin and metofluthrin, possible loss of insecticide content in coated bottles during the 24-h drying time might explain the variation in test results, especially when low concentrations were used (the LC_{100} was 0.5–1.5 µg AI/bottle for metofluthrin and transfluthrin and 15 µg AI/bottle for prallethrin). The results nevertheless confirmed that the WHO bottle bioassay is suitable for establishing a DC to monitor phenotypic resistance.

Previous attempts were made to establish DCs for clothianidin in *Anopheles* spp. *(16, 17, 19)*, but with different test methods and test conditions (e.g. different holding times, no additives); therefore, interpretation and comparison of the results with this study was difficult. In this study, we standardized the test protocols for generating concentration–response data, used a large sample size (≈ 45 000 mosquitoes) and cross-validated the results in independent laboratories, providing confidence in the study outcomes. In contrast to the other studies, no increase in mortality rates was reported after 24–48-h or 72-h post-exposure; consequently, a holding period of 24 h was considered adequate for testing clothianidin in WHO bottle bioassays.

Use of MERO as a surfactant to prevent crystallization of clothianidin and flupyradifurone in coated glass bottles proved challenging. Some centres reported high mortality rates (> 20%) in control batches of *Anopheles* spp. when MERO was used at the manufacturer-recommended concentration of 1500 ppm. During the bioassay, some mosquitoes were observed sticking to the walls of coated bottles, possibly because of the presence of fine droplets of MERO or humidity, causing their death. Reduction of the concentration of MERO to 800 ppm for all *Anopheles* spp. except *An. albimanus*, which required 200 ppm, was found to reduce mosquito mortality in control bottles. Furthermore, it was found that the absence of visible droplets in bottles before introducing mosquitoes was essential to the success of bioassays. It was recommended that, if droplets remained visible after the bottles have been dried for the specified time, each bottle should be rolled manually for at least 10 min in front of a fan (or for a minimum of 20 min when a laboratory roller machine is used) and dried under a fume hood in a horizontal position overnight. It was suggested that acetone and surfactant not be used at cold temperatures to avoid condensation of water vapour inside bottles.

Chlorfenapyr caused 100% mortality of *Anopheles* species at concentrations of 12.5–125 µg AI/bottle; however, a plateau of mortality was not always achieved. Temperature was shown to affect the results significantly, and bioassays could be validated only when the test temperature was maintained at 27 °C ± 2 °C and relative humidity at 75% ± 10%. The results were also more consistent when the holding period was extended beyond 24 h and up to 72 h, in agreement with previous observations *(15–17)*. No abnormal mortality was reported in controls up to 72 h. Data from both WHO- and non-WHO-supervised trials *(14)* showed that a DC of 100 µg/bottle was suitable, and this DC was adopted for all *Anopheles* species at the third WHO consultation. In view of the technical difficulties encountered in bottle bioassays with chlorfenapyr, specific instructions and guidance will be required for monitoring insecticide resistance in wild mosquito populations in the field (see section 7.2.1 and the SOP for bottle bioassays *(14)*).

The WHO bottle bioassay was shown in many centres to be effective and reproducible and could be used to establish a DC to monitor the sterilizing properties of pyriproxyfen. The percentage OI was selected as the end-point, and a DC of 100 µg AI/bottle was adopted for *An. gambiae, An. funestus* and *An. stephensi*; bioassay results were inconclusive for other *Anopheles* species. The test requires that 7 days be allowed for females to lay eggs in a chambering system, which requires maintenance of suitable testing and holding conditions (27 °C ± 2 °C and 80% ± 10% relative humidity). Less than 20% mortality in control bottles 72 h after a 1-h exposure and > 30% oviposition rates in control mosquito chambers must be ensured to validate the test.

Inconsistent data and lack of reproducibility within and among laboratories were reported for three insecticides, imidacloprid, indoxacarb and dinotefuran. These compounds will probably require addition of a suitable surfactant to coat the bottles in order to achieve reproducible outcomes for concentration–response curves. Extension of the holding period to 72 h might be necessary to reduce the variation and inconsistency, as observed for other slow-acting compounds. In view of the promising prospects offered by these compounds for vector control, their manufacturers are encouraged to develop suitable methods and suggest end-points for determining DCs.

To facilitate monitoring with the WHO bottle bioassay, WHO should coordinate provision of pre-weighed vials of aliquots of technical-grade active ingredients and solvents to institutions and countries for coating bottles at their WHO-recommended DC.

8.3 Challenges and the way forward

In view of the wide variation in test results among laboratories, with differences in the distribution of lethal concentrations, further investigation is necessary to determine whether the variation is consistent for all mosquito species/insecticide combinations and whether three repetitions of tests, as used in this study, is sufficient to reduce the variation. Studies should also be conducted to determine whether the variation is significantly different in the bottle and filter paper bioassays.

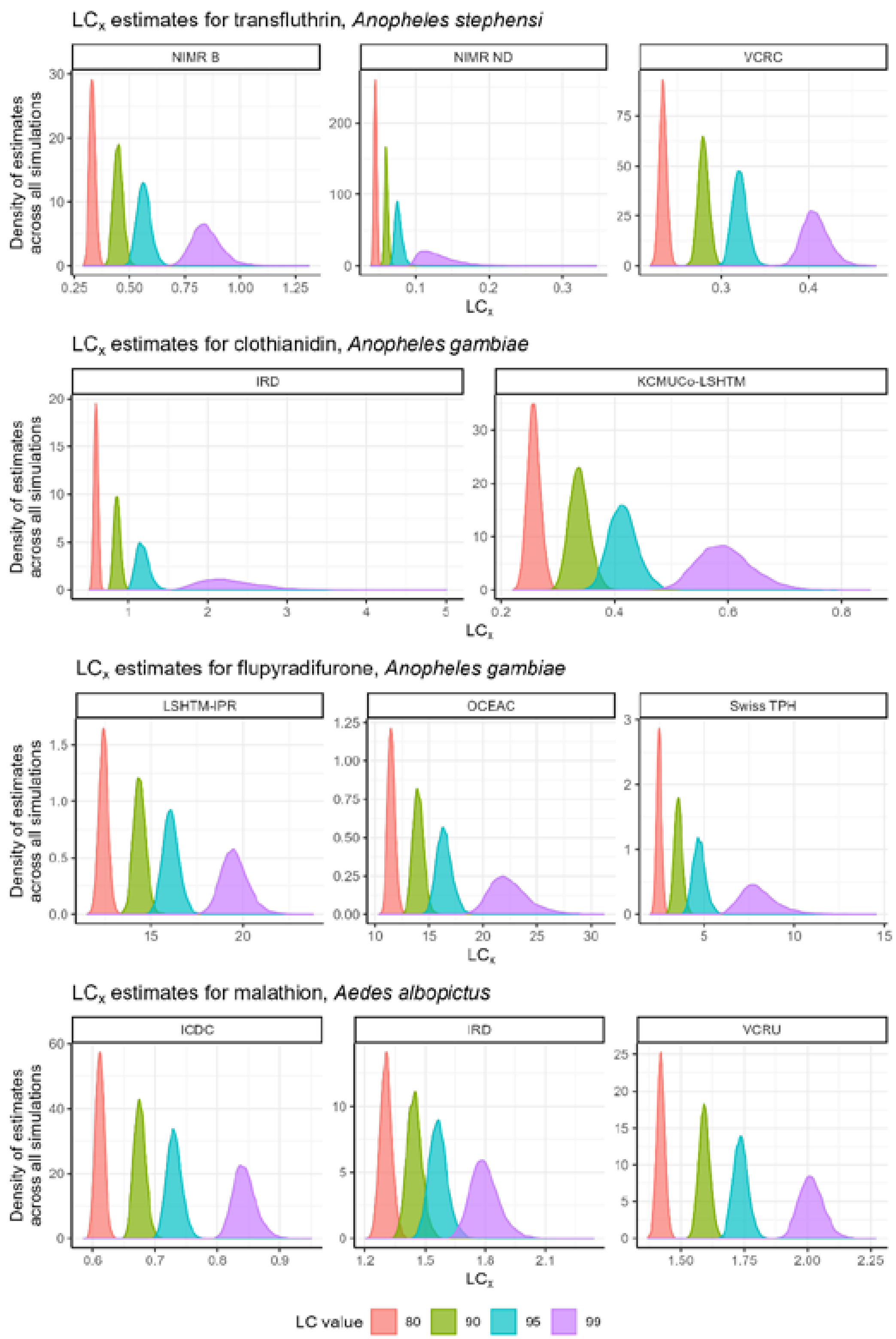
In this study, TDCs were established by doubling the highest LC_{99} found by three participating laboratories for each mosquito species or group of species where appropriate; LC_{100} was rarely used because it was not achieved at all test sites. Use of twice the LC_{99} values to estimate the DC was debated at the WHO consultation to overcome the uncertainty and margin of error at the top of the concentration–response curve. A Bayesian binomial model developed at Imperial College, London, to assess concentration–response statistics showed that the variation or uncertainty was much lower when lower LC values (e.g., LC_{95}, LC_{90} or LC_{80}) were used, regardless of the test method, type of insecticide or species (Fig. 37). Better understanding of variations in mortality at high concentrations of insecticides is essential, as this metric is used to determine an insecticide's DC. More robust and reproducible statistical analyses should be found to determine DCs, especially in small data sets, and to address the sources of the intra- and inter-laboratory variation in test results.

The suitability of treating filter papers or coating bottles with 5 times and 10 times concentrations of DCs of certain compounds is currently unknown. For bottle bioassays, further investigations are necessary to ensure that these test compounds do not crystallize at the higher concentrations used in intensity assays. For the filter paper tests, the stability and suitability of using 5 times and 10 times concentrations of pirimiphos-methyl should be carefully investigated, as no carrier oil is used for impregnating filter papers.

The link between resistance frequency, intensity and vector control failure in the field is unknown, and the results of intensity bioassays should be correlated with resistance mechanisms and operational significance. This will help in interpreting test results and guide selection of insecticides and management of insecticide resistance.

In the testing of pyriproxyfen, the experts raised concern about the difficulty in getting wild female mosquitoes to lay eggs in laboratory settings. To address this problem, the test protocol should be validated with field-caught populations in various settings. Dissection of ovaries to detect eggs could be an alternative approach to assessing mosquito resistance to pyriproxyfen when tests with oviposition end-points cannot be performed.

Fig. 37. Example of variations in estimates of lethal concentrations as measured in a Bayesian binomial model with a five-parameter logistic function



Source: Tom Churcher and Mara Kont, Imperial College, London, based on data from this study. "Tighter" density represents less variation in the population, whereas "wider" density indicates more variation in the laboratory population.

8.4 Research priorities for WHO tube tests

- The shelf-life of Whatman no. 1 filter papers treated with the newly recommended DCs of insecticides is unknown for most compounds and should be investigated. This is particularly important for pirimiphos-methyl-treated papers that do not contain a carrier oil.
- The dynamics of PBO-oxidases-pyrethroids in mosquitoes are not well understood. Better understanding could improve the method and criteria for insecticide–synergist bioassays and facilitate collection and interpretation of more useful data. Key factors to be considered include simultaneous versus sequential exposure to PBO, use of different concentrations and addition of surfactants or adjuvants.

8.5 Research priorities for WHO bottle bioassays

- More information is needed on how long treated bottles can be stored and how many times bottles treated with DCs of new compounds can be used and reused for bioassays. The length of storage of stock solutions should also be determined, especially for group B compounds.
- Better guidance is needed on bottle drying times and procedures, especially for volatile compounds that may evaporate more quickly than other insecticides.
- Studies should be conducted on the role and capacity of surfactants, such as MERO, in facilitating cuticular penetration of insecticide and any impact this might have on bioassay outcomes.
- The suitability of WHO bottle bioassays for alternative surfactants or additives should be confirmed, because MERO is currently produced by a single manufacturer (Bayer CropScience). A study by IRD sponsored by Sumitomo Chemical Co. Ltd. showed that SPAN 80 (used at 800 ppm against Anopheles spp. and at 1500 ppm against Aedes spp.) was suitable for conducting bottle assays with clothianidin (V. Corbel, IRD, unpublished data, 2021). More studies with various mosquito species and compounds should be conducted to determine the suitability of SPAN 80 for WHO bottle bioassays with other mosquito species and insecticide compounds.

References

1. Golding N, Wilson AL, Moyes CL, Cano J, Pigott DM, Velayudhan R et al. Integrating vector control across diseases. BMC Med. 2015;13:249 (doi.org/10.1186/s12916-015-0491-4).

2. Wilson AL, Courtenay O, Kelly-Hope LA, Scott TW, Takken W, Torr SJ et al. The importance of vector control for the control and elimination of vector-borne diseases. PLoS Negl Trop Dis. 2020;14(1): e0007831 (doi: 10.1371/journal.pntd.0007831).

3. Corbel V, Durot C, Achee N, Chandre F, Coulibaly MB, David JP et al. Second WIN International Conference on "Integrated approaches and innovative tools for combating insecticide resistance in vectors of arboviruses", October 2018, Singapore. Parasit Vectors. 2019;12(1):331 (doi: 10.1186/s13071-019-3591-8).

4. Kleinschmidt I, Bradley J, Knox TB, Mnzava AP, Kafy HT, Mbogo C et al. Implications of insecticide resistance for malaria vector control with long-lasting insecticidal nets: a WHO-coordinated, prospective, international, observational cohort study. Lancet Infect Dis. 2018;18(6):640–9 (doi.org/10.1016/S1473-3099(18)30172-5).

5. World malaria report: 2019. Geneva: World Health Organization; 2019 (https://apps.who.int/iris/handle/10665/330011).

6. Corbel V, Achee N, Chandre F, Coulibaly MB, Dusfour I, Fonseca DM et al. Tracking insecticide resistance in mosquito vectors of arboviruses: the Worldwide Insecticide resistance Network (WIN). PLoS Negl Trop Dis. 2016;10(12):e0005054 (doi: 10.1371/journal.pntd.0005054).

7. Moyes CL, Vontas J, Martins AJ, Ng LC, Koou SY, Dusfour I et al. Contemporary status of insecticide resistance in the major Aedes vectors of arboviruses infecting humans. PLoS Negl Trop Dis. 2017;11(7):e0005625 (doi: 10.1371/journal.pntd.0005625).

8. Global vector control response 2017–2030. Geneva: World Health Organization; 2017 (https://www.who.int/publications/i/item/9789241512978).

9. Global plan for insecticide resistance management in malaria vectors. Geneva: World Health Organization; 2012 (https://apps.who.int/iris/handle/10665/44846).

10. Test procedures for insecticide resistance monitoring in malaria vector mosquitoes. 2nd ed. Geneva: World Health Organization; 2016 (https://apps.who.int/iris/handle/10665/250677).

11. Monitoring and managing insecticide resistance in *Aedes* mosquito populations: interim guidance for entomologists. Geneva: World Health Organization; 2016 (https://apps.who.int/iris/handle/10665/204588).

12. Report of the WHO informal consultation. Test procedures for insecticide resistance monitoring in malaria vectors, bio-efficacy and persistence of insecticides on treated

surfaces. Geneva: World Health Organization; 1998 (https://apps.who.int/iris/handle/10665/64879).

13. Guideline for evaluating insecticide resistance in vectors using the CDC bottle bioassay. Atlanta (GA): Center for Global Health, Division of Parasitic Diseases and Malaria; 2012 (https://stacks.cdc.gov/view/cdc/21777).

14. Standard operating procedures for insecticide resistance testing in mosquitoes. Geneva: World Health Organization; 2022 (https://www.who.int/teams/control-of-neglected-tropical-diseases/vector-ecology-and-management/vector-control/insecticide-resistance).

15. Oxborough RM, Seyoum A, Yihdego Y, Chabi J, Wat'senga F, Agossa FR et al. Determination of the discriminating concentration of chlorfenapyr (pyrrole) and *Anopheles gambiae* sensu lato susceptibility testing in preparation for distribution of Interceptor® G2 insecticide-treated nets. Malar J. 2021;20:316 (https://doi.org/10.1186/s12936-021-03847-3).

16. Dagg K, Irish S, Wiegand RE, Shililu J, Yewhalaw D, Messenger LA. Evaluation of toxicity of clothianidin (neonicotinoid) and chlorfenapyr (pyrrole) insecticides and cross-resistance to other public health insecticides in *Anopheles arabiensis* from Ethiopia. Malar J. 2019;18:49 (doi.org/10.1186/s12936-019-2685-2).

17. Agumba S, Gimnig JE, Ogonda L, Ombok M, Kosgei J, Munga S et al. Diagnostic dose determination and efficacy of chlorfenapyr and clothianidin insecticides against *Anopheles* malaria vector populations of western Kenya. Malar J. 2019;18:243 (doi.org/10.1186/s12936-019-2858-z).

18. Lees R, Praulins G, Davies R, Brown F, Parsons G, White A et al. A testing cascade to identify repurposed insecticides for next-generation vector control tools: screening a panel of chemistries with novel modes of action against a malaria vector. Gates Open Res. 2019;3:1464 (doi: 10.12688/gatesopenres.12957.2).

19. Oxborough RM, Seyoum A, Yihdego Y, Dabire R, Gnanguenon V, Wat'senga F et al. Susceptibility testing of *Anopheles* malaria vectors with the neonicotinoid insecticide clothianidin; results from 16 African countries, in preparation for indoor residual spraying with new insecticide formulations. Malar J. 2019;18:264 (doi.org/10.1186/s12936-019-2888-6).

Annex. Participating institutions and mosquito strains available

Only representative strains were selected from the list after discussion at the WHO consultation in February 2017.

Institution or laboratory	Country	Susceptible colony strains available
Institut de Recherche pour le Développement (IRD)	France	*Aedes aegypti* (Bora Bora) *Aedes aegypti* (SBE) *Aedes albopictus* (Perols) *Anopheles gambiae* (Kisumu) *Culex quinquefasciatus* (S-laboratory)
National Environmental Agency (NEA)	Singapore	*Aedes aegypti* (NEA-EHI) *Aedes albopictus* (NEA-EHI) *Aedes aegypti* (Bora Bora) *Culex quinquefasciatus* (NEA-EHI)
Indian Council of Medical Research – National Institute of Malaria Research – New Delhi (NIMR-D)	India	*Anopheles stephensi* (Nadiad) *Anopheles culicifacies* (Dehra) *Aedes aegypti* (Delhi)
Fundação Oswaldo Cruz (Fiocruz – IOC)	Brazil	*Aedes aegypti* (Rockefeller) *Aedes albopictus* (NEA-EHI) *Anopheles aquasalis* (IOC) *Culex quinquefasciatus* (IBEx, JPA)
Vector Control Reseach Unit, Universiti Sains Malaysia (VCRU)	Malaysia	*Aedes aegypti* (VCRU) *Aedes aegypti* (IMR) *Aedes aegypti* (Bora Bora) *Aedes albopictus* (VCRU) *Culex quinquefasciatus* (VCRU) *Culex quinquefasciatus* (IMR) *Anopheles sinensis* (Penaga)
Institut de Recherche en Sciences de la Santé (IRSS)	Burkina Faso	*Anopheles gambiae* (Kisumu) *Aedes aegypti* (Bora Bora)
London School of Hygiene & Tropical Medicine – Institut Pierre Richet (LSHTM-IPR)	Côte d'Ivoire	*Anopheles gambiae* (Kisumu) *Culex quinquefasciatus* (S-laboratory)

Institution or laboratory	Country	Susceptible colony strains available
Organisation de coordination et de coopération pour la lutte contre les grandes endémies en Afrique Centrale (OCEAC)	Cameroon	*Anopheles gambiae* (Kisumu) *Anopheles gambiae* (Ndokayo) *Anopheles coluzzii* (Ngousso) *Anopheles coluzzii* (Youpwe) *Aedes aegypti* (Douala and Yaoundé) *Aedes albopictus* (Douala and Yaoundé) *Culex quinquefasciatus* (Yaoundé)
Centers for Disease Control and Prevention (CDC)	USA	*All strains available at MR4 including An. albimanus, Anopheles minimus and Anopheles funestus* https://www.beiresources.org/
Liverpool School of Tropical Medicine (LSTM)	UK	*Anopheles gambiae* s.s. (Kisumu) *Anopheles arabiensis* (Moz) *Aedes aegypti* (New Orleans) *Anopheles funestus* (Fang) *Anopheles colluzzi N'gusso* (Cameroon) *Aedes aegypti* (New Orleans)
National Institute for Communicable Diseases (NICD)	South Africa	*Anopheles arabiensis* (KGB) *Anopheles coluzzii* (SUA) *Anopheles quadriannulatus* (SANGWE) *Anopheles funestus* (FANG)
National Institute of Health (NIH)	Colombia	*Anopheles albimanus* (Buenaventura) *Aedes aegypti* (Rockefeller)
National Institute of Health (NIH Peru)	Peru	*An. albimanus* (Sanarate) *Aedes aegypti* (Rockefeller)
Kasetsart University of Agriculture (KU-AG)	Thailand	*Anopheles minimus* (TM) *Aedes aegypti* (Bora Bora)
Mahidol University (MU)	Thailand	*Anopheles minimus* (TM) *Anopheles dirus* (TM) *Aedes aegypti* (Bora Bora) *Aedes albopictus* (Rayong) *Culex quinquefasciatus* (Nont)

Institution or laboratory	Country	Susceptible colony strains available
Indian Council of Medical Research – National Institute of Malaria Research – Benguluru (NIMR-B)	India	*Anopheles stephensi* (Sonepat) *Aedes aegypti* (Benguluru)
Indian Council of Medical Research – Vector Control Research Centre (VCRC)	India	*Anopheles stephensi* (Puducherry) *Aedes aegypti* (Puducherry)
London School of Hygiene & Tropical Medicine – Centres de Recherches Entomologiques de Cotonou (LSHTM–CREC)	Benin	*Anopheles gambaie* (Kisumu) *Aedes aegypti* (ROCK)
London School of Hygiene & Tropical Medicine – Kilimanjaro Christian Medical University College (LSHTM-KCMUCo)	Unted Republic of Tanzania	*Anopheles gambaie s.s.* (Kisumu) *Culex quinquefasciatus* (TPRI) *Aedes aegypti* (LSHTM)
Universidad Autónoma de Nuevo León (UANL)	Mexico	*Aedes aegypti* (New Orleans) *Aedes aegypti* (Rockefeller) *Culex quinquefasciatus* (S-laboratory)
Swiss Tropical and Public Health Institute (Swiss TPH)	Switzerland	*Aedes aegypti* (ROCK) *Anopheles gambiae* (Kisumu)
Chinese International Center for Disease Control and Prevention (ICDC)	China	*Anopheles sinenis* (Shanghai) *Anopheles dirus* (Yunnan) *Aedes aegypti* (Hainan) *Aedes albopictus* (Chengdu) *Culex quinquefasciatus* (Guangdon)
Malaria Research and Training Centre (MRTC)	Mali	*Anopheles gambaie* (Kisumu) *Anopheles coluzzii* (N’Gaba) *Aedes aegypti* (Bora Bora)

9789240045200

9 789240 045200