

REPORT OF THE FIFTEENTH
WHOPES
WORKING GROUP MEETING

WHO/HQ, GENEVA
18–22 JUNE 2012

Review of:

OLYSET® PLUS
INTERCEPTOR® LN
MALATHION 440 EW
VECTOBAC® GR



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1. INTRODUCTION

The fifteenth meeting of the WHOPES Working Group, an advisory group to the WHO Pesticide Evaluation Scheme (WHOPES), was convened at WHO headquarters in Geneva, Switzerland, from 18 to 22 June 2012. The objective of the meeting was to review Olyset® Plus (Sumitomo Chemical, Japan) and Interceptor® (BASF, Germany) long-lasting insecticidal mosquito nets (LN) for malaria prevention and control; malathion 440 emulsion oil-in-water (EW) (Cheminova, Denmark) for space spraying against mosquitoes; and VectoBac® granules (GR) (Valent BioSciences, USA) for mosquito larviciding. The meeting also addressed issues and challenges related to procedures, criteria and requirements for testing and evaluation of public health pesticides, and made appropriate recommendations.

The meeting was attended by 16 scientists (see Annex I: List of participants). Professor Dr Marc Coosemans was appointed as Chairman and Dr John Gimnig as Rapporteur. The meeting was convened in plenary and group sessions, in which the reports of the WHOPES supervised trials and relevant published literature and unpublished reports were reviewed and discussed (see Annex II: References). Recommendations on the use of the above-mentioned products were made.

Declaration of interest

All invited experts completed a *Declaration of interests for WHO experts*, which was submitted and assessed by the WHO Secretariat prior to the meeting. The following interests were declared:

Dr Rajendra Bhatt and Dr Kamaraju Raghavendra's institute has received prescribed standard fees from eight manufacturers of pesticide products (BASF India, Bayer CropScience India, Bestnet Insect Controls Pvt Ltd India, Chemtura India, Clarke Mosquito Control USA, Sumitomo Chemical India, Syngenta Crop Protection India and Vestergaard Frandsen India) in order to meet the costs of product evaluation.

Dr Fabrice Chandre's institute has received prescribed standard fees from Sumitomo Chemical Japan, Bayer CropScience Germany and SPCI France in order to meet the costs of evaluating their respective LNs. In addition, his travel to a malaria meeting in Nairobi in 2009 was paid for by Bayer Environmental Science France.

Dr Marc Coosemans' research unit has received grants from the European Union for mapping insecticide resistance in the Mekong Region, and from the Bill & Melinda Gates Foundation for studying the impact of repellents on malaria in Cambodia. The unit has also received repellents free of charge from SC Johnson & Son USA for use in the latter study.

Dr Vincent Corbel's national partner institute, Centre de Recherches Entomologiques de Cotonou (CREC), has received grants from DART (a joint venture of Vestergaard Frandsen, the Acumen Fund and Dr Richard Allan) and Vestergaard Frandsen for testing and evaluation of their durable wall lining products. In addition, CREC has received grants from Sumitomo Chemical Japan for testing its Olyset Plus LN.

Dr John Gimnig's research unit has received LNs from Clarke Mosquito Control, BASF, Sumitomo Chemical, Tana Netting and Vestergaard Frandsen for use in field evaluations of such nets undertaken by its partner institutions in Kenya and Malawi.

Dr Raphael N'Guessan and Dr Mark Rowland's unit has received grants from the Innovative Vector Control Consortium (IVCC) for testing and evaluation of various pesticide products manufactured by BASF Germany, Dow AgroSciences, Dupont, Sumitomo Chemical Japan, Syngenta Switzerland and Vestergaard Frandsen Switzerland.

Dr Olivier Pigeon's research centre has received prescribed standard fees from Sumitomo Chemical Japan and BASF in order to meet the costs of physico-chemical studies of pesticide products manufactured by the respective companies.

The interests declared by the experts were assessed by the WHO Secretariat. With the exception of Dr Vincent Corbel's declared interest on the part of his national partner institute, the declared interests were not found to be directly related to the topics under discussion at the meeting. It was therefore decided that all of the above-mentioned experts (with the exception of Dr Corbel) could participate in all evaluations, subject to the public disclosure of their interests.

In view of the declared interest on the part of his national partner institute, Dr Corbel did not participate in the evaluation of Sumitomo Chemical's Olyset Plus LN.

2. REVIEW OF OLYSET[®] PLUS

Olyset Plus is a long-lasting insecticidal mosquito net (LN) manufactured by Sumitomo Chemical, Japan. The product is made of 150 denier high-density mono-filament polyethylene yarn (weight 40 g/m²), containing technical permethrin (40:60 *cis:trans* isomer ratio) 2% (w/w) as an active ingredient (AI), corresponding to 20 g AI/kg (about 800 mg of AI/m²), and piperonyl butoxide (PBO) 1% (w/w), as synergist, corresponding to 10 g PBO/kg (about 400 mg of PBO/m²). Permethrin and the synergist are incorporated into filaments and migrate through them by diffusion.

Olyset Plus is made of wide mesh (the average number of complete holes in 100 cm² shall be not less than 645 and the lowest value shall be not less than 600 holes/100 cm²) with minimum bursting strength of 250 kPA. The manufacturer has confirmed that the permethrin used in making the LN complies with WHO specification 331/TC (March 2009), and that the PBO complies with WHO specification 33/TC (September 2011) and is solely from the source supported by the WHO specification (Endura Fine Chemicals, Italy).

2.1 Safety assessment

On behalf of WHOPES, the Finnish Institute of Occupational Health (FIOH, 2011) assessed the risk to public health of washing and use of permethrin plus PBO (incorporated into filaments) LN provided by the manufacturer. The WHO generic risk assessment model for insecticide treatment of mosquito nets and their subsequent use¹ was used as a guiding document.

The assessment of health risks of washing and use of Olyset Plus reported by Sumitomo Chemical deviates considerably from the WHO generic risk assessment model. However, applying the

¹ *A generic risk assessment model for insecticide treatment and subsequent use of mosquito nets*. Geneva, World Health Organization, 2004 (WHO/CDS/WHOPES/GCDPP/2004.6, WHO/PCS/04.1; available at http://www.who.int/whopes/resources/resources_2004/en/index.html).

hazard data developed by the FAO/WHO Joint Meeting on Pesticide Residues (JMPR), acceptable daily intake (ADI) for sleeping under the net, and acute reference dose (ARfD) for net washing, using the default assumptions and values of the model and assuming that:

- the maximal observed release in the CIPAC washing procedure² is equal to that in the user's washing, and the average observed release is equal to the surface concentration available for the dermal contact when sleeping under the net and for release during chewing of the net by a child or infant;
- the washing volume is 2L;
- dermal absorption is lower than the default, as demonstrated by the proposer's experimental data for both permethrin and PBO (oral absorption is 100% for both); and
- for dermal contact (and hand-to-mouth transfer), the model default 2.5% is actually transferred from the net onto skin;

it may be estimated that:

during the washing of the net, the exposure to permethrin is approximately 1–2% of the JMPR ARfD, and exposure to

² Currently (2012), the Collaborative International Pesticide Analytical Council (CIPAC) is developing a washing method to determine the retention behaviour of long-lasting insecticidal nets. Copies of the method are available from the CIPAC web site (<http://www.cipac.org>) prior to its publication in a handbook. This method is a further standardization of the WHO washing method published in the WHO *Guidelines for laboratory and field testing of long-lasting insecticidal mosquito nets* published by the World Health Organization, Geneva in 2005 (document WHO/CDS/WHOPES/GCDPP/2005.11). Briefly, the retention index is determined by analysing net samples in triplicate, representing wash points 0 and 4 for total active ingredient content, and calculating the average retention index per wash using the equation for a free migration stage behaviour. A retention index per wash of 0.95 indicates that 95% of the insecticide present in samples washed 1 to 3 times is still present after an additional wash step. The retention index applies to the average obtained from triplicate tests performed on samples removed from the same net or batch of netting.

PBO is approximately 1% of the dose derived from the JMPR short-term NOAEL (no-observed adverse effect level) by dividing by the JMPR default uncertainty factor of 100. From sleeping under the net, the exposure to permethrin and PBO is 25.2% or less than that of the JMPR ADI, except for the exposure to permethrin of the newborn. In this case, the estimated exposure is 107% of the JMPR ADI. Mouthing, chewing and sucking comprises >99% of the total systemic dose for the infant sleeping under the net; the estimate is based on the release of all available (released to soap water) permethrin in a 50 cm² piece of the net. The assumed 100% release is a worst-case scenario: saliva may not dissolve permethrin to the same extent as soap. The size of 50 cm² is for a child of all ages, and probably represents the upper limit for the newborn. As the estimated dose only exceeds the ADI by 7%, and falls below the 100% limit within a few weeks with the growth of the child, it may be concluded that, even for the infant, sleeping under the net does not constitute a risk of adverse health effects.

It is therefore concluded that Olyset Plus® LNs, when used as instructed, do not pose undue hazards to the user.

2.2 Efficacy – background and supporting documents

Duchon et al (2010) carried out a laboratory study (phase I) commissioned by Sumitomo Chemical at the Laboratoire de Lutte contre les Insectes Nuisibles (LIN/IRD) in Montpellier, France, to confirm the regeneration time of Olyset Net as a reference net for the Olyset Plus using pre-cut samples of 25 x 25 cm (*n*=4) from two Olyset Nets. To estimate the regeneration time, the two net samples were washed and dried three times consecutively on a given day to deplete the concentration of insecticide on the net surface. After washing, a range of bioassays (cone, circular chamber and tunnel tests) were conducted using susceptible *Anopheles gambiae* Kisumu strain at regular intervals (1, 3, 5, 7, 10 and 14 days). The bio-efficacy (knock-down (KD) and mortality) curves were established and compared with those of the net samples before washing. Different values of the regeneration time

were recorded depending on the test method used. In cone tests, the regeneration time was 5–7 days based on mortality outcomes. The measurement of time to KD in the circular chamber gave a regeneration time of 5 days, whereas the tunnel test that involved behavioural consideration and overnight testing gave 3 days. Since the assessment of an LN and its wash resistance capacity is based upon WHO cone tests, the authors considered 7 days as the regeneration time for the Olyset Net.

Bouraima et al (2010) conducted a second preliminary study under laboratory and semi-field conditions in experimental huts in Benin and Cameroon to evaluate two candidate LNs (S-4201 and S-4553) from the manufacturer Sumitomo Chemical. The manufacturer has confirmed to WHOPES that S-4201 is the code for Olyset Plus and that S-4553 is the Olyset Plus but without PBO.

The laboratory experiment aimed at determining in cone and tunnel tests the bio-efficacy of the candidate LNs unwashed and washed (three consecutive washes) and after 7 days storage of the nets. At both locations, pyrethroid-susceptible (Kisumu colony strain) and resistant *An. gambiae* s.l. (adults from wild larvae) were used. Polymerase chain reaction and biochemical analysis conducted in 2010 on the resistant *An. gambiae* s.s. populations from Benin showed high *kdr* allele frequency (0.9) plus enhanced monooxygenases P450 activity (Djègbè et al., 2011³), whereas *An. arabiensis* from Cameroon exhibited increased P450 and esterases activities (Etang et al., 2007⁴).

The semi-field trial involved the release and recapture of four replicates of 50–75 females of susceptible or pyrethroid-resistant *An. gambiae* s.l. in experimental huts containing the candidate LNs,

³ Djègbè I et al. Dynamic of multiple insecticide resistance in malaria vectors in Benin: first evidence for the presence of *L1014S* *kdr* in *An. gambiae* from West Africa. *Malaria Journal*, 2011, 10:261.

⁴ Etang J et al. Spectrum of metabolic - based resistance to DDT and pyrethroids in *Anopheles gambiae* s.l. populations from Cameroon. *Journal of Vector Ecology*, 2007, 32:123–133.

unwashed and washed. The LNs were washed according to a protocol adapted from the standard WHO washing procedure used in phase I⁵. As in the cone and tunnel tests, LNs were washed three times consecutively, then tested after 7 days of storage. The performance of the two LNs (S-4201 and S-4553) was compared with that of the Olyset Net. The outcomes measured were blood-feeding inhibition, induced exophily and mortality.

Results for the inhibition of blood-feeding by *An. gambiae s.l.* in tunnel tests are reported in Table 1.

There were some statistically significant differences, although not substantial (<10%), between the LNs in blood-feeding inhibition, with the exception of the washed LNs against the resistant *An. gambiae* in Cotonou. There was no significant improvement in protection provided by Olyset Plus over S-4553 (Olyset Plus without PBO; 87% versus 80%).

Overall, the mortality followed the same trend as that of the blood-feeding inhibition. However, a more pronounced impact against the pyrethroid-resistant *An. gambiae s.l.* was observed (Table 2).

Against the pyrethroid-resistant specimens from Cotonou and Pitoa, there was a significant improvement of efficacy of Olyset Plus over the Olyset Net regardless of the wash status. However, the difference in mortality induced by Olyset Plus and S-4553 (Olyset Plus without PBO) was only significant against pyrethroid-resistant *An. gambiae s.s.* from Cotonou, in particular with the washed Olyset Plus (56% versus 18%).

⁵ WHO (2005). *Guidelines for laboratory and field testing of long-lasting insecticidal mosquito nets*. Geneva, World Health Organization, 2005 (WHO/CDS/WHOPES/GCDPP/2005.11; available at <http://www.who.int/whopes/guidelines/en/>; accessed July 2012).

2.3 Efficacy – WHOPES supervised trials

2.3.1 Laboratory studies

Rossignol et al (2011) determined the regeneration time of Olyset Plus with that of Olyset Net in laboratory studies (WHOPES phase I) according to WHOPES guidelines using the susceptible Kisumu strain of *An. gambiae* s.s.

WHO cone tests were conducted first on unwashed samples of both LNs to record initial mortality. The netting samples were then washed and dried consecutively three times and stored at 30 °C. Bioassays were conducted on the washed samples at different intervals of time (1, 2, 3, 5 and 7 days) until bio-efficacy reached a plateau. Time elapsed until the plateau is reached was considered as the regeneration time. In total, six netting samples (4 Olyset Plus and 2 Olyset Net) were washed and bioassayed at each interval of time.

Mortality was maximal (100%) for the unwashed Olyset Plus, but only 63% for Olyset Net. After three consecutive washes of Olyset Plus and storing the net for 1 day, mortality decreased to 64%. It then increased to a plateau value around 83% between day 2 and day 7. The regeneration time was considered to be 2 days.

The median knock-down time (MKDT) was also used to estimate the peak of bioavailability of insecticide on the nets using the circular chamber test method previously described by Skovmand et al. in 2008.⁶ The investigators observed no significant differences between the average values of MKDT for Olyset Plus samples unwashed and washed 3 times.

The study did not provide a clear regeneration time for Olyset Net during the 7-day study period.

⁶ Skovmand O et al. Median knock-down time as a new method for evaluating insecticide-treated textiles for mosquito control. *Malaria Journal*, 2008, 7:114.

Using standard cone bioassays, the bio-efficacy of Olyset Plus netting was also assessed in terms of KD effect and mortality after different numbers of washes (1, 3, 5, 10, 15, 20 and 25 washes) (Rossignol et al., 2011) (Table 3). The assessed regeneration time of 2 days was applied between washes of samples.

The KD rates were 99–100% up to 15 washes. There was a slight decrease after 20 washes but values still exceeded the WHO threshold (95% KD). Mortality showed a downward trend with a significant fall of Olyset Plus under WHO threshold just after 3 washes (76%) and 10 washes (21%). Mortality between 20 and 25 washes was still lower and did not exceed 16%.

Pigeon (2011b) performed chemical analyses on LN samples bio-assayed at LIN/IRD, Montpellier, to determine regeneration time and wash resistance. For the regeneration time study, the analyses were performed on Olyset Plus and Olyset Net samples washed 0 and 3 times consecutively. Per wash cycle, 2 pieces (25 cm x 25 cm) from 2 nets of Olyset Net and 4 pieces (25 cm x 25 cm) from 4 nets of Olyset Plus were analysed for determination of permethrin and/or piperonyl butoxide.

For the wash resistance study, chemical analyses were performed on Olyset Plus samples washed 0, 1, 3, 5, 10, 15, 20 and 25 times. After each wash cycle, 4 pieces (25 cm x 25 cm) from 4 nets were analysed to determine the content of permethrin and/or piperonyl butoxide. The analysis was done using CIPAC method 331/LN/M/3, which involves extracting permethrin and piperonyl butoxide from the net sample, by dipping the sample in a water bath (heated to 85–90°C) for 45 minutes, adding heptane in the presence of triphenyl phosphate as an internal standard and determining content by gas chromatography with flame ionisation detection (GC-FID).

The permethrin content (20.2–20.3 g AI/kg) in the unwashed Olyset Nets complied with the target dose of 20 ± 3 g AI/kg; the between-net variation, expressed as the relative standard deviation (RSD) of the content found on the 2 pieces, was 0.3% and 0.0% respectively, showing good homogeneity of the active substance's distribution over the net.

The permethrin content (19.3 and 19.2 g AI/kg) in the unwashed Olyset Plus complied with the target dose of 20 ± 5 g AI/kg; the between-net variation, expressed as the RSD of the content found on the 4 pieces, was 0.6% and 1.5% respectively, showing good homogeneity of the active substance's distribution over the net.

The permethrin between-net variation on Olyset Plus samples washed 1–25 times remained low (RSD = 1.3–3.8%). The average permethrin content was 16.0–16.1 g AI/kg after 3 washes (lower than for Olyset Net, which was 19.8 g/kg after 3 washes), 13.9 g AI/kg after 10 washes and 12.3 g AI/kg after 20 washes. The differential permethrin AI load between Olyset Plus and Olyset Net was due to the bleed rate of the ingredient onto the net surface, which is higher in Olyset Plus than in Olyset Net. The overall permethrin retention after 20 washes was 64.1%, corresponding to an average retention per wash of 97.8%.

The piperonyl butoxide content (9.2 and 9.1 g PBO/kg) in the reference and unwashed Olyset Plus complied with the target dose of 10 ± 2.5 g PBO/kg. The between-net variation, expressed as the relative standard deviation (RSD) of the content found on the 4 pieces was 0.4% and 1.9% respectively, showing good homogeneity of the synergist's distribution over the net. The piperonyl butoxide between-net variation on Olyset Plus samples washed 1–25 times remained low (RSD = 1.8–7.0%). The average piperonyl butoxide content was 6.5 g PBO/kg after 3 washes, 5.2 g PBO/kg after 10 washes and 4.0 g PBO/kg after 20 washes. The overall piperonyl butoxide retention after 20 washes was 44.2%, corresponding to an average retention per wash of 96.0% (Table 3, Figure 1).

2.3.2 Experimental hut studies

WHOPES supervised three field studies that were conducted in Malanville in northern Benin (Bouraima et al., 2012), Muheza in the United Republic of Tanzania (Tungu et al., 2012b) and Odisha in East-Central India (Gunasekaran et al., 2012). The overall aim of the three studies was to assess in experimental huts the effect

of washing Olyset Plus on mosquito behaviour, compared to the Olyset Net and a polyester conventionally treated net (CTN) washed to just before exhaustion. The trial was done between September and December 2011 in Benin and from November 2011 up to the beginning of February 2012 in India. The trial in the United Republic of Tanzania ran from March to June 2012. In Benin, the main malaria vector was *An. gambiae* s.s., M form, showing permethrin resistance (22% mortality to permethrin in WHO cylinder assays). Resistance mechanisms included enhanced P450 and a high frequency of the 1014F *kdr* mutation (0.5 in 2010)⁷. In the United Republic of Tanzania, *An. gambiae* s.s. was the main vector in the area and showed susceptibility to pyrethroids. In India, *An. fluviatilis* was the main vector and was susceptible to pyrethroids.

At all field sites, the design of experimental huts and evaluation methods followed WHOPES guidelines.⁸

Treatment design in all three study sites included (i) unwashed Olyset Plus; (ii) Olyset Plus washed 20 times; (iii) unwashed Olyset Net; (iv) Olyset Net washed 20 times; (v) polyester net conventionally treated with permethrin EC at 500 mg Al/m² (CTN) and washed to just before exhaustion, defined as the last wash providing mortality $\geq 80\%$ or KD ≥ 95 ; and (vi) untreated polyester net.

The nets were washed as per the standard WHO procedures for phase II (WHO, 2005). In all sites, the regeneration time (RT) was set at 2 days for the Olyset Plus and 7 days for the Olyset Net, as determined previously in laboratory assays at LIN/IRD, Montpellier, France (Rossignol et al, 2011). All nets had six square size holes

⁷ Djègbè I et al. Dynamic of multiple insecticide resistance in malaria vectors in Benin: first evidence for the presence of *L1014S kdr* in *An. gambiae* from West Africa. *Malaria Journal*, 2011, 10:261.

⁸ *Guidelines for laboratory and field testing of long-lasting insecticidal mosquito nets*. Geneva, World Health Organization, 2005 (WHO/CDS/WHOPES/GCDPP/2005.11; available at <http://www.who.int/whopes/guidelines/en/>; accessed July 2012).

(4 cm x 4 cm each) deliberately made in each net: two on each length panel, and one on each width.

The treatment arms were rotated weekly and sleepers were rotated daily among the huts on the basis of a Latin square scheme at the three sites. Six nets were used per treatment arm; each was tested one night during the week. The huts were carefully cleaned and aired at the end of each week to remove potential contamination. At all sites, 12 weeks were necessary to complete two Latin square designs and obtain sufficient numbers of mosquitoes for statistical analysis. The outcome measures were deterrence (the reduction in the number of mosquito in huts with treated nets relative to the control hut); induced exophily (the proportion of mosquitoes exiting and caught in the veranda trap of huts with treated nets relative to the control hut); blood-feeding inhibition (the reduction in blood-feeding rates in huts with treated nets compared with those in the control hut); and induced mortality (the proportion of mosquitoes killed, corrected for control). Cone and/or tunnel bioassays were conducted at all field sites.

Chemical analysis was performed on all unwashed and washed treated nets before and after the field trial. Five pieces (25 cm x 25 cm) were cut from each net according to the WHO sampling method for LNs and pooled for chemical analysis. The average permethrin and/or PBO content was determined using the CIPAC method 331/LN/M/3. This method involved extraction of the active ingredient and synergist from the net samples in a water bath (85–90°C) for 45 minutes with heptane in the presence of triphenyl phosphate as internal standard and determination by gas chromatography with flame ionization detection (Pigeon 2012b, c and d).

The analysis of numeric data of the hut trial (hut entry rates) was carried out using the Kruskal–Wallis non-parametric test in Benin, and the negative binomial regression in India and the United Republic of Tanzania. The proportional outcomes (exophily, blood-feeding and mortality rates) were analysed and compared using logistic regression at all sites.

Benin

The cone bioassay results before field testing the nets showed that all treated nets, unwashed or washed, induced 100% KD in the exposed mosquitoes whereas KD for the Olyset Net washed 20 times was below the WHO threshold (77%). Under cone bioassays, washing the nets 20 times significantly reduced mortality of *An. gambiae*, from 100% to 42% for Olyset Plus and from 37% to 19% for Olyset Net. The CTNs washed to cut-off showed KD within the WHO criteria (95% KD, 79% mortality).

In experimental huts, there were no significant differences in the numbers of *An. gambiae s.l.* entering the different huts, but all treatments induced significantly higher exophily (from 147% to 225%) than the untreated net. Rates of blood-feeding inhibition were significantly higher for all treatments compared with those of the control.

After washing the nets 20 times, the inhibition of *An. gambiae* blood-feeding for Olyset Plus (79%) was similar to CTNs washed to cut-off (74%) but significantly higher than for Olyset Net (60%) (Table 4).

All treatments killed significantly more *An. gambiae s.l.* (from 36% to 81%) than the untreated net (0%). Mortality rates of resistant *An. gambiae s.l.* with unwashed and washed Olyset Plus (81% and 67%) were significantly higher than for unwashed and washed Olyset Net (42% and 36%). Olyset Plus washed 20 times induced blood-feeding inhibition and mortality rates similar to the CTNs washed to just before exhaustion (Tables 4 and 5).

The investigators further analysed and presented data for culicine mosquitoes, although no information on insecticide resistance status and resistance mechanisms in these mosquitoes was provided. The trend in efficacy of treatments against culicines mirrored that of *An. gambiae s.l.*, except for the proportions of mosquitoes exiting by dawn to the verandas of the different huts.

There were no significant differences in entry rates (deterrence) and exit rates (exophily) of culicines between treatments and the control hut. Blood-feeding inhibition rates for all treatments were

high ($\geq 93\%$) compared with the control; there was no significant difference between treatments (Table 4). All treatments caused higher mortality rates of culicine mosquitoes (85–96%) compared with the untreated net (2%). For *An. gambiae* s.l., the Olyset Plus washed 20 times induced mortality similar to the CTN washed to just before exhaustion. Both washed and unwashed Olyset Plus induced significantly higher mortality of Culicidae than did Olyset Net before and after washes.

There were no perceived adverse effects reported by the sleepers concurrent with the use of either LNs or the CTNs.

At the end of the trial, one net per treatment arm was randomly sampled from the huts and bio-assayed. The results indicated that Olyset Plus washed 20 times remained fully effective against susceptible *An. gambiae* Kisumu (100% mortality) after field use, whereas a significant drop in activity was observed for the CTN washed to just before exhaustion (86% mortality) and the 20 times washed Olyset Net (64% mortality). Both washed Olyset Plus and washed Olyset Net showed higher insecticidal activity after field testing (100% and 64% respectively) than before field testing (42% and 19% respectively). This suggests that further diffusion of permethrin and/or PBO to the surface of the net occurred during the trial period.

The permethrin content in three samples of unwashed Olyset Net (19.7, 19.6 and 20.0 g AI/kg) complied with the target dose of 20 ± 3 g AI/kg. The permethrin content was 16.7 g AI/kg after 20 washes, corresponding to an overall permethrin retention of 85% (Pigeon 2012b).

The permethrin content in three samples of unwashed Olyset Plus (18.6, 18.6 and 19.0 g AI/kg) complied with the target dose of 20 ± 5 g AI/kg. The permethrin content was 14.5 g AI/kg after 20 washes, corresponding to an overall permethrin retention of 78%. The piperonyl butoxide content in three samples of unwashed Olyset Plus (8.7, 8.8 and 9.0 g PBO/kg) complied with the target dose of 10 ± 2.5 g PBO/kg. The piperonyl butoxide content was 4.51 g/kg after 20 washes, corresponding to an overall piperonyl butoxide retention of 51%.

The unwashed CTN contained 341 mg AI/m² (11.0 g AI/kg) permethrin. The CTN washed to just before exhaustion contained 261 mg AI/m² (7.7 g AI/kg) permethrin, corresponding to a retention rate of 70%.

After the experimental hut study, the permethrin and or piperonyl butoxide content in the tested Olyset Net and Olyset Plus did not decrease significantly.

India

The bioassay results before washing of all LNs gave 100% mortality of blood-fed *An. stephensi*. After washing but before testing the nets in experimental huts mortality of this species dropped to 90% for the Olyset Plus and to 62% for Olyset Net. On *An. fluviatilis*, mortality before field testing of LNs was 100% for all washed or unwashed LNs except for the CTN washed to cut-off level (86%).

All treatments strongly deterred entry of *An. fluviatilis* into huts (82–89%). Deterrence was 89% in huts with unwashed and washed Olyset Plus and 82–84% in huts with washed and unwashed Olyset Net.

Natural exophily of *An. fluviatilis* from the control hut was 44%. This rate significantly increased to 56–83% with all treatments except for the unwashed Olyset Plus.

The rate of blood-feeding inhibition for washed Olyset Plus (60%) was similar to that of unwashed Olyset Net (61%) but lower than the CTN washed to just before exhaustion (91%) (Table 4). All treatments induced high mortality of *An. fluviatilis* (96–100%). The differences between them were not significant ($P>0.05$).

Bioassays conducted after the hut trial on unwashed and washed LNs still produced 100% mortality of susceptible *An. fluviatilis*, while the CTN washed to cut-off killed slightly less (96%).

The permethrin content in two samples of unwashed Olyset Net (19.9 g AI/kg and 20.0 g AI/kg) complied with the target dose of 20

g AI/kg \pm 3 g/kg. The permethrin content was 17.7 g AI/kg after 20 washes, indicating that 88% of the original target dose was still present (Pigeon, 2012c).

The permethrin content in two samples of unwashed Olyset Plus (19.1 g AI/kg and 18.8 g AI/kg) complied with the target dose of 20 g AI/kg \pm 5 g AI/kg. The permethrin content was 14.1 g AI/kg after 20 washes, indicating that 75% of the original target dose remained. The piperonyl butoxide content in two samples of unwashed Olyset Plus (9.0 g PBO/kg and 8.8 g PBO/kg) complied with the target dose of 10 g PBO/kg \pm 2.5 g PBO/kg. The piperonyl butoxide content was 4.0 g PBO/kg after 20 washes, indicating that 45% of the original target dose of PBO remained.

The unwashed CTN contained 509 mg AI/m² (15.3 g AI/kg) permethrin. The CTN washed to just before exhaustion contained 370.3 mg AI/m² (11.4 g AI/kg) permethrin, corresponding to a retention rate of 74%.

After the experimental hut study, the content of permethrin and piperonyl butoxide in the tested Olyset Net and Olyset Plus was similar to that before the study (Tables 6 and 7).

United Republic of Tanzania

Cone bioassays conducted against the resistant *Culex quinquefasciatus* MASIMBANI strain (*kdr* and oxidases) indicated initially higher toxicity of unwashed Olyset Plus (85%) than Olyset Net (30%). After 20 washes, the overall mortality with Olyset Plus declined below 20%, but was still higher compared with the Olyset Net washed 20 times. Against *An. gambiae*, the additional mortality induced by Olyset Plus relative to Olyset Net was limited owing to the inherent high susceptibility (hence high mortality) of the *An. gambiae* strain to permethrin.

In the experimental hut trial, only the unwashed Olyset Net gave significant deterrence (81%) against *An. gambiae* s.s. The rate of blood-feeding inhibition with the Olyset Plus washed 20 times (100%) exceeded that of the Olyset Net washed 20 times (88%) and the CTN washed to just before exhaustion (83%). Both Olyset

Plus and Olyset Net caused high rates of mortality against *An. gambiae* at 0 washes (100% and 98% respectively) (Table 5). After 20 washes, the mortality rate with the Olyset Plus (90%) exceeded that of the CTN washed to just before exhaustion (78%), which was similar to the percentage mortality with the Olyset Net washed 20 times (75%) (Table 4).

The permethrin content in three samples of unwashed Olyset Net (19.8, 19.9 and 19.7 g AI/kg) complied with the target dose of 20 ± 3 g AI/kg). The permethrin content was 16.5 g AI/kg after 20 washes, corresponding to 83% of the original permethrin content (Pigeon, 2012d).

The permethrin content in three samples of unwashed Olyset Plus (19.1, 18.4 and 19.0 g AI/kg) complied with the target dose of 20 ± 5 g AI/kg. The permethrin content was 13.9 g AI/kg after 20 washes, corresponding to an overall permethrin retention of 76%. The piperonyl butoxide content in three samples of unwashed Olyset Plus (8.7, 8.4 and 9.0 g PBO/kg) complied with the target dose of 10 ± 2.5 g PBO/kg. The piperonyl butoxide content was 3.2 g PBO/kg after 20 washes, corresponding to 38% of the original content.

The CTN washed to just before exhaustion contained 87 mg AI/m² (2.6 g AI/kg) permethrin or about one fifth of the target dose (500 mg AI/m²).

2.4 Conclusions and recommendations

Olyset Plus is a long-lasting insecticidal net manufactured by Sumitomo Chemical. The net is made of mono-filament polyethylene yarn, containing 2% (w/w) technical permethrin (40:60 *cis:trans* isomer ratio) as active ingredient (AI), corresponding to 20 g AI/kg (about 800 mg AI/m²), and 1% (w/w) piperonyl butoxide (PBO), as synergist, corresponding to 10 g PBO/kg (about 400 mg PBO/m²). Permethrin and the synergist are incorporated into filaments and diffuse to the surface.

WHO's assessment of the manufacturer's compliance with the assessment of exposure to and risks of washing and sleeping under an Olyset Plus was in line with its generic risk assessment model; when used as instructed, the net does not pose undue risk to the user.

The contents of permethrin and PBO in unwashed Olyset Plus samples tested in phase I and II studies complied with their target doses of 20 ± 5 g AI/kg and 10 ± 2.5 g PBO/kg respectively. The between-net variation of the permethrin and PBO contents was within the limits specified by the WHO guidelines and showed good homogeneity.

The bioassays and chemical analysis from phase I wash resistance studies showed an increased release rate of permethrin and a shorter regeneration time of 2 days of Olyset Plus compared with Olyset Net.

In both laboratory and field experiments, Olyset Plus washed 20 times was at least as effective as the conventional permethrin-treated net washed to just before exhaustion against both susceptible and pyrethroid-resistant mosquitoes. Laboratory wash resistance tests of Olyset Plus showed good KD effect, but rates of mosquito mortality declined significantly after washing. However, no such decline in activity was observed in the experimental huts trials.

In experimental hut trials (phase II), Olyset Plus showed significant improvement over the Olyset Net, reflected by higher mortality and lower blood-feeding rates.

It is not clear to what degree the improved performance of the Olyset Plus in phase I and phase II studies is the result of increased rates of permethrin release or the addition of PBO. Tests of Olyset Plus without PBO as an additional positive control in experimental huts are required to better understand the role of increased permethrin release rates versus the addition of PBO.

The Olyset Plus fulfilled the requirements of WHOPES phase I and phase II studies for LNs.

Considering the safety, efficacy and wash-resistance of Olyset Plus, the meeting recommended that:

- a time-limited interim recommendation be given for the use of Olyset Plus in the prevention and control of malaria;
- WHOPES should coordinate large-scale field studies of Olyset Plus to confirm its long-lasting efficacy and assess its physical integrity in diverse settings, as a basis for developing full recommendations on the use of this product.

Note: WHO recommendations on the use of pesticides in public health are valid ONLY if linked to WHO specifications for their quality control.

Table 1. **Overview of blood-feeding (%) and blood-feeding inhibition (% shown in bold) induced by three insecticidal nets according to wash status after release of *An. gambiae* s.l. with different resistance mechanisms (values in the same row sharing the same superscript letter do not differ significantly; P>0.05)**

Wash status	Pyrethroid resistance status (origin)	Un- treated net	Olyset Net	S-4201	S-4533
Before washing	susceptible	84 ^a	0 ^b	0 ^b	0 ^b
	(Kisumu strain)		100	100	100
3 washes + 7 d storage	susceptible	84 ^a	3 ^{bc}	0 ^b	4 ^c
	(Kisumu strain)		95	100	95
Before washing	Kdr+metabolic*	77 ^a	12 ^{b,c}	14 ^b	6 ^c
	(Cotonou)		85	82	92
3 washes + 7 d storage	Kdr+metabolic*	86 ^a	26 ^b	11 ^c	18 ^{bc}
	(Cotonou)		70	87	80
Before washing	Metabolic**	85 ^a	2 ^b	0 ^b	0 ^b
	(Pitoa)		97	100	100
3 washes + 7 d storage	Metabolic**	82 ^a	10 ^b	3 ^c	5 ^{bc}
	(Pitoa)		88	96	94

* *An. gambiae* s.s. M form with increased P450 oxidases, F(kdr)=0.9 (Djègbè et al., 2011).

** *An. arabiensis* showing enhanced P450 oxidases and esterases activity.

S-4201 is the Olyset Plus and S-4553 is the Olyset Plus without piperonyl butoxide.

Table 2. **Overview of mortality (%) induced by three insecticidal nets according to wash status after release of *An. gambiae* s.l. with different resistance mechanisms (values in the same row sharing the same letter superscript do not differ significantly; P>0.05)**

Wash status	Pyrethroid resistance status (origin)	Un-treated net	Olyset Net	S-4201	S-4533
Before washing	susceptible (Kisumu strain)	2.6 ^a	99.5 ^b	100 ^b	100 ^b
3 washes + 7 d storage	susceptible (Kisumu strain)	0.5 ^a	92.3 ^b	100 ^c	98.8 ^c
Before washing	Kdr+metabolic* (Cotonou)	2.7 ^a	78.1 ^b	92.31 ^c	80.5 ^b
3 washes + 7 d storage	Kdr+metabolic* Cotonou	1.6 ^a	11.4 ^b	55.6 ^c	18.2 ^b
Before washing	Metabolic** (Pitoea)	0.6 ^a	75.8 ^b	97.5 ^c	96.3 ^{c,d}
3 washes + 7 d storage	Metabolic** (Pitoea)	0.5 ^a	38.0 ^b	68.6 ^c	52.4 ^d

* *An. gambiae* s.s. M form with increased P450 oxidases, F(kdr)=0.9 (Djègbè et al., 2011);

** *An. arabiensis* showing enhanced P450 oxidases and esterases activity;

S-4201 is the Olyset Plus and S-4553 is the Olyset Plus without piperonyl butoxide.

Table 3. Wash resistance test: knock-down and mortality (%) of *An. gambiae* in relation to permethrin and PBO content and retention of Olyset Plus (WHOPES phase I wash resistance study). Target dose and tolerance limit for permethrin in baseline Olyset Plus = 20 ± 5 g AI/kg. Target dose and tolerance limit for PBO in baseline Olyset Plus = 10 ± 2.5 g PBO/kg.

No. of washes	% Knock-down	Corrected mortality %	PMT content (g/kg)	Between net RSD (%)	PMT retention (% of wash 0)	Average PMT retention (% at each wash)	PBO content (g/kg)	Between net RSD (%)	PBO retention (% of wash 0)	Average PBO retention (% at each wash)
0	100.0	100.0	19.2	1.5	-	-	9.1	1.9	-	-
1	100.0	100.0	18.1	1.6	94.2	94.2	7.4	2.9	81.6	81.6
3	100.0	75.9	16.1	1.4	83.5	94.2	6.5	1.8	71.4	89.4
5	99.5	42.9	14.9	1.3	77.4	95.0	5.8	1.6	64.5	91.6
10	98.5	21.5	13.9	2.8	72.2	96.8	5.2	5.4	57.0	94.5
15	99.0	35.6	13.0	2.1	67.7	97.4	4.6	3.6	50.9	95.6
20	95.4	15.3	12.3	2.8	64.1	97.8	4.0	7.0	44.2	96.0
25	95.4	15.8	11.4	3.8	59.2	97.9	3.8	5.8	41.8	96.6

KD = knock-down; PMT = permethrin; PBO = piperonyl butoxide; RSD = relative standard deviation

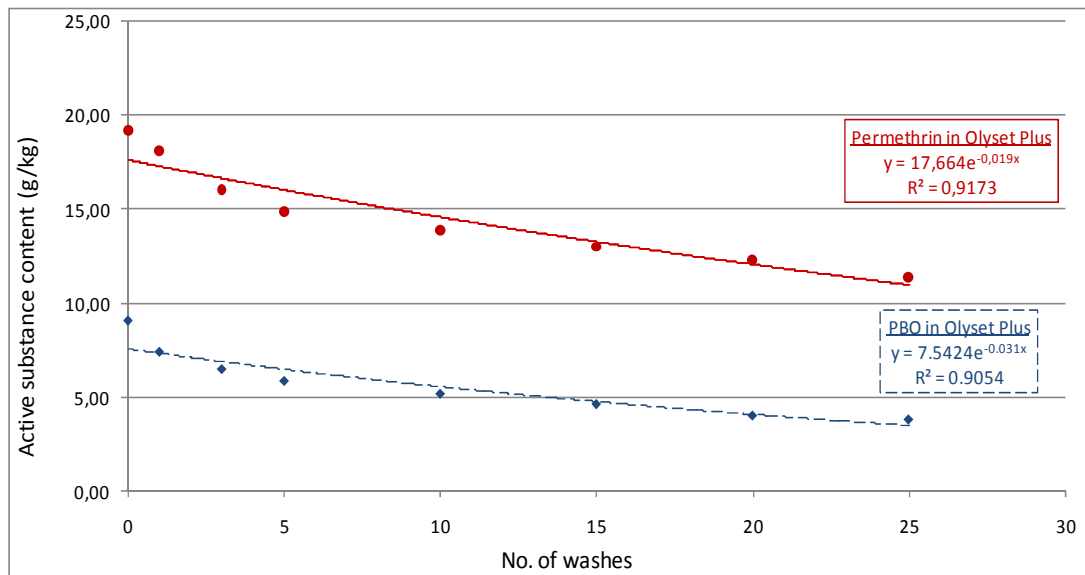


Figure 1. **Permethrin and piperonyl butoxide (PBO) content and retention (wash curve) for Olyset Plus (WHOPES phase I)**

Table 4. Overview of blood-feeding (%) and blood-feeding inhibition (% in bold) induced by Olyset Plus compared with Olyset Net and conventionally treated nets (CTN) washed to just before exhaustion in three study sites (values in the same row sharing the same superscript letter do not differ significantly; P>0.05)

Study sites (number of mosquitoes collected in the control hut and species)	Pyrethroid resistance status	Un-treated net	CTN washed to just before exhaustion	Olyset Plus unwashed	Olyset Plus 20 washes	Olyset Net unwashed	Olyset Net washed 20 times
Muheza - United Republic of Tanzania (68 <i>An. gambiae</i>)	Permethrin susceptible	72 ^a -	12 ^b 74	9 ^b 88	0 ^c 100	0 ^c 100	9 ^b 88
Malanville - Benin (69 <i>An. gambiae</i> s.l.)	<i>Kdr</i> + metabolic** Oxidases+	62 ^a	16 ^{b,c} 74	10 ^b 83	13 ^b 79	11 ^b 82	25 ^c 60
Malanville - Benin (821 Culicidae)*	Unknown	65 ^a -	3 ^b 96	3 ^{b,c} 95	3 ^{b,c} 95	3 ^b 96	5 ^c 93
Odisha - India (303 <i>An. fluviatilis</i>)	Permethrin susceptible	78 ^a -	7 91	19 75	31 60	27 65	30 61

* 10% *Anopheles* spp., 1% *Culex* spp. and 89% *Mansonia* spp.

** *An. gambiae* s.s. M Form, with enhanced oxidases, F(kdr) = 0.5 in 2010 (Djègbè et al., 2011).

Note: In Muheza, United Republic of Tanzania, initial dose of permethrin on CTN was 1/5th of the target dose.

Table 5. **Overview of mortality (%) and corrected mortality (% in bold) induced by Olyset Plus compared with Olyset Net and permethrin conventionally treated nets (CTN) washed to just before exhaustion in three study sites (values in the same row sharing the same superscript letter do not differ significantly; P>0.05)**

Study sites (number of mosquitoes collected in the control huts and species)	Pyrethroid resistance status	Un-treated net	CTN washed to just before exhaustion	Olyset Plus unwashed	Olyset Plus 20 washes	Olyset Net unwashed	Olyset Net washed 20 times
Muheza-United Republic of Tanzania (68 <i>An. gambiae</i>)	Permethrin susceptible	9 ^a	79 ^{c,d} 76	98 ^b 98	90 ^{b,d} 90	100 ^e 100	74 ^c 72
Malanville – Benin (69 <i>An. gambiae</i>)	<i>Kdr</i> + metabolic** oxidases+	0 ^a	55 ^{b,d} 55	81 ^c 81	67 ^{b,c} 67	42 ^{d,e} 42	36 ^e 36
Malanville - Benin (821 Culicidae *)	Unknown	2 ^a	91 ^b 91	96 ^c 96	90 ^b 90	88 ^{b,d} 87	85 ^d 85
Odisha - India (303 <i>An. fluviatilis</i>)	Permethrin susceptible	2 ^a	100 ^b 100	100 ^b 100	97 ^b 97	100 ^b 100	96 ^b 96

* 10% *Anopheles* spp., 1% *Culex* spp. and 89% *Mansonia* spp.

***An. gambiae* s.s. M Form, with enhanced oxidases, F(kdr)= 0.5 in 2010 (Djègbè et al., 2011).

Note: In Muheza-United Republic of Tanzania, initial dose of permethrin on CTN was 1/5th of the target dose.

Table 6. Permethrin content and retention in Olyset Plus (WHOPES phase II study). Target dose and tolerance limit for permethrin in baseline Olyset Net = 20 g/kg \pm 3 g AI/kg; target dose and tolerance limit for permethrin in baseline Olyset Plus = 20 \pm 5 g AI/kg

Treatment	Benin				India				United Republic of Tanzania			
	PMT content (g/kg) before washing	PMT content (g/kg) after washing	AI retention (% of wash 0)	PMT content (g/kg) after trial	PMT content (g/kg) before washing	PMT content (g/kg) after washing	AI retention (% of wash 0)	PMT content (g/kg) after trial	PMT content (g/kg) before washing	PMT content (g/kg) after washing	AI retention (% of wash 0)	PMT content (g/kg) after trial
Olyset Net 0 wash	19.7	20.0	-	19.7	19.9	-	-	19.3	19.8	19.7	-	19.2
Olyset Net 20 washes	19.6	16.7	85	17.1	20.0	17.7	88	17.6	19.9	16.5	83	16.6
Olyset Plus 0 wash	18.6	19.0	-	17.9	19.1	-	-	17.6	19.1	19.0	-	16.9
Olyset Plus 20 washes	18.6	14.5	78	14.4	18.8	14.1	75	13.8	18.4	13.9	76	13.4
CTN, exhausted	11.0	7.7	70	3.9	15.3	11.4	74	10.4	2.7	2.6	96	3.3
Untreated net	< 1	< 1	-	< 1	< 1	< 1	-	< 1	-	-	-	-

Determination of permethrin content “before washing” implies baseline data on receipt of nets; assay of nets “after washing” refers to those taken to experimental hut trials on completion of washing procedure.

CTN = conventionally treated polyester net; PMT = permethrin.

Table 7. Piperonyl butoxide (PBO) content and retention in Olyset Plus (WHOPES phase II study). Target dose and tolerance limit for PBO in baseline Olyset Plus = 10 ± 2.5 g PBO/kg

Treatment	Benin				India				United Republic of Tanzania			
	PBO content (g/kg) before washing	PBO content (g/kg) after washing	AI retention (% of wash 0)	PBO content (g/kg) after testing	PBO content (g/kg) before washing	PBO content (g/kg) after washing	AI retention (% of wash 0)	PBO content (g/kg) after testing	PBO content (g/kg) before washing	PBO content (g/kg) after washing	AI retention (% of wash 0)	PBO content (g/kg) after testing
Olyset Plus 0 wash	8.7	9.0	-	8.1	9.0	-	-	8.3	8.7	9.0	-	6.0
Olyset Plus 20 washes	8.8	4.5	51	4.3	8.8	4.0	45	3.8	8.4	3.2	38	2.6

Determination of PBO content “before washing” implies baseline data on receipt of nets; assay of nets “after washing” refers to those taken to experimental hut trials on completion of washing procedure.

3. REVIEW OF INTERCEPTOR LN

Interceptor LN is a long-lasting insecticidal mosquito net manufactured by BASF Germany. The net is treated with alpha-cypermethrin (coated onto filaments) at a target dose of 6.7 g AI/kg of netting material for 75-denier yarn or 5.0 g AI/kg for 100-denier yarn, corresponding to 200 mg of alpha-cypermethrin per square metre of the polyester fabric.⁹ Safety assessment and WHO interim recommendations for the product were published in 2006;¹⁰ WHO interim specifications for its quality control and international trade were published in October 2009.¹¹ The present assessment includes a review of relevant background information as well as the results of WHOPES supervised large-scale studies as requirements for development of full recommendations.

3.1 Efficacy – background and supporting documents

Uganda

Killian et al (2011) measured the efficacy of Interceptor LN over three years in Uganda in an area with meso- to hyper-endemic malaria. A total of 200 Interceptor LNs and 100 conventionally treated nets (CTNs) were provided to or prepared for the study. The CTNs were dipped by a team of trained staff to achieve a

⁹ The tolerance limits for active ingredient content in Interceptor LN are $\pm 25\%$ of the target dose, i.e. 5.025–8.375 g AI/kg for 75-denier yarn and 3.75–6.25 g AI/kg for 100-denier yarn.

¹⁰ *Report of the 10th WHOPES Working Group Meeting – Review of Spinosad 0.5% GR and 12% SC, Lambda-Cyhalothrin 10% CS, K-O TAB 1-2-3[®] Interceptor[®]. 11–14 December 2006.* Geneva, World Health Organization, 2006 (WHO/CDS/NTD/WHOPES/2007.1; available at <http://www.who.int/whopes/recommendations/wgm/en/>; accessed July 2012).

¹¹ *WHO specifications and evaluations for public health pesticides: alpha-cypermethrin long-lasting (coated onto filaments) insecticidal net.* Geneva, World Health Organization, 2009 (available at http://www.who.int/whopes/quality/Alphacypermethrin_Interceptor_specs_eval_WHO_Oct_2009.pdf; accessed July 2012).

target dose of 25 mg AI/m². The nets were dipped individually in basins using alpha-cypermethrin 6% (FENDONA, BASF, Midrand, South Africa). All nets were white, 75-denier, polyester nets. A total of 10 Interceptor LNs and 10 CTNs were randomly selected for baseline bioassays and chemical analysis. The remaining 190 Interceptor LNs and 90 CTNs were randomly allocated a unique code and distributed to households in five different villages in May 2006. Only the principal investigator had the allocation list.

A baseline survey was conducted in May 2006 to assess demographic and socio-economic characteristics. After distribution of nets, surveys were conducted at approximately 6-month intervals. At each follow-up survey, all remaining nets were assessed for usage, dirtiness, and frequency and methods of washing. Each net was also assessed for the number and size of holes, categorized as finger size (<2 cm in maximum diameter),¹² hand size (>2 cm but <10 cm in maximum diameter) or head size (>10 cm). The proportionate hole index (pHI) was estimated as number of finger-size holes plus number of hand-size holes multiplied by 9 plus number of head-size holes multiplied by 56. The weights were selected to reflect the approximate surface areas of each hole size and divided by the surface area of the smallest hole size. Including the baseline survey, 8 surveys were conducted over 3.5 years.

At months 6, 12, 24, 36 and 42 post-distribution, nets were randomly selected, removed from the study and replaced with new LNs that were not included as part of the study. A target of 40 LNs was sampled at each survey, except at the 42-month follow up when only 21 nets remained. Forty CTNs were sampled at 6 months and 12 months; all remaining CTNs were sampled at 24 months. In the laboratory, sampled nets were carefully inspected for the number and sizes of holes. Two pieces of netting were then removed from the long side of the net for bioassays and

¹² This study was conducted before the publication of *WHO Guidelines for monitoring the durability of long-lasting insecticidal mosquito nets under operational conditions* (available at <http://www.who.int/whopes/guidelines/en/>; accessed July 2012) and holes <0.5 cm were therefore included in the estimates of physical integrity.

chemical analysis. The piece removed for bioassays was 30 cm x 30 cm and the piece removed for chemical analysis was 10 cm x 10 cm.

Samples for chemical analysis were measured and weighed, and alpha-cypermethrin was extracted by heating under reflux for 60 min in 40 ml of xylene. The extract was transferred to a 50 ml volumetric flask and filled to volume with xylene. A 10x dilution of the extract was analysed to determine alpha-cypermethrin content by capillary gas chromatography with ^{63}Ni electron capture detection using an external standard calibration.¹³

Bioassays were conducted using *An. gambiae* Kisumu strain that was susceptible to pyrethroid insecticides. For the first four follow ups, bioassays were carried out by exposing mosquitoes in plastic cones using standard WHO procedures. Four plastic cones were fixed to a piece of netting and 5 unfed, female mosquitoes, 2–4 days old were introduced into the cones. After 3 min, mosquitoes were removed from the cones and placed in cups with access to honey solution. The process was repeated to allow 40 mosquitoes to run against each net sample. For the 42-month follow up, the bioassays were transferred to a different laboratory because resistance had been detected within the original mosquito strain. For that round, mosquitoes were exposed to the nets for 3 min inside WHO tubes for susceptibility testing, with the netting material replacing the papers. Knock-down (KD) was recorded at 60 min and mortality at 24 h post-exposure. Mosquitoes were considered dead if they could not fly or stand upright. Those that had lost legs but could fly or remain standing were considered alive.

¹³ Chemical residual analysis was done at the Walloon Agricultural Research Centre (WHO Collaborating Centre for quality control of pesticides) using an internal method. This method was accredited according to ISO 17025. The method preceded the publication of the CIPAC method for determination of alpha-cypermethrin content in Interceptor LN. Before the analysis of samples, the method was successfully validated for its specificity, linearity of chromatographic response, repeatability and accuracy.

The primary outcome of net efficacy was based on the bioassay results. Geometric means of KD and mortality were estimated at each time point. In addition, individual nets were categorized as having optimal efficacy, minimal efficacy or lack of efficacy. Nets were considered to have optimal efficacy if they had $\geq 95\%$ KD (60 minutes) or $\geq 80\%$ mortality. Nets were considered to have minimal efficacy if they had $\geq 75\%$ KD (60 minutes) or $\geq 50\%$ mortality.

At baseline, there were no statistically significant differences in demographic or socio-economic variables between households that received an Interceptor LN or a CTN. Interceptor LNs sampled at baseline had a median alpha-cypermethrin dosage of 6.5 g AI/kg, while the CTN had a median alpha-cypermethrin dosage of 0.7 g AI/kg. Both of these values were within 3% of the target doses of 6.7 AI/kg for the Interceptor LN and 25 mg AI/m² for the CTNs. At baseline, both the Interceptor LN and the CTN had full efficacy in bioassays.

Over the course of the study, a total of 16 nets (14 Interceptor LNs and 2 CTNs) were lost to follow up. Net use was high during the first two follow-up surveys, with over 93% of nets used every night. Net use decreased over time to 81.2% of nets reported used every night during the final two surveys. There was no difference in net use between the Interceptor LN and the CTN. Nets were washed approximately 0.9 times per year during the first year. Washing increased to 1.2 times per year at 24 months and to 1.4 washes per year at 36 and 42 months. Washing was mostly done in cold water in a basin with local soap. Nets were dried outside either lying flat or hanging. There was no difference in washing and drying patterns between the Interceptor LN and the CTNs.

The proportion of nets with at least one hole was 19.8% after 6 months and 33.7% after 12 months. By 36–42 months, 77.4% of nets had at least one hole. The pHI increased linearly, with an estimated 3.8 pHI units per month, equivalent to 15 cm² per month or 182 cm² per year (Table 8). Adjusting for time to follow up, there were no differences in physical condition between the Interceptor LN and the CTN.

The alpha-cypermethrin content on Interceptor LNs fell from a median of 6.5 g AI/kg at baseline to a median of 2.1 g AI/kg after 36 months, and to 1.9 g AI/kg after 42 months. At 36 months, 94% of the Interceptor LNs had more than 0.5 g AI/kg of alpha-cypermethrin (Table 9). The rate of loss was estimated at 20% per year. In contrast, the CTNs had lost 93% of their initial alpha-cypermethrin content by 24 months.

In bioassays of sampled nets at 36 months, the Interceptor LN induced >90% KD at 60 min exposure. Knock-down fell to 71.4% after 42 months. Mortality remained >85% at 24 months; it fell to 79.5% after 36 months and 68.3% after 42 months. More than 80% of all Interceptor LNs were considered to have optimal efficacy at 36 months, while at 42 months, 71.4% had optimal efficacy (Table 10).

3.2 Efficacy – WHOPES supervised trials

India

Bhatt et al (2012) conducted two trials of Interceptor LN at two rural sites in India. In the State of Gujarat, four villages with a population of 2117 in 436 households were selected. Most people lived in brick houses with plastered mud walls and tiled roofs. The economy of the villages was centered around agricultural activities; the main crops included rice, groundnut, cotton, millet, banana, tobacco and potato. At the start of the study, households owned an average of 0.5 nets. In the State of Chhattisgarh, seven villages with a population of 2109 in 439 households were selected for the study. Most houses had brick walls with mud plastering and tiled or thatched roofs. The primary economic activity at the study sites was rice cultivation. At the start of the study, there were 1.4 nets per household.

Nets distributed included the Interceptor LN and CTNs. The Interceptor LNs were white, 75-denier, polyester nets measuring 180 cm x 160 cm x 150 cm. The CTNs were white polyester, 100-denier nets of the same size and shape as the Interceptor LNs. The CTNs were treated using an appropriate quantity of alpha-cypermethrin SC 10% (BASF, Agri Production, Genay, France) to

achieve the target dose of 40 mg AI/m². The treatment was done by experienced staff who dried the nets in the shade before packing them in polyethylene bags for distribution. A master list of nets was prepared and each house was randomly assigned to receive two nets of either Interceptor LN or CTN. Each net was given a unique code, which was marked on the net in indelible ink.

A total of 30 Interceptor LNs and 30 CTNs were randomly sampled at 6-month intervals after distribution. Interceptor LNs were sampled up to 36 months while the CTNs were sampled up to 12 months after which all CTNs were replaced with Interceptor LNs. Nets that were sampled were replaced with a new Interceptor LN. The sampled nets were examined for physical integrity and subjected to bioassays. Holes were classified into three sizes: smaller than a thumb,¹⁴ larger than a thumb but smaller than a fist or larger than a fist. A hole index was calculated as the sum of the values derived by multiplying separate number of holes in the three hole-size categories by 1, 23 and 196 respectively. During sampling, a questionnaire was administered to assess frequency of net use and net washing habits. At yearly intervals, all households were visited and were asked about net use and washing habits.

At each study site, 30 Interceptor LNs and 30 CTNs were randomly sampled at baseline for chemical analysis. Another 30 CTNs were sampled at the end of year one and subjected to chemical analysis, while 30 more Interceptor LNs were sampled at the end of year 3 and subjected to chemical analysis. Four samples of 30 cm x 30 cm per net (positions 2 to 5 as per WHOPES guidelines) were cut for chemical analysis. The sample from the lowest portion of each net (position 1) was excluded. Alpha-cypermethrin was extracted by heating under reflux with tetrahydrofuran. Diethyl phthalate was added as an internal standard and alpha-cypermethrin content was

¹⁴ This study was conducted before the publication of *WHO Guidelines for monitoring the durability of long-lasting insecticidal mosquito nets under operational conditions* (available at <http://www.who.int/whopes/guidelines/en/>; accessed July 2012) and holes <0.5 cm were therefore included in the estimates of physical integrity.

determined using gas chromatography with flame ionization detection (GC-FID)¹⁵ (Pigeon 2009, 2010a and 2011a).

Five pieces of netting (30 cm x 30 cm) were cut from each net according to the WHOPES recommendations¹⁶ for bioassays. Bioassays were conducted on all five pieces of netting. A cone was attached to each piece of netting and 5 *An. culicifacies* from a colony susceptible to alpha-cypermethrin were introduced into each cone and exposed for 3 min. The mosquitoes were removed and the process was repeated a second time to expose a total of 50 mosquitoes. After exposure, mosquitoes were transferred to plastic cups and provided glucose solution. Knockdown was recorded at 60 minutes after exposure and mortality at 24 hours post-exposure. Control bioassays were done using untreated netting material. If control mortality was 5–20%, the data were corrected using Abbott's formula. If control mortality was >20%, the data were discarded and tests repeated.

If the KD rate was <95% and bioassay mortality was <80%, the nets were subjected to a tunnel test. The test was performed according to WHOPES guidelines¹⁷ with the exception that a rabbit was used as the bait. One hundred mosquitoes were introduced into the long end of the tunnel at 18:00 and observed at 09:00 the following morning. Mosquitoes were scored according to whether they were dead and/or blood-fed. Blood-feeding inhibition was estimated by comparison with an untreated control net in parallel

¹⁵ CIPAC method 454/LN/M/3.1, CIPAC Handbook M, p.40, 2009.

¹⁶ *Report of the 12th WHOPES Working Group Meeting – Review of Bioflash[®] GR, PermaNet[®] 2.0, PermaNet[®] 3.0, PermaNet[®] 2.5, lambda-cyhalothrin LN. 8 – 11 December 2008.* Geneva, World Health Organization, 2009 (WHO/HTM/NTD/WHOPES/2009.1; available at <http://www.who.int/whopes/recommendations/wgm/en/>; accessed July 2012).

¹⁷ *Guidelines for laboratory and field testing of long-lasting insecticidal mosquito nets.* Geneva, World Health Organization, 2005 (WHO/CDS/WHOPES/GCDPP/2005.11; available at <http://www.who.int/whopes/guidelines/en/>; accessed July 2012).

tunnel tests. Nets were considered effective if mortality was $\geq 80\%$ or blood-feeding inhibition was $\geq 90\%$.

A total of 436 houses were enrolled in Gujarat and 439 in Chhattisgarh. Between the two sites, a total of 1020 Interceptor LNs and 730 CTNs were distributed. Annual rates of loss of Interceptor LNs was $<5\%$ in Gujarat. In Chhattisgarh, the rate of loss accelerated over time: 3.1% was lost in the first year, 8.4% in the second year and 17.7% in the third year. Rates of net use varied seasonally. In Gujarat, the proportion of Interceptor LNs reported used every night and year round ranged from 36.7% to 96.6%. Net use was higher in Chhattisgarh: the proportion reported used every night and year round ranged from 75.0% to 94.6%. There were no differences in net use between the Interceptor LNs and the CTNs. Most nets were washed 1–4 times per year; 12.4% of owners washed their nets once per year, 22.5% twice per year, 29.4% three times per year and 11.5% four times per year. Most people washed their nets using locally available soap powder.

In Gujarat, the proportion of Interceptor LNs with at least one hole rose from 33.3% at 6 months to 87.0% at 36 months. The hole index was 33.6 at 6 months post-distribution and then ranged between 119.1 and 176.8 from 12 months to 30 months post-distribution. At 36 months, the hole index rose to 377.8. In Chhattisgarh, 6.7% of Interceptor LNs had at least one hole after 6 months, while 93.3% had at least one hole after 36 months. The hole index was 7.3 after 6 months and then ranged between 33.9 and 86.8 from 12 months to 30 months post-distribution. At 36 months post-distribution, the hole index was 116.4 (Table 8). In both sites, most of the holes were located on the lower sides and were in the smallest size category. A low proportion of holes were found repaired by the users.

Chemical analysis of the CTNs at baseline indicated that average alpha-cypermethrin concentrations were 30.5 mg Al/m² (0.9 g Al/kg) in Gujarat and 43.9 mg Al/m² (1.4 g Al/kg) in Chhattisgarh (Pigeon 2009). After one year, the alpha-cypermethrin content on nets had declined by 55% in Gujarat and by 74% in Chhattisgarh (Pigeon 2010a).

At baseline, the average alpha-cypermethrin content of the Interceptor LNs was 8.4 g AI/kg in Gujarat and 7.8 g AI/kg in Chhattisgarh (Pigeon 2009). In Gujarat, the mean value exceeded the upper tolerance limit of 8.375 g AI/kg. In Gujarat, 17 of 30 samples were above the upper tolerance limit while 8 of 30 exceeded the upper tolerance limit in Chhattisgarh (Figure 2). No Interceptor LNs were below the lower tolerance limit. After 3 years of use (Pigeon 2011a), the average alpha-cypermethrin content on the Interceptor LNs had declined by 79% in Gujarat and by 85% in Chhattisgarh (Table 9).

Cone bioassays on CTNs showed that mortality after 6 months was 80.8% in Gujarat and 76.1% in Chhattisgarh. After one year, the CTNs failed to meet the WHOPES criteria for knockdown and mortality, and all the remaining CTNs were replaced with Interceptor LN.

In cone bioassays, the Interceptor LNs caused 97.8% knockdown and 98% mortality at baseline. After 36 months, average knockdown declined to 83.3% in Gujarat and 84.3% in Chhattisgarh; mortality declined to 85.0% in both sites. At 12 months, all Interceptor LNs met the WHOPES criteria for mortality and knockdown. At 18 months until the end of the study, a total of 52 Interceptor LNs failed to meet the WHOPES criteria for knockdown and mortality in the cone bioassay and were therefore subjected to the tunnel test. In Gujarat, the proportion of nets meeting WHOPES criteria by either the cone test or the tunnel test was 100% at 18 months, 93% at 24 months, 100% at 30 months and 97% at 36 months. In Chhattisgarh, the proportion of nets meeting WHOPES criteria was 90% at 18 and 24 months, 93% at 30 months and 73% at 36 months (Table 10). However, the efficacy of the Interceptor LN over time may have been influenced by the high proportion of nets exceeding the tolerance limits for alpha-cypermethrin content at baseline. This is particularly true for Gujarat, where alpha-cypermethrin content in more than half of Interceptor LNs tested at baseline was above the tolerance limits.

In a survey carried out one month after distribution, 34% of users in Gujarat and 27% in Chhattisgarh reported transient skin irritations. No adverse effects were observed in subsequent surveys.

United Republic of Tanzania

Tungu et al (2012a) conducted a trial comparing Interceptor LNs with CTNs in Muheza District in the United Republic of Tanzania. The study included three villages with a total population of 4374 in 934 households. A baseline census was conducted between July and October 2008, and nets were distributed door to door in November and December 2008. A total of 1953 Interceptor LNs were distributed along with 1593 CTNs. The nets were randomly distributed to households; each household received enough nets to cover all sleeping spaces. All nets were marked with a unique code number using permanent marker.

Thirty nets of each type were randomly sampled at baseline and at 6 and 12 months post-distribution. The 30 Interceptor LNs were also randomly sampled at 18, 24, 30 and 36 months post-distribution. At the time of net collection, a questionnaire was administered to households that were sampled to assess net use and acceptability, washing practices and any adverse effects. In this study, attrition (loss of nets) was not reported.

The randomly selected nets were assessed for physical durability by draping the nets over a wooden frame and counting the number of holes. Each hole was categorized into four hole sizes: size 1 (smaller than a thumb),¹⁸ size 2 (larger than a thumb but smaller than a fist), size 3 (larger than a fist but smaller than a head) and size 4 (larger than a head). However, no size 4 holes were observed. A hole index was calculated using WHOPES recommendations by counting the number of holes of sizes 1, 2

¹⁸ This study was conducted before the publication of WHO *Guidelines for monitoring the durability of long-lasting insecticidal mosquito nets under operational conditions* (available at <http://www.who.int/whopes/guidelines/en/>; accessed July 2012), and holes <0.5 cm were therefore included in the estimates of physical integrity.

and 3, and multiplying by 1, 23 or 196 respectively. The hole area was calculated by assuming size 1 holes were 0.8 cm², size 2 holes were 28.3 cm² and size 3 holes were 78.5 cm².

At baseline and 12 months post-distribution, five pieces of netting (30 cm x 30 cm) were cut from each of the 117 sampled nets (60 Interceptor and 57 CTNs) for chemical assays. This was also done for 30 each of the Interceptor LNs sampled at 24 and 36 months post-distribution. The piece from the lowest part of the net (position 1) was excluded according to WHOPES guidelines. Net pieces were cut and weighed, and alpha-cypermethrin was extracted by heating under reflux with tetrahydrofuran. Dioctyl phthalate was added as an internal standard; alpha-cypermethrin content was determined using gas chromatography with flame ionization detection (GC-FID)¹⁹ (Pigeon 2010b).

Five netting pieces (25 cm x 25 cm) were cut from each sampled net according to WHOPES guidelines for bioassays. Cone bioassays were conducted using 2–5 day-old, unfed, female *An. gambiae* s.s. (Kisumu strain). Twenty mosquitoes were exposed to each piece of each net (total of 100 mosquitoes per net) for 3 min in a standard WHO plastic cone. After exposure, mosquitoes were held in paper cups at 26 °C and 80% relative humidity with access to cotton wool soaked in 10% glucose solution. Knockdown was measured at 3 min, and then mortality 60 min and 24 h post-exposure. When knockdown at 60 min was <95% and mortality was <80%, the net was subjected to a tunnel test. Only the net piece closest to average mortality was used for the tunnel test.

During net sampling, households were asked about house characteristics, net use and net washing practices. Most houses (50–64%) had palm thatched roofs; the remainder had corrugated iron roofs. Most residents (43–97%) were farmers and most (65–79%) had received 7 or more years of primary-school education; but <10% had received secondary education or beyond.

¹⁹CIPAC method 454/LN/M/3.1, CIPAC Handbook M, p.40, 2009.

Reported net use was high throughout the study. At 12, 24 and 36 months post-distribution, 100% of respondents indicated using their nets every night throughout the year.

Interceptor LNs were washed on average four times per year. Washing frequencies were not different from the CTNs. Nets were soaked by 20–37% of respondents; soaking times ranged from 10 min to 2 hours. Most nets (68–90%) were rinsed after washing and most (75–95%) were dried outside. Nets were reported washed using commercial bar soap (53–62%), commercial detergent powder (17–27%) or both (8–30%).

Among Interceptor LNs, 63% had acquired at least one hole after 6 months and 60% had at least one hole at 12 months post-distribution. By 36 months, 83% of nets had at least one hole. Among the CTNs, 83% had at least one hole at 6 months, although this declined to 67% after one year as different nets were sampled at each time point. The average pHI of the Interceptor LN was 139 at 6 months after distribution, 442 at 30 months and 332 at 36 months (Table 8). The median pHI was 2 at both 6 months (IQR = 0–83) and 12 months (IQR = 0–68). The median pHI rose to 78 (IQR = 3–533) at 24 months and 126 (IQR = 30–549) at 36 months. The mean pHI of the CTN was 121 at 6 months and 205 at 12 months. The median pHI of the CTNs was 4 (IQR = 1–60) at 6 months and 6 (IQR = 0–87) at 12 months. The mean hole area was 83 cm² in the Interceptor LNs and 91 cm² in the CTNs after 6 months. The mean hole area was 88 cm² in the Interceptor LNs and 134 cm² in the CTNs after 12 months. At 36 months, the mean hole area was 229 cm² in the Interceptor LNs. The median hole area on the Interceptor LNs rose from 2 cm² (IRQ = 0–69) at 6 months, to 84 cm² (IQR = 3–404) at 24 months and 102 cm² at 36 months (IQR = 33–346). The median hole area on the CTNs was 4 cm² (IQR = 1–60) at 6 months and 6 cm² (IQR = 0–87) at 12 months.

Mean alpha-cypermethrin content at baseline was 6.5 g Al/kg for the Interceptor LN (corresponding to 204.4 mg/m²) and 1.0 g Al/kg (corresponding to 31.9 mg/m²) for the CTN (Pigeon 2010b). One of the 30 Interceptor LNs was outside the acceptable range for Al content (Figure 2). At 12 months, the mean alpha-cypermethrin

content had fallen to 3.3 g AI/kg for the Interceptor LN (corresponding to 109.8 mg/m²) and 0.8 g AI/kg (corresponding to 28.5 mg/m²) for the CTN. Alpha-cypermethrin content on the Interceptor LN was 2.0 g AI/kg at 24 months and 1.2 g AI/kg at 36 months (Pigeon 2012a) (Table 9).

At baseline, knockdown was 100% and mortality was >99% for all nets. After 6 months, the mean percentage mortality was 92% on the Interceptor LNs and 80% on the CTNs (P<0.001). Similarly, knockdown was 95% on the Interceptor LNs compared with 85% on the CTNs (P<0.001). Two of the Interceptor LNs and 10 of the CTNs failed to meet the WHOPES criteria for the cone test. When the tunnel test was applied, all Interceptor LNs and all but two of the CTNs met the WHOPES criteria. At 12 months, 97% of the Interceptor LNs but only 63% of the CTNs met the WHOPES criteria for cone and tunnel tests.

The CTNs were removed from the study after 12 months and replaced with uncoded Interceptor LNs; all subsequent tests were done on the coded Interceptor LNs only. At 18 months, 97% of Interceptor LNs met the WHOPES criteria by either the cone or the tunnel test. This figure declined to 90% at 24 months, 83% at 30 months and 87% at 36 months (Table 10).

Very few adverse effects were reported by net users. At 12 months post-distribution, 8.4% of respondents reported experiencing adverse effects during the first few days of use. The most common events were tingling (2%), headache (1.6%) and irritation (1.2%). Adverse effects were slightly higher among users of the Interceptor LN compared to CTNs (11.5% versus 5%). No adverse effects were reported in any of the subsequent surveys.

3.3 Conclusions and recommendations

Interceptor LN is a long-lasting insecticidal mosquito net manufactured by BASF Germany. The net is treated with alpha-cypermethrin (coated onto filaments) at a target dose of 6.7 g AI/kg of netting material for 75-denier yarn or 5.0 g AI/kg for 100-denier

yarn, corresponding to 200 mg of alpha-cypermethrin per square metre of the polyester fabric.

WHOPES published interim recommendations for Interceptor in 2006 based on phase I laboratory testing and phase II experimental hut studies. Full WHO recommendations require further evidence of the efficacy, durability and acceptability of Interceptor LN under routine household use over a period of 3 years. Longitudinal, randomized household trials to evaluate its efficacy, longevity and fabric integrity over a period of 3 years are required as part of phase III testing. WHOPES guidelines recommend that after 3 years of routine household use at least 80% of nets tested meet the cut-off criteria²⁰ for either the WHO cone bioassay test or the tunnel test. However, criteria for fabric integrity have yet to be established.

This report reviews the data from phase III testing of the Interceptor LN in four sites in three countries.

The loss of physical integrity was measured by the percentage of nets with holes and the hole index. Among the four sites, the percentage of Interceptor LNs with at least one hole after 6 months of use ranged from 6.7% to 63%. After 36 months of use, that percentage ranged between 77.4% and 93.3%. The mean hole index ranged between 7.3 and 139 after 6 months of use and between 116.4 and 377.8 after 36 months of use (Table 8). The methods used in these studies differed slightly from those recommended in WHO's *Guidelines for monitoring the durability of long-lasting insecticidal nets under operational conditions*,²¹ because holes of <0.5 cm in diameter were included in the estimates of the proportion of nets with holes and the hole indices.

The alpha-cypermethrin content declined over the 3 years of use in each study site. Among the different trials, the loss of insecticidal

²⁰ *Guidelines for laboratory and field testing of long-lasting insecticidal mosquito nets*. Geneva, World Health Organization, 2005 (WHO/CDS/WHOPES/GCDPP/2005.11; available at <http://www.who.int/whopes/guidelines/en/>; accessed July 2012).

²¹ Available at <http://www.who.int/whopes/guidelines/en/>.

content ranged from 68% to 85% over the course of 36 months (Table 9).

After 36 months, the percentage of nets that met WHOPES criteria by either the cone test or the tunnel test was 83.3% in Uganda, 97.6% in one site in India (Gujarat), 73.3% at a second site in India (Chhattisgarh) and 87% in the United Republic of Tanzania (Table 10). However, the validity of the data from Gujarat cannot be confirmed as 56% of nets exceeded the acceptable threshold for alpha-cypermethrin content at baseline.

In the two trials where the Interceptor LNs were in the acceptable range for alpha-cypermethrin content, the Interceptor LN did meet the WHOPES efficacy criteria after 3 years of use.

Noting the above, the meeting recommended:

- that based on existing WHOPES guidelines, which are largely based on efficacy criteria, and noting the overall bio-efficacy of the Interceptor LN, full recommendation is granted;
- a need for an improved manufacturing quality assurance to ensure that LNs comply with WHO specifications.

The meeting also recommended:

- that national programmes monitor and evaluate the performance of LNs, including the Interceptor LN, under local conditions following procedures recommended in WHO guidelines,²² to select the most suitable LN for their local setting;

²² *Guidelines for monitoring the durability of long-lasting insecticidal mosquito nets under operational conditions*. Geneva, World Health Organization, 2011 (WHO/HTM/NTD/WHOPES/2011.5; available at <http://www.who.int/whopes/guidelines/en/>; accessed July 2012)

- that the relationship between hole index and personal protection be investigated to better inform policies and strategies for distribution and replacement of LNs.

Note: WHO recommendations on the use of pesticides in public health are valid ONLY if linked to WHO specifications for their quality control.

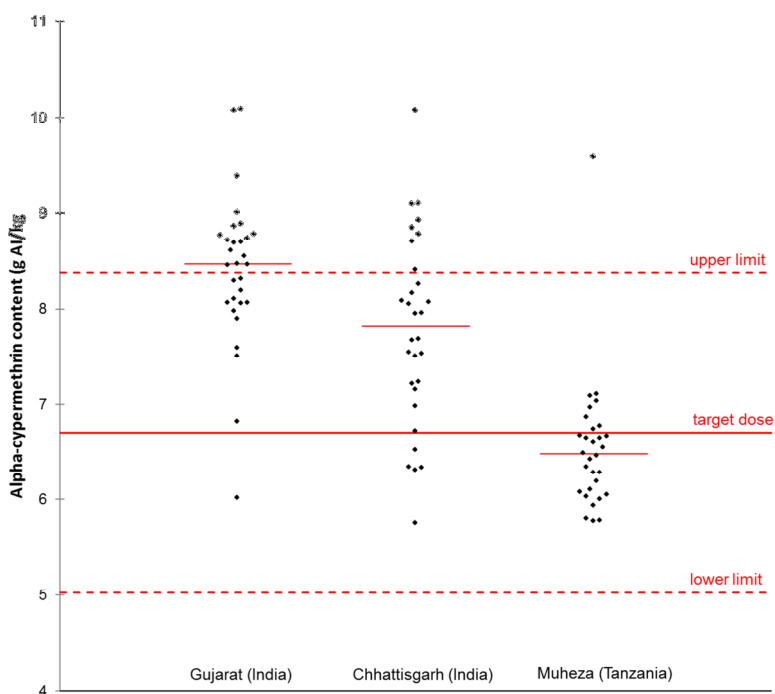


Figure 2. Scatterplot of alpha-cypermethrin content in g AI/kg on individual net samples at baseline, India and the United Republic of Tanzania. The target dose (6.7 g AI/kg) and the upper (8.375 g AI/kg) and lower (5.025 g AI/kg) limits are for alpha-cypermethrin content, indicated as solid and dashed lines, respectively.

Table 8. Physical integrity of Interceptor LNs over time in three study sites. For each site, the number of nets examined (N), the percentage with any holes and the mean proportionate hole index (pHI) (standard deviation in bracket) are presented.

		Months after distribution					
		6	12	18	24	30	36
Uganda*	N	---	447	---	239	---	122
	% with any holes	---	26	---	47	---	63
	pHI	---	13 (50)	---	46 (99)	---	92 (160)
India	N	30	30	30	30	30	30
Gujarat	% with any holes	33	63	67	77	77	87
	pHI	34 (150)	177 (674)	119 (371)	137 (323)	173 (375)	378 (858)
India	N	30	30	30	30	30	30
Chhattisgarh	% with any holes	7	33	67	63	77	93
	pHI	7 (36)	58 (190)	34 (88)	87 (207)	68 (36)	116 (157)
United Republic of Tanzania	N	30	30	30	30	30	30
	% with any holes	63	60	---	83	---	83
	pHI	139 (351)	170 (630)	---	442 (696)	---	332 (442)

* Note: Uganda results were combined across years such that the 12-month follow up represents the combined results from the 6 and 12 month follow ups, the 24 month follow up represents the combined results from the 18- and 24-month follow up, and the 36-month follow up represents the combined results from the 30- and 36-month follow ups. The standard deviations for the Uganda pHI values were back calculated from the mean, 95% confidence limits and the sample size. The pHI was calculated using different conversions for the Uganda study.

Table 9. **Mean (95% confidence limits) alpha-cypermethrin content in Interceptor LNs in g AI/kg and percent AI lost over time in three study sites**

Site	Test	Months after distribution				
		0	6	12	24	36
Uganda*	N	10	38	37	37	36
	Median	6.5 (5.3-7.1)	6.5 (5.5-7.0)	4.8 (3.2-5.9)	4.5 (2.7-6.1)	2.1 (1.5-3.0)
	% Lost	---	0	26	31	68
India	N	30	---	---	---	30
Gujarat	Mean	8.4 (8.1-8.7)	---	---	---	1.8 (1.2-2.3)
	% Lost	---	---	---	---	79
India	N	30	---	---	---	30
Chhattisgarh	Mean	7.8 (7.4-8.2)	---	---	---	1.2 (0.7-1.7)
	% Lost	---	---	---	---	85
United Republic of Tanzania	N	30	---	30	30	30
	Mean	6.5 (6.3-6.8)	---	3.3 (2.6-4.0)	2.0 (1.6-2.4)	1.2 (0.8-1.6)
	% Lost	---	---	49	69	82

* For Uganda, the median alpha-cypermethrin content was reported in both g AI/kg and mg/ AI/m² at baseline but in mg AI/m² only for months 6, 12 and 24. These were converted to g AI/kg using the net density that was calculated at baseline.

Table 10. Number and percentage of Interceptor LNs meeting WHOPES criteria according to the cone test or the tunnel test in three study sites (for each site, the total number (N), the number passing the cone test (Cone) and the number passing the tunnel test (Tunnel) are presented; the percentage passing according to either the cone test or the tunnel test are presented in the bottom row for each site (%))

		Months after distribution					
		6	12	18	24	30	36
Uganda	N	40	33	---	37	---	36
	Cone	40	33	---	35	---	30
	Tunnel	---	---	---	---	---	---
	%	100	100	---	95	---	83
India Gujarat	N	30	30	30	30	30	30
	Cone	30	30	26	27	26	20
	Tunnel	---	---	4	1	4	9
	%	100	100	100	93	100	97
India Chhattisgarh	N	30	30	30	30	30	30
	Cone	30	30	25	27	24	13
	Tunnel	---	---	2	0	4	9
	%	100	100	90	90	93	73
United Republic of Tanzania	N	30	30	30	30	30	30
	Cone	28	29	29	19	25	23
	Tunnel	2	0	0	8	0	3
	%	100	97	97	90	83	87

4. REVIEW OF MALATHION 440 EW

Malathion 440 EW is an emulsion, oil-in-water formulation, containing 440 g of active ingredient per litre. The product is a water-based formulation marketed for outdoor space spraying as either thermal fog or cold aerosol against mosquitoes.

Malathion ultra-low volume liquid (UL) has previously been evaluated by WHO and is recommended for outdoor, thermal or cold fog space spraying at the dosage of 112–600 g AI/ha.²³ WHO specifications for malathion technical material and UL formulation,²⁴ developed under the new procedure, are based on Cheminova's data package and were published in July 2004.

The present review assesses the efficacy of malathion EW (Fyfanon® 440 EW of Cheminova, Denmark) comparing with the previously published WHO recommendations for the UL formulation.

4.1 Safety assessment

The human and environmental risk assessment of malathion 440 g AI/L EW for outdoor space spraying, provided by the manufacturer, was assessed by the Finnish Institute of Occupational Health (FIOH, 2011) on behalf of WHOPES. The WHO *Generic risk assessment model for indoor and outdoor space spraying of insecticides* was used as a guiding document.

The following assumptions were made in the assessment as per WHO recommendations, that:

- malathion 440 g/L EW is only used for outdoor space spraying;

²³ Available at:

http://www.who.int/whopes/Insecticides_for_space_spraying_nov_2011.pdf.

²⁴ Available at: http://www.who.int/whopes/quality/en/Malathion_july04.pdf.

- WHO guidance for space spray application of insecticides for vector and public health pest control²⁵ and the manufacturer's label instructions are strictly followed;
- the product complies with the WHO specification, notably with reference to the impurity profile;
- the acceptable daily intake (ADI) of 0.3 mg/kg_{bw} per day and the acute reference dose (ARfD) of 2.0 mg/kg_{bw} adequately reflect the toxicity of malathion; and
- the environmentally acceptable concentration of 30 µg/L adequately reflects the toxicity of malathion to *Daphnia*.

FIOH concluded that the characterization of the risks performed by the proposer closely follows the WHO generic risk assessment model and where default assumptions are not accepted, justification is presented. The conclusion, in line with the generic model, is that exposure from space spraying with malathion EW using vehicle-mounted or hand-held sprayer:

- does not cause untoward health effects to the operator, or to bystanders of different ages; toddlers should not stay in the spray cloud;
- does not cause untoward effects on soil function or terrestrial vertebrates; but
- causes a medium risk to aquatic organisms, notably fish. Drift of spray onto waterways should be avoided.

4.2 Efficacy – background and supporting documents

Published reports on the use of malathion EW for space spraying are limited. Four unpublished reports were considered by the meeting as background documents: two trials carried out by the Institute of Vector Reservoir Control, Research and Development, Java, Indonesia (2009a and 2009b) and two studies conducted by the Institute for Medical Research, Kuala Lumpur, Malaysia (Chen Chee Dhang et al., 2009a and 2009b). The studies compared the

²⁵ Available at:
http://whqlibdoc.who.int/hq/2003/WHO_CDS_WHOPES_GCDPP_2003.5.pdf.

application of malathion 440 EW as outdoor cold and thermal fog applications against *Aedes aegypti* and *Culex quinquefasciatus*.

The Indonesian reports did not provide critical information relating to the test procedure, such as the type of sprayer used for cold fogging, meteorological data, duration of exposure and the mosquito susceptibility status. Noting that these omissions may have affected the outcome or interpretation of the results, the studies were not included as part of this assessment.

The Malaysian studies provided adequate detail on the application procedure. However, the duration of exposure used (60 min) was far longer than that recommended by WHO²⁶ and raised the possibility that efficacy was overestimated as a result of prolonged contact of mosquitoes with insecticide deposits on the cages. The use of stationary magnesium oxide coated slides to collect airborne droplets rather than a slide rotating device would not have collected a representative sample.

4.3 Efficacy – WHOPES supervised trials

Malaysia

Zairi et al (2012) evaluated the efficacy of space spray formulations of malathion EW and malathion UL in small-scale field trials in Penang, Malaysia, against four species of susceptible, laboratory bred mosquitoes (*Ae. aegypti*, *Ae. albopictus*, *An. sinensis* and *Cx. quinquefasciatus*). All species showed full susceptibility to malathion (100% mortality) using the WHO diagnostic dosages of 0.8% for *Ae. aegypti* and *Ae. albopictus*²⁷ and 5.0% for *An. sinensis* and *Cx. quinquefasciatus*.

²⁶ *Guidelines for efficacy testing of insecticides for indoor and outdoor ground-applied space spray applications*. Geneva. World Health Organization, 2009 (WHO/CDS/NTD/WHOPES/2009.2; available at <http://www.who.int/whopes/guidelines/en/>; accessed July 2012).

²⁷ Currently, there is no WHO-recommended diagnostic dosage for *Aedes albopictus*, and the recommended diagnostic dosage for *Ae. aegypti* has been used.

The space spraying trials were carried out during the evening from 19:00 onwards in an open, outdoor arena measuring 200 m x 100 m. The EW formulation was mixed with water and the UL formulation was mixed with diesel and heavy aromatic naphtha (HAN) at the ratio 1:1. The spray application equipment used were a LECO ULV Model 1800 cold fogger and a vehicle-mounted TIFA Model 100E thermal fogger.

The formulations were sprayed with the nozzle directed upwards at 30° horizontal; the vehicle travelled the length of the arena at a speed of 6–9 km/hour. The target dosages applied were 132, 180, 198, 218 and 264 g AI/ha. The cold fog treatments were applied at 0.6 litres/min and the thermal fog at 3.2 litres/min. Water was sprayed as a control.

Sampling stations were positioned at 25 m, 50 m, 75 m and 100 m perpendicular to the line of application. The Teflon-coated slides were placed at each sampling station on slide rotors 1.5 m above the ground to record the presence and distribution of droplet sizes. The rotator was operated 15 min before spraying and 15 min after spraying.

The distribution of droplet size was determined by microscopy using image analysis software. Insecticidal efficacy was determined by bioassay using batches of 20 female mosquitoes of each species, 2–5 days old, held in nylon mesh-framed cylindrical cages (diameter 10 cm, height 15 cm, 1.2 mm mesh) hung 1.5 m above the ground at each station.

The mosquitoes were exposed for 15 min post application, KD was recorded, then they were transported to a laboratory maintained at 26–28 °C and 65–80% relative humidity (RH), and transferred from the cages to plastic cups and provided with 10% sugar solution. Knockdown was recorded 60 min post exposure and mortality after 24 h. Abbott's formula was used to adjust for control mortality. Control mortality was reported as less than 10%.

Each concentration of the formulations plus controls were tested three times. During the malathion 440 EW cold and thermal fog applications, the temperature ranged from 23.6 °C to 29.0 °C, the

relative humidity from 62% to 90% and the wind velocity from 0.4 m/sec to 1.8 m/sec. During the malathion ULV applications, the temperature ranged from 24.2 °C to 28.0 °C, the relative humidity from 62 to 97% and the wind velocity from 0.5 to 4.8 m/sec.

The objective was to determine the dosage of active ingredient per hectare to achieve an average of least 90% mortality across all four stations.²⁸ Cold fogging with malathion 440 EW against the four indicator mosquito species registered 98–100% mortality at all sampling stations for the higher application rates of 218 and 264 g AI/ha (Table 11). The minimum effective dosage to achieve $\geq 90\%$ mortality was 198 g AI/ha for *An. sinensis* and *Cx. quinquefasciatus*, and 180 g AI/ha for *Ae. aegypti* and *Ae. albopictus*. Thermal fogging with malathion 440 EW registered 100% mortality against the four indicator mosquito species at all sampling stations for the application rate of 198 g AI/ha. This was also the minimum effective dosage for thermal fogging. The efficacy of thermal fogging with malathion 440 EW showed some differences to that of cold fogging for certain species, but overall the efficacy was similar.

Cold fogging with malathion UL (965 g AI/l) registered 100% mortality against the four indicator mosquito species at all sampling stations for the application rate of 264 g AI/ha. The minimum effective dosage was 132 g AI/ha for *Ae. aegypti*, *Ae. albopictus* and *An. sinensis* and was 180 g AI/ha for *Cx. quinquefasciatus* (Table 11).

Thermal fogging with malathion UL registered 99–100% mortality against the four indicator mosquito species at all sampling stations for the application rate of 218 g AI/ha. The minimum effective dosage was 180 g AI/ha for *Ae. aegypti*, *An. sinensis* and *Cx. quinquefasciatus* and was 132 g AI/ha for *Ae. albopictus*. The efficacy of cold fogging with malathion UL was similar to that of thermal fogging (Table 12).

²⁸ Guidelines for efficacy testing of insecticides for indoor and outdoor ground-applied space spray applications. Geneva, World Health Organization, 2009 (WHO/CDS/NTD/WHOPES/2009.2; available at <http://www.who.int/whopes/guidelines/en/>; accessed July 2012).

The evidence from lower application rates suggests that in this site, cold and thermal fogging were for each species more effective with UL than with EW.

USA

Bonds and Latham (2012) carried out open field trials to compare the efficacy of space spray formulations of malathion EW and malathion UL in Florida, USA, against three laboratory-bred species of mosquitoes (*Ae. albopictus*, *An. quadrimaculatus* and *Cx. quinquefasciatus*). *An. quadrimaculatus* and *Cx. quinquefasciatus* showed full susceptibility to malathion (100% mortality) using the WHO diagnostic concentration of malathion 5%. *Ae. albopictus* tested against 0.8% malathion (i.e. the diagnostic concentration for *Ae. aegypti*) produced a mortality less than 10%. Tests with a wide range of concentration against *Ae. albopictus* produced an LC90 of 13%, indicating malathion resistance.

Two application methods were used: cold aerosol and thermal fogging. The spray application equipment used were a vehicle-mounted cold fogger Model Grizzly (Clarke Mosquito Control, Illinois, USA) and a portable thermal fogger Model SN50 (Swingtec GmbH, Isny, Germany).

For deciding on application rates of malathion space spray treatment, reference was made to three authoritative sources: WHO recommendations (112–600 g AI/ha), manufacturers' label rates for malathion 440 EW (264–352 g AI/ha) and label rates of the United States Environment Protection Agency for malathion UL (33–65 g AI/ha for cold fogging and 89–123 g AI/ha for thermal fogging). Malathion 440 EW was applied undiluted as a cold fog at five application rates (33.5, 67, 123, 198 and 264 g AI/ha) and as a thermal fog (at 90, 123, and 198 g AI/ha). Malathion UL was applied undiluted as a cold fog (at 33.5, 67 and 264 g AI/ha) and as a thermal fog (at 123 and 264 g AI/ha) but at the 90 g AI/ha rate the malathion UL was diluted with a mixture of one part heavy aromatic naphtha 150 (HAN) and two parts of diesel.

The application was sampled in three rows, 25 m apart, with sampling stations perpendicular to the line of application located at 25, 50, 75 and 100 m downwind, giving 12 sampling stations per

spray run. Two control stations were located at least 50 m upwind of the spray application route. The fog was applied when the wind speed was between 1.6 and 16 km/hour, and after the temperature inversion was formed indicative of stable atmosphere.

Droplets were sampled using slide spinners (speed 5.6 m/s) with two 3 mm wide Teflon-coated slides placed at each of the sampling stations and two control sites for 15 min after completion of the spray application.

Insecticidal efficacy was determined by bioassay using batches of 25 female mosquitoes of each species, 2–7 days old, held in nylon mesh-framed cages (diameter 9 cm, height 12 cm, 1.3 mm mesh) positioned 1.5 m above the ground at each station. The mosquitoes were exposed for 15 min post application, given light CO₂ anesthesia, transferred to the laboratory and held in holding cups with sugar solution at 25 ± 2 °C for 24 h. The mortality was read at 1 hour and 24 h post-exposure. Abbott's correction was made when the mortality in the control was 5–20%; above 20% the result was rejected.

Cold fogging with malathion 440 EW against two indicator species (*An. quadrimaculatus* and *Cx. quinquefasciatus*) registered 100% mortality at all sampling stations for the application rates of 132 g AI/ha and above. The lowest dosage applied (33.5 g AI/ha) gave 90% mortality (Table 11).

Thermal fogging with malathion 440 EW against *An. quadrimaculatus* and *Cx. quinquefasciatus* registered 100% mortality at all sampling stations for the application rate of 90 g AI/ha. Lower application rates were not tested (Table 11).

Cold fogging with malathion UL against *An. quadrimaculatus* and *Cx. quinquefasciatus* registered 98–100% mortality for the application rate of 33.5 g AI/ha and all higher dosages. Lower rates were not tested (Table 12).

Thermal fogging with malathion UL against *An. quadrimaculatus* showed 98–100% mortality at all application rates (90 g AI/ha and above). *Cx. quinquefasciatus* did not produce a clear dose-dependent trend with increasing application rates: 98–100%

mortality was registered for the application rates of 90 g AI/ha and 264 g AI/ha, whereas the application rate of 123 g AI/ha recorded 89% mortality (Table 12).

Mortality of *Ae. albopictus* was consistently lower than the other two indicator species. Neither the two formulations nor the two different application techniques killed 100% of this species probably as a result of resistance to malathion. In this site, this species does not fulfill the susceptibility requirement of the test procedure, and these results were not considered in the development of recommendations.

It was not possible to differentiate between the efficacy of the two formulations because both induced high mortality of the susceptible species at the lowest dosages tested (>98%).

4.4 Conclusions and recommendations

Malathion 440 EW (Fyfanon 440 EW of Cheminova, Denmark) is an emulsion, oil-in-water formulation, containing 440 g active ingredient per litre. The product is a water-based formulation marketed for outdoor space spraying as either thermal or cold fogging for the control of mosquitoes.

The present review assessed the efficacy of malathion EW and compared it with the UL formulation for which WHO recommendations have previously been published. Malathion UL is recommended for outdoor, thermal or cold fog space spraying at the dosage of 112–600 g AI/ha.²⁹

The human and environmental risk assessment of malathion 440 g/l EW for outdoor space spraying, provided by the manufacturer, was assessed, using the procedures and criteria of the WHO generic risk assessment for indoor and outdoor space spraying of insecticides. It was concluded that exposure from space spraying

²⁹ Available at:

http://www.who.int/whopes/Insecticides_for_space_spraying_nov_2011.pdf.

with malathion EW using vehicle-mounted or hand-held sprayer does not cause untoward health effects to the operators, or to bystanders of different ages. However, toddlers should not stay in the spray cloud. Moreover, it does not cause untoward effects on soil function or terrestrial vertebrates, but causes a medium risk to aquatic organisms, notably fish. Therefore, drift of spray onto waterways should be avoided.

Efficacy of malation EW was assessed with that of malathion UL in outdoor applications of cold and thermal fog in Penang, Malaysia and Florida, USA. Malathion-susceptible *Ae. aegypti*, *Ae. albopictus*, *An. sinensis* and *Cx. quinquefasciatus* were used in Malaysia, while malathion-susceptible *An. quadrimaculatus* and *Cx. quinquefasciatus* were used in Florida (Tables 11 and 12).

The effective dosage to achieve >90% average mortality at stations up to 100 m from the sprayer in the Malaysian study were 180–198 g AI/ha for cold and thermal fogging with the EW, but with the UL they ranged from 132 g AI/ha to 180 g AI/ha.

The minimum effective dosage in the Florida study could not be determined because the lowest dosage applied caused >90% against susceptible species.

Full effectiveness was achieved with application rates much lower than the maximum dosage recommended for UL formulations, indicating that thresholds could be reduced for susceptible species using modern application equipment under good weather conditions.

Current label recommendations of malathion UL, which vary in different countries, and the comparable efficacy of EW with UL formulation allows use of EW at similar dosages for outdoor thermal and cold fog applications.

Considering the safety of malathion 440 EW and its comparable efficacy with malathion UL formulation, the meeting recommended:

- the use of malathion 440 EW for outdoor cold and thermal fog application, using the previously recommended WHO

dosage of 112–600 g AI/ha; the dosage should be adapted to the local target mosquito population and local setting under operational conditions.

Note: WHO recommendations on the use of pesticides in public health are valid ONLY if linked to WHO specifications for their quality control.

Table 11. Percentage 24-hour mortality (\pm standard deviation) of mosquitoes exposed to malathion 440 EW cold and thermal fog (average of 25, 50, 75 and 100 m sampling stations downwind)

Application	Dosage	Malaysia				Florida		
		<i>Ae. aegypti</i>	<i>Ae. albopictus</i>	<i>An. sinensis</i>	<i>Cx. quinquefasciatus</i>	<i>Ae. albopictus</i>	<i>An. quadrimaculatus</i>	<i>Cx. quinquefasciatus</i>
Cold fog	33.5	NE*	NE	NE	NE	64.9 \pm 8.6	99.2 \pm 1.1	92.6 \pm 10.0
	67	NE	NE	NE	NE	81.0 \pm 20.2	97.8 \pm 9.1	96.0 \pm 15.6
	132	68.3 \pm 20.5	61.7 \pm 10.1	75.8 \pm 10.2	46.7 \pm 12.6	72.3 \pm 19.5	100.0 \pm 0	100.0 \pm 0
	180	98.7 \pm 1.3	94.2 \pm 5.8	88.8 \pm 71.3	84.6 \pm 14.2	NE	NE	NE
	198	99.2 \pm 0.8	97.1 \pm 1.8	90.4 \pm 3.3	94.6 \pm 1.1	96.1 \pm 8.9	100.0 \pm 0	100.0 \pm 0
	264	100.00 \pm 0	100.0 \pm 0	100.0 \pm 0	100.0 \pm 0	95.0 \pm 8.2	100.0 \pm 0	99.9 \pm 0.7
Thermal fog	89.6	NE	NE	NE	NE	81.7 \pm 21.7	100.0 \pm 0	100.0 \pm 0
	123	NE	NE	NE	NE	91.9 \pm 16.1	100.0 \pm 0	100.0 \pm 0
	132	56.7 \pm 23.3	53.8 \pm 2.9	52.3 \pm 24.6	45.0 \pm 21.4	NE	NE	NE
	180	74.6 \pm 4.8	77.1 \pm 4.2	88.3 \pm 3.4	76.7 \pm 2.1	NE	NE	NE
	198	100.0 \pm 0	100.0 \pm 0	100.0 \pm 0	100.0 \pm 0	78.7 \pm 16.7	99.4 \pm 3.5	99.4 \pm 3.7
	218	100.0 \pm 0	100.0 \pm 0	100.0 \pm 0	100.0 \pm 0	NE	NE	NE
	264	100.0 \pm 0	100.0 \pm 0	100.0 \pm 0	100.0 \pm 0	NE	NE	NE

NE = not examined.

Table 12. **Percentage 24-hour mortality (\pm standard deviation) of mosquitoes exposed to malathion UL cold and thermal fog (average of 25, 50, 75 and 100 m sampling stations downwind)**

Application	Dosage	Malaysia				Florida		
		<i>Ae. aegypti</i>	<i>Ae. albopictus</i>	<i>An. sinensis</i>	<i>Cx. quinquefasciatus</i>	<i>Ae. albopictus</i>	<i>An. quadrimaculatus</i>	<i>Cx. quinquefasciatus</i>
Cold fog	33.5	NE	NE	NE	NE	76.5 \pm 26.0	100.0 \pm 0	98.3 \pm 5.5
	67	NE	NE	NE	NE	74.5 \pm 18.8	99.6 \pm 1.9	99.7 \pm 1.1
	132	98.8 \pm 1.3	98.8 \pm 1.3	99.2 \pm 0.8	82.1 \pm 12.2	NE	NE	NE
	180	97.1 \pm 1.3	90.8 \pm 6.3	90.8 \pm 6.3	95.4 \pm 2.9	NE	NE	NE
	198	98.8 \pm 0.7	97.9 \pm 1.1	97.2 \pm 1.5	90.0 \pm 5.5	NE	NE	NE
	218	100.0 \pm 0	99.6 \pm 0.4	98.3 \pm 0.8	97.1 \pm 1.1	NE	NE	NE
Thermal fog	264	100.0 \pm 0	100.0 \pm 0	100.0 \pm 0	100.0 \pm 0	99.7 \pm 1.1	100.0 \pm 0	100.0 \pm 0
	50	72.1 \pm 2.2	75.8 \pm 1.1	85.0 \pm 3.8	78.3 \pm 1.5	NE	NE	NE
	89.6	NE	NE	NE	NE	70.8 \pm 14.0	100.0 \pm 0	97.8 \pm 9.4
	90	87.1 \pm 1.7	85.8 \pm 2.5	84.6 \pm 0.8	85.4 \pm 0.4	NE	NE	NE
	123	NE	NE	NE	NE	43.8 \pm 21.1	98.0 \pm 7.0	89.1 \pm 25.3
	132	88.3 \pm 3.3	91.3 \pm 0.7	88.3 \pm 2.9	91.7 \pm 1.5	NE	NE	NE
	180	95.8 \pm 0.4	92.9 \pm 1.1	95.0 \pm 1.3	93.8 \pm 0.7	NE	NE	NE
	198	99.6 \pm 0.4	98.3 \pm 1.1	97.9 \pm 0.8	97.1 \pm 1.7	NE	NE	NE
	218	100.0 \pm 0	100.0 \pm 0	100.0 \pm 0	100.0 \pm 0	NE	NE	NE
	264	NE	NE	NE	NE	90.9 \pm 17.4	99.4 \pm 2.9	96.9 \pm 11.4

NE = not examined.

5. Review of VectoBac GR

VectoBac GR (Valent BioSciences, USA) is a granule formulation of a bacterial larvicide, the active ingredient of which is composed of viable *Bacillus thuringiensis israelensis* (*Bti*) strain AM65-52 endospores and delta-endotoxin crystals, produced by fermentation of this bacterium. The biopotency of GR formulation is 200 international toxic units (ITU) per mg and can be applied to mosquito breeding sites by hand or granule spreaders. The product should not be mixed with sand for application.

VectoBac GR is not intended for the control of container-breeding mosquitoes. The formulation is designed for good penetration down through vegetation and immediate release of *Bti* active ingredients into water.

The manufacturer has informed WHOPES that VectoBac GR is similar to the VectoBac custom granules (CG) and to VBC-060216 in performance but replacing the corn cob carrier with a new carrier. The manufacturer has further clarified that the three formulations are alternative names used in the registration of these formulations by the United States Environmental Protection Agency; therefore, published reports on VectoBac CG and VBC-060216 are the suitable supporting documentation relating to the performance of VectoBac GR. The product label recommends the use of the GR formulation at the rate of 2.5–10 lb/acre (0.28–1.12 g/m²; 2.8–11.2 kg/ha), with 10–20 lb/acre in heavily polluted water (e.g. sewage lagoons).

The human and environmental safety of *Bti* strain AM65-52 for mosquito larviciding has been assessed and published by WHO.^{30,31}

³⁰ *Microbial pest control agent, Bacillus thuringiensis*. Geneva, World Health Organization, 1999 (Environmental Health Criteria 217).

³¹ *Report of the 4th WHOPES Working Group Meeting, WHO/HQ, Geneva, 2–4 December 2003*. Geneva, World Health Organization, 2004 (available at <http://www.who.int/whopes/recommendations/wgm/en/>; accessed July 2012)

The current review assesses the efficacy of VectoBac GR against anopheline and culicine mosquitoes in different aquatic habitats. The efficacy of this product has also been compared with that of the VectoBac custom granule formulation to further bridge efficacy data related to these two formulations that are different only in use of carriers.

5.1 Efficacy – background and supporting documents

Madagascar

Romi et al (1993) evaluated the field efficacy of *Bacillus thuringiensis* H-14 and *B. sphaericus* (strain 2362) against *An. arabiensis* in five types of larval habitats in a village in the highlands of Madagascar using two formulations of *Bti*: VectoBac® GR (200 ITU/mg) and a flowable concentrate VectoBac® 12AS (1200 ITU/mg). Both formulations were manufactured by Abbott Laboratories, North Chicago, IL, USA. The *B. sphaericus* was a granular formulation (ABG 6185) derived from strain 2362 (5×10^{10} spores/g, Abbott Laboratories). The trial was carried out in 1991 and 1992. Vectobac GR was applied by hand in (i) four small natural pools exclusively breeding for *An. arabiensis* with 14.2 larvae/dip, treated at 2 to 8 kg/ha; (ii) five ditches, made by the local population to produce bricks, having rainwater rich with organic matter and vegetation with larval density of 5.6/dip, treated at 3 to 10 kg/ha; (iii) two small rice fields with clear water without vegetation with larval density of 3.8/dip, treated at 5 and 10 kg/ha; (iv) two rice fields having clear water with 60 cm high plants and larval density of 1.4/dip, treated at 2.5 and 5 kg/ha; and (v) one fallow rice field with clear water with density of 1.1 larvae/dip, treated at 2.5 kg/ha. Collected anopheline larvae were counted and identified. In rice fields, *An. arabiensis* larvae were found breeding with those of *An. coustani* and *An. squamosus*.

Untreated habitats of each type were taken as control. Larval samples from all treated and control areas were taken using the standard 250 ml dipper immediately before the treatment and then from one to 7 days after treatment.

VectoBac GR gave complete larval control in small pools by providing 100% reduction within 24 h at all rates of application. In rainwater ditches, it gave complete larval control after 24 h at rates of 4, 6 and 10 kg/ha. The rate of 3 kg/ha gave 93% reduction within 24 h. The application rate of 5 kg/ha gave only 87% reduction, probably because of the presence of thick vegetation.

In different types of rice fields, all dosages applied (2.5, 5 and 10 kg/ha) gave 100% reduction in larval counts after 24 h. The residual activity of VectoBac GR and other formulations was very short, as dips taken between 5 and 7 days after treatment indicated a quick and continual colonization of all treated sites by early instars of anophelines. However, VectoBac GR was found to be more efficient than *B. sphaericus* granular formulation (ABG 6185) and flowable concentrate (VectoBac 12AS) as it could afford greater penetration through vegetation into larval habitats.

Kenya

Fillinger and Lindsay (2006) determined the contribution larviciding could make to reduce abundance of larval and adult mosquitoes by conducting a trial of microbial larvicides in a 4.5 km² area in rural Kenya on the shores of Lake Victoria. The study was carried out over a period of 52 months beginning July 2001, which involved the 12 months of baseline monitoring followed by an intervention period of 28 months of microbial larval control and a post-intervention period of a further 12 months. All larval breeding habitats were identified, visited on a weekly basis and classified. The presence or absence of larvae was scored by taking 6–10 dips per site with a 250 ml capacity larval dipper.

Changes in larval density over time were measured in 26 sentinel sites previously selected and surveyed, of which 23 sites were frequently colonized by *Anopheles* larvae. Indoor resting mosquito collections were made by pyrethroid spray catches from 12 randomly selected houses at fortnightly intervals throughout the study. *Anopheles gambiae* s.l., *An. funestus* and *An. coustani* were identified and separated from other mosquito species using morphological criteria.

VectoBac (*Bti*; 3000 ITU/mg) water-dispersible granules (WG) and corn granules (CG) manufactured by Valent BioSciences Corporation, IL, USA, and VectoLex (*B. sphaericus*; 650 ITU/mg) water-dispersible granules (WDG) were evaluated for mosquito larval control.

Vectobac WG and CG were applied at 0.2 and 5 kg product/ha. Vectolex WDG and CG were applied at 1 and 15 kg/ha. WG/WDG formulations were applied with knapsack compression sprayer as liquid application while corn granules were dispersed by hand. Most habitats were treated with liquid formulations, whilst difficult to access areas were treated with granular formulations.

The first four rounds of *B. sphaericus* (VectoLex) were conducted at approximately 2-week intervals, while *Bti* applications were made at weekly intervals. In a total of 50 rounds of application, VectoBac CG was applied during 27 rounds (54%) with a consumption of 40 kg (32%) in a total of 125 kg of larvicides used. On average, 149 sites (95% CI: 143.4, 154.9) were treated with VectoBac formulations at an average interval of 11 days (CI: 9.8, 12.8) between treatments. The preferred control agents were VectoLex formulations because of their longer residual effect. VectoBac was applied to prevent the emergence of insecticide resistance at regular intervals and exclusively during periods of heavy rains.

Larval density in treated habitats during the intervention period declined by 95% and 97% when compared with the pre- and post-intervention levels, respectively. There was a marked reduction in the proportion of habitats with late-instar larvae per dip in the intervention period (average 24–26% to 5%) with late-instar densities declining by 99% compared with the combined non-intervention periods.

In general, VectoLex (*B. sphaericus*) applications showed residual effect of three weeks, extending up to 13 weeks during dry periods. There was a 92% reduction in the density of blood-fed malaria vectors per person resting indoors during the intervention period compared with the pre-intervention period and a 93% reduction

when compared with the post-intervention period. Measurement of the impact of Vectobac GR formulation alone is difficult, noting the specific design of the study as described above.

5.2 Efficacy – WHOPES supervised trials

Benin, West Africa

Djènontin et al (2012) carried out a simulated field trial at the Centre de Recherches Entomologiques de Cotonou in Benin to determine the optimum dose of VectoBac GR against susceptible strains of *An. gambiae* (Kisumu) and F₁ progeny of wild *Cx. quinquefasciatus* from Cotonou. Following this study, phase III trials were undertaken in natural breeding habitats of *An. gambiae* and *Cx. quinquefasciatus*.

For the simulated field trial, rectangular cement containers of 60 x 30 x 30 cm size simulating breeding habitats were constructed under shade and periodically filled with tap water to a depth of 15 cm. The containers were covered with netting pieces to avoid oviposition from wild female mosquitoes.

In two separate experiments, cohorts of 50 second-instar larvae of *An. gambiae* and *Cx. quinquefasciatus* were added to each container every 7 to 10 days depending upon the larval development time. Dry cat food (0.5–1 g) was added to each container at each cohort. After 2–3 h of larval acclimation, VectoBac GR (*Bti* strain AM65-52) was dispersed manually on the water surface, taking safety precautions at 0.6, 0.9 and 1.2 g of product/m² doses against *An. gambiae* and at 1, 1.5 and 2 g/m² against *Cx. quinquefasciatus* in four replicates each at each dose and compared with untreated controls. Temperature of water and pH were recorded daily. Pupae were removed daily and transferred to plastic cups containing water. Adult mosquitoes that emerged from pupae of each container were recorded daily per treatment to determine emergence rates. The study was conducted in June–July 2011.

The average temperature and pH during the trial were respectively 26.5 °C and 7.5 (*An. gambiae*) and 26.5 °C and 6.9 (*Cx.*

quinquefasciatus). The mean ambient temperature at the study site was 27 °C (24.7–29.2 °C) and relative humidity ranged from 75% to 93%.

The emergence rate of *An. gambiae* in the control ranged from 90% to 96% on different days during the trial. Emergence inhibition rates (EIR) were >80% for all dosages up to 19 days post-treatment; after 26 days post-treatment, this rate fell below the WHO threshold of 80% (at 44%, 55% and 63% at 0.6, 0.9 and 1.2 g/m² dosages respectively) (Table 13). Pair-wise comparison using the logistic regression model to predict the emergence rate according to dose showed that 1.2 g/m² of VectoBac GR caused greater emergence inhibition than 0.9 g/m², while there was no difference in EIR between 0.6 and 0.9 g/m² doses. Based on these findings, the dose of 1.2 g/m² was selected for the field trial. From the logistic regression model, the time for which Vectobac GR treatment would remain effective (emergence rates <20%) was estimated to be 15, 17 and 21 days for 0.6, 0.9 and 1.2 g/m² dosage, respectively.

The emergence rate of *Cx. quinquefasciatus* in the control ranged from 90% to 99% during the trial. EIR was 100% at day 11 at all dosages, decreasing to below the WHO threshold of 80% after 19 days for 1 g/m², 26 days at 1.5 g/m² and 34 days at 2 g/m² (Table 13). Pair-wise comparison using the logistic regression model to predict the emergence rate according to dose showed that 1.5 g/m² caused greater EIR than 1 g/m² but lower EIR than 2 g/m². Based on these findings, the dose of 2 g/m² was selected for the field trial. The time for which Vectobac GR treatment would remain effective (emergence rates <20%) was estimated to be 19, 22 and 28 days for 1, 1.5 and 2 g/m², respectively.

Field trials were conducted to assess the efficacy of VectoBac GR against *An. gambiae* and *Cx. quinquefasciatus* in their natural breeding habitats. The trial against *An. gambiae* was conducted in a rice field in Lélé (Cové district), while the trial against *Cx. quinquefasciatus* was conducted in cesspits in Cotonou.

The rice field used for the trial was delimited with natural silt and converted into 30 ponds of 2 m x 4 m size, of which 15 were treated by manual dispersal with VectoBac GR at 1.2 g/m²; the

remaining 15 were kept as untreated control ponds. A total of three replicates were run for this study (corresponding to 45 treated and 45 untreated ponds). The level of water was maintained manually in the ponds to allow oviposition by mosquitoes. Before treatment, each pond was divided into quadrants and sampled twice to determine larval and pupal density. Post-treatment sampling was done on days 1, 2, 3, 7 and every third day thereafter until the density in the treated and control ponds equalized. Larvae were sampled from the treated and control ponds by taking three dips by the same operator and number of first-, second-, third- and fourth-instar larvae and pupae were counted. Reduction in density of young (L1 + L2) and late (L3 + L4) instars and pupae after treatment was estimated using Mulla's formula.³²

The average temperature and pH recorded in ponds during the trial were respectively 35.1 °C (range: 28–41.7 °C) and 6.6 (range: 5.1–8.8) and did not vary significantly between experimental and control ponds (ANOVA, $P > 0.05$). No rain was recorded during the trial.

For the reduction in density of late-instar larvae (L3 + L4), the efficacy of Vectobac GR at 1.2 g/m² was >80% up to 2 days post-treatment. After 3 days post-treatment, reduction in density was 73%, which was below the WHO efficacy threshold of $\geq 80\%$, and then reached zero after day 7. The reduction in density of young-instar larvae (L1 + L2) was below the 80% cut-off level during the first 3 days. There was no reduction by day 7. According to the regression model, the time for which the density reduction of late-instar larvae would reach 80% and 50% was 2 (1–3) and 5 (4–6) days, respectively.

The trial against *Cx. quinquefasciatus* was conducted in cesspits rich with organic matter. Fifteen of 30 selected cesspits were randomly assigned for treatment with VectoBac GR at 2 g/m²; the remainders were kept as untreated control. The surface area of the selected cesspits ranged from 0.14 m² to 3.46 m². VectoBac

³² WHO (2005). *Guidelines for laboratory and field testing of mosquito larvicides*. Geneva, World Health Organization (document WHO/CDS/WHOPES/GCDPP/2005.13, available at <http://www.who.int/whopes/guidelines/en/>).

GR was applied manually so as to uniformly cover the water surface. A total of three replicates of 15 cesspits each were run for the study, corresponding to 45 treated and 45 untreated cesspits. The plan for sampling immature mosquitoes remained the same as that of rice field ponds.

The average water temperature and pH during the trial were respectively 27.1 °C (range: 25.1–32.2 °C) and 6.8 (range: 5.7–8.1) and did not vary significantly between treated and control cesspits (ANOVA, $P>0.05$).

For late-instar larvae, the reduction in density caused by Vectobac GR was >80% up to 2 days post-treatment and fell below WHO threshold after 3 days, reaching zero after day 16. According to the regression model, the time for which the reduction in density of late-instar larvae would reach 80% and 50% was 3 (2–5) and 9 days (8–10), respectively.

Goa, India

Kumar et al (2012) evaluated VectoBac GR in Candolim, Goa, India against anopheline and culicine mosquitoes breeding in clean and polluted water habitats, respectively. To bridge data, the VectoBac CG (custom granule) formulation was also evaluated in phase II in clean and polluted water habitats.

To determine optimum application dosages for a large-scale trial, initially a small-scale (phase II) trial was undertaken for control of the breeding of *An. stephensi* in clean waters and *Cx. quinquefasciatus* in polluted water habitats. Following these studies, a phase III study was carried out with VectoBac GR using optimum dosages.

The clean water habitats used for the trial included water collections used for curing concrete at construction sites and rainwater collections on flat roofs of buildings. The polluted water breeding habitats for *Cx. quinquefasciatus* included surface drains receiving domestic wastewater in periurban settings.

Pre-treatment counts of larvae and pupae were made twice a week up to 2 weeks. Five dips using a standard 350 ml larval dipper were taken from each habitat, and samples of larvae and pupae were counted by stages. After counting, larvae and pupae were returned to the same habitats. Habitats from each type with comparable pre-treatment densities of immature mosquitoes were randomly assigned to either treatment or control. Replicates treated with each dose covered the entire range of pre-treatment larval densities. In small-scale trials, the two formulations were applied by hand dispersion at 0.5, 1.0, 1.5 and 2.0 g/m² of surface area.

For the evaluation against *Cx. quinquefasciatus* in drains, the entire length of each selected drain was treated with a single dose. Separate drains were selected for each dose as well as a control. Within each drain, every segment of 10 m length was considered as a replicate.

Post-treatment sampling was done usually on days 1, 2, 3 and 7, and thereafter twice weekly until the reduction of third- and fourth-instar larval density fell below 80% in the treated habitats in comparison with the control. Where the efficacy was low during the first three observations post-treatment, densities were monitored for at least 7 days. The percentage reduction in larval and pupal densities on post-treatment days was calculated for each replicate of each treatment using Mulla's formula.

Water temperature and pH were recorded on the day of sampling. Data on ambient temperature, relative humidity and rainfall were collected from a local meteorological station. The range of mean minimum ambient temperature was 21–27 °C; the mean maximum temperature was 28–40 °C in the study area. The water temperature ranges were: curing waters 26–29 °C; drains 26–32 °C; roof top collections 23–26 °C. The pH of curing waters was 6–14, of water in drains was 6–8 and of rainwater collections on roofs was 7.

In curing waters at construction sites, a single application of VectoBac GR gave >80% reduction in the densities of third- and fourth-instar larvae of *An. stephensi* for 37–67 days, 10–17 days,

10–18 days and 27–66 days at 0.5, 1.0, 1.5 and 2.0 g/m², respectively. Based on the results of the phase II trial, 0.5 g/m² and 1 g/m² dosages of VectoBac GR were selected for the application in clean water habitats in the phase III trial.

The application of VectoBac CG at 0.5 g/m² gave effective control (>80% reduction) of third- and fourth-instar larvae of *An. stephensi* from day 1 to 49 post-treatment. At 1.0 g/m², effective control was found up to 31 days. At 1.5 g/m², effective control was found up to 49 days, while at 2 g/m² effective control was found up to 38 days post-treatment.

In phase III studies, twelve replicates of curing-water habitats each were treated at 0.5 g/m² and 1 g/m² with VectoBac GR. A similar number of controls were run in parallel. More than 80% reduction of third- and fourth-instar larvae was achieved in 2 days post-treatment and remained until about six weeks (Table 14).

Rainwater collections on building roofs with mixed breeding of *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* were also treated at 0.5 g/m² and 1 g/m² with Vectobac GR. More than 80% reduction of late-instar larvae was observed from day 1 until 17 days post-treatment.

In polluted water drains, VectoBac GR was found to provide effective control (>80% reduction) of late-instar larvae of *Cx. quinquefasciatus* for 2 days and 2–17 days at 0.5 and 1 g/m², respectively in the phase II trial. The dosage of 1.5 and 2 g/m² provided a maximum reduction of 74% and 63% of late-instar larvae over one week of monitoring post-treatment. This could have been due to greater flow of water or higher pH or organic matter in these drains. Hence, dosages of 1 and 2 g/m² of VectoBac GR were further tested in the phase III trial. The pre-treatment densities of *Cx. quinquefasciatus* were monitored. Eight drains each were treated at 1 and 2 g/m² with VectoBac GR. Four drains were taken as controls. At the two dosages evaluated, the reduction in density of third- and fourth-instar larvae was respectively a maximum of 71% and 28% during 13 days of monitoring post-treatment.

Cuddalore, Tamil Nadu, India

Sadanandane et al (2012) carried out small-scale field trials of VectoBac GR in an urban area against *Cx. quinquefasciatus* breeding prolifically in cesspits and open drains and disused wells with high organic matter. Cesspits are dug just outside houses to receive domestic wastewater. Elsewhere, cement-lined U-shaped open drains carry domestic wastewater throughout the year and also drain rainwater during the monsoon period. In the absence of proper gradient and cleaning, drains are often found choked with debris and silt leading to stagnation of water that supports profuse breeding of *Cx. quinquefasciatus*. Unused wells polluted with floating debris and garbage are also sources of breeding of *Cx. quinquefasciatus*.

Immature *Cx. quinquefasciatus* were sampled from drains and cesspits using enamel dippers (350 ml) and from abandoned wells using a galvanized iron bucket (2 L) tied with a rope. Three dips were taken from each habitat replicate and immature mosquitoes were counted by stages and later returned to the habitats after counting them. VectoBac GR was applied manually over the water surface in selected habitats at 0.5, 1, 1.5 and 2 g product per m². Five replicates of cesspits and drains were selected for each dose with an equal number of control replicates. Three replicates of abandoned wells were taken for each treatment and control. Drains were divided in 10 m long segments; each segment was taken as a replicate.

Larval and pupal counts were made twice a week for 1–2 weeks prior to application of VectoBac GR formulation, and on days 1, 2, 3 and 7 and 14 post-treatment. Larval and pupal counts in abandoned wells were also made up to day 21. The reduction of larval and pupal densities during the post-treatment period was estimated by comparing the pre- and post-treatment densities in the treated habitats with the corresponding densities in the untreated habitats using Mulla's formula.³³

³³Mulla MS et al. Control of chironomid midges in recreational lakes. *Journal of Economic Entomology*, 1971, 62:300–307.

In cesspits, the pupal density declined between 5% and 56% up to day 14 post-treatment with all four dosages applied. The reduction of late (L3 + L4) and early (L1 + L2) instar larval densities ranged between 0% and 18% and 0% and 11%, respectively during the post-treatment period. Application of VectoBac GR in drains at 0.5, 1 and 1.5 g/m² caused up to 52% reduction of pupal density during 14 days of monitoring post-treatment. At 2 g/m², the formulation produced 50–75% reduction in pupal density from days 2 to 14 post-treatment. The density of late and early instar larvae declined by 3–64% and 6–46%, respectively during 14 days of observation post-treatment. In abandoned wells with organic matter, the VectoBac GR formulation reduced pupal density by 1–63% in different replicates during 21 days of observation post-treatment at all four dosages tested. There was no obvious dose-dependent effect of the VectoBac GR formulation. The reduction of late- and early-instar larval densities ranged from 0–74% and 0–69% respectively at all dosages tested.

5.3 Conclusions and recommendations

VectoBac GR (Valent BioSciences, USA) is a granule formulation of a bacterial larvicide containing viable *Bacillus thuringiensis israelensis* (*Bti*) strain AM65-52 endospores and delta-endotoxin crystals. The GR formulation contains biopotency of 200 international toxic units (ITU) per mg and can be applied to mosquito breeding sites by hand or granule spreaders. The product is not intended to be mixed with sand for application, or used for the control of container-breeding mosquitoes. The formulation is designed for good penetration into water with emergent vegetation and immediate release of *Bti* active ingredient into water. VectoBac GR is similar to the VectoBac custom granules (CG) and to VBC-060216, but replacing the corn cob carrier with a new carrier. The product label recommends the use of the GR formulation at the rate of 2.5–10 lb of formulated product per acre (0.28–1.12 g/m²; 2.8–11.2 kg/ha), with 10–20 lb/acre (1.12–2.24 g/m²; 11.2–22.4 kg/ha) in heavily polluted water (e.g. sewage lagoons).

Vectobac GR was evaluated in polluted waters such as cesspits, drains and disused wells with breeding of *Cx. quinquefasciatus* at

the dosage of 0.5 to 2 g product/m². While in the simulated studies under controlled conditions the product showed >80% inhibition of adult emergence from day 11–26 post-treatment at dosages of 1–2 g/m², its application in natural breeding habitats of *Cx. quinquefasciatus* achieved effective control of late-instar and pupae for 1–3 days in Benin. At the other two sites, Vectobac GR did not yield effective control at dosages ranging from 0.5 g to 2 g of the formulated product per m².

Vectobac GR was evaluated in simulated conditions against *An. gambiae* at 0.6–1.2 g of the formulated product per m². It was evaluated against *An. arabiensis*, *An. gambiae* and *An. stephensi* at dosages of 0.5–10 g of the formulated product per m² in clean water habitats such as small natural pools, rainwater ditches, different types of rice fields (nurseries, fields with early crop and fallows), curing water collections at construction sites and rainwater collections on building roofs.

In the simulated tests, effective control (>80% inhibition of adult emergence) was recorded up to 19 days post-treatment. In trials in natural habitats, Vectobac GR gave an effective control of *An. arabiensis* and *An. gambiae* for up to one week and of *An. stephensi* for 1–6 weeks post-treatment within the label recommended dose range of 0.5 g to 2 g formulated product per m².

VectoBac custom granule (CG) performed as well as Vectobac GR in trials in non-polluted waters but did not give effective control of *Cx. quinquefasciatus* in organically polluted water up to 2 g of the formulated product per m² dose tested.

Considering the above, and noting the safety and efficacy of VectoBac GR, the meeting:

- recommended the use of VectoBac GR (*Bti* with biopotency of 200 international toxic units per mg) in open water bodies such as small natural pools, rainwater ditches, rice fields, curing and other type of water collections in urban settings, and rainwater collections on

building roofs at 0.5–2 g formulated product per m² with an expected duration of efficacy of one week.

- does not recommend the use of VectoBac GR for control of *Cx. quinquefasciatus* in polluted water with high levels of organic matter such as cesspits and drains as well in disused wells with organic matter.

Note: WHO recommendations on the use of pesticides in public health are valid ONLY if linked to WHO specifications for their quality control.

Table 13. **Emergence inhibition rates of *An. gambiae* and *Cx. quinquefasciatus* in simulated field trial of VectoBac GR in Cotonou, Benin**

Day post- treatment	<i>An. gambiae</i>			<i>Cx. quinquefasciatus</i>		
	0.6g/m ²	0.9g/m ²	1.2g/m ²	1g/m ²	1.5g/m ²	2g/m ²
11	99	99	99	100	100	100
19	83	85	91	74	85	99
26	44	55	63	47	56	84
34	–	–	–	38	47	57
35	8	9	37	–	–	–
42	–	–	–	8	10	22
43	5	6	22	–	–	–

Table 14. Efficacy of VectoBac GR formulation as tested against mosquito larvae in field trials in various habitats

Country and location	Species	Habitats	Dose in g/m ²	Percent reduction in late instar larval density	Duration of effective control in days ¹
Benin, Cotonou	<i>An. gambiae</i>	Rice field ponds	1.2	80–91	1–2
	<i>Cx. quinquefasciatus</i>	Cesspits	2.0	83	1
India, Candolim (Goa)	<i>An. stephensi</i>	Clean water pools	0.5	81–100	1–29
			1.0	87–100	1–43
	Culicidae ²	Rainwater collections on roof	0.5	83–100	1–17
			1.0	87–100	1–7
	<i>Cx. quinquefasciatus</i> ³	Drains	1.0	0–74.5	0
			2.0	0–63.3	0
India, Cuddalore (Tamil Nadu)	<i>Cx. quinquefasciatus</i> ⁴	Cesspits	0.5	0–2	0
			1.0	0–5.5	0
			1.5	0–17.6	0
			2.0	1.8–13.2	0
		Drains	0.5	29.7–38.4	0
			1.0	18.6–38.5	0
			1.5	3.1–46.7	0
			2.0	5.4–64.4	0
		Disused wells	0.5	0–43.2	0
			1.0	0–70.9	0
			1.5	20–69.7	0
			2.0	2.3–48.5	0

¹ Duration of >80% reduction of density of third and fourth instar larvae beginning with the day when such reduction was first observed;

² Mixed breeding of *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti*; ³ Based on observations made for 1–2 weeks; ⁴ Based on observations made for 2 weeks in cesspits and drains, and 3 weeks in wells.

6. GENERAL RECOMMENDATIONS

The fourteenth meeting of the WHOPES Working Group³⁴ drafted some general recommendations on testing methods, including methods for the evaluation of new products containing novel public-health pesticides. These were circulated for comments, and a wide range of suggestions and feedback were received, including comments from industry and members of the Roll Back Malaria Vector Control Working Group.

The fifteenth meeting considered these comments and produced the amended version presented below.

It should be noted that these guidelines can be further adapted according to mode of action, product specificity and manufacturers' claims about new products, as has been a normal practice with new developments in the past, for example in the introduction of insecticide-treated nets.

I. Definition of knock-down and mortality for adult mosquitoes

For the purpose of insecticide bioassays, the definition of knock-down³⁵ and mortality involves not only the state of the insect but also the time at which the observation is made.

³⁴ *Report of the 12th WHOPES Working Group Meeting – Review of Bioflash[®] GR, PermaNet[®] 2.0, PermaNet[®] 3.0, PermaNet[®] 2.5, lambda-cyhalothrin LN, 8–11 December 2008.* Geneva, World Health Organization, 2009 (WHO/HTM/NTD/WHOPES/2009.1; available at <http://www.who.int/whopes/recommendations/wgm/en/>; accessed July 2012).

³⁵ Note that the criteria for knock-down and mortality are applicable not only to pyrethroids but also to other insecticides. For example, the criteria specified in the *Guidelines for laboratory and field testing of long-lasting insecticidal mosquito nets* (available at <http://www.who.int/whopes/guidelines/en/>) require minimum levels of knock-down and or mortality, not knock-down and mortality.

A mosquito is classified as dead or knocked down if it is immobile or unable to stand or take off (Table 15). The distinction between knocked down and dead is defined only by the time of observation. The assessment of knock-down is made within 60 min post-exposure. Mortality is determined at least 24 h post-exposure. The holding container may be tapped a few times before a final determination is made.

In the case of slow-acting insecticides, the recovery period may be extended beyond 24 h. Control mortality should be measured over the same recovery period. Mortality after 24 h should be recorded and, in some cases, repeated observations may be appropriate.

Table 15. Classification of adult mosquitoes as alive, knocked down or dead in bioassays

Alive	Knocked down after 60 minutes or dead after 24 hours of exposure	
	Moribund	Dead
Can both stand on and fly in a coordinated manner	<ul style="list-style-type: none"> Any mosquito that cannot stand (e.g. has 1 or 2 legs) Any mosquito that cannot fly in a coordinated manner A mosquito that lies on its back, moving legs and wings but unable to take off A mosquito that can stand and take off briefly but falls down immediately 	No sign of life; immobile; cannot stand

II. Amendments to the existing WHOPES guidelines for efficacy testing of pyrethroid-treated LNs

The meeting proposed the following amendments to the existing WHOPES guidelines for testing the efficacy of LNs based on experience gained in the evaluation of such products.

- Following the publication by Skovmand et al (2008),³⁶ WHOPES has studied median knock-down time (MKDT) as a supplementary test to determine regeneration time of washed LNs, including both coating and incorporation technologies (reports of the 13th and 14th WHOPES Working Group Meetings³⁷ and unpublished data). Based on the evidence to date, no additional benefit was found in the determination of the MKDT over %KD or %mortality from the cone bioassay.
- Given the challenges in proper treatment of mosquito nets in the field, the determination of exhaustion point, the experiences gained and information available on WHOPES-recommended LNs, and the desire to better standardize experimental hut studies, it was recommended to use WHOPES recommended LNs as positive controls in place of conventionally treated nets in the trials.
- In phase II studies, efficacy of candidate LNs is normally compared with a positive control in experimental huts. The positive control was previously a conventionally treated net washed until just before exhaustion. The Group now proposes that the positive control should be a reference WHOPES-recommended LN, unwashed and washed 20 times.

³⁶ Skovmand O et al. Median knock-down time as a new method for evaluating insecticide-treated textiles for mosquito control. *Malaria Journal*, 2008, 7:114.

³⁷ Available at <http://www.who.int/whopes/recommendations/wgm/en/>; accessed 12 July 2011.

It should be noted that using a reference LN as a positive control does not change the definition of a LN, e.g. to retain biological activity for at least 20 standard WHO washes under laboratory conditions and three years of recommended use under field conditions. This means that the performance of the candidate LN will be tested on its own in phase II and phase III studies. Also, as there is no absolute threshold for mortality and blood-feeding inhibition in phase II, a reference LN that underperforms relative to a candidate LN in phase II studies would not be considered a failed LN product and would not lose its existing WHOPES recommendation.

- It is recommended to standardize the study arms to include as a minimum the following:
 1. Untreated net, preferably of the same material as the candidate LN; if not, a polyester net
 2. Unwashed candidate LN
 3. Candidate LN washed 20 times
 4. An unwashed reference LN as a positive control LN (a WHOPES-recommended LN similar to the candidate in terms of fabric, active ingredient and/or treatment technology)
 5. The reference LN washed 20 times.

Additional arms with candidate LNs washed more than 20 times (according to the manufacturer's claim) may also be included.

- The reference LN should be the one that has been used to develop WHO recommendations and specifications. For logistic and practical reasons during testing, a reference LN with maximum acceptable regeneration time of three days should only be used. Additional arms with candidate LNs washed according to the manufacturer's claim may also be included.
- It is recommended to conduct phase II studies in areas of pyrethroid susceptibility. However, it is recognized that

pyrethroid resistance is expanding rapidly and that areas with fully susceptible vector populations may not always be available in the future. Studies conducted in areas with pyrethroid-resistant mosquitoes can provide equally valuable information, as the comparison would be with positive control LNs.

- In sites where phase II studies are to be carried out, the wild vector population must be tested regularly for the presence or absence of resistance, using conventional WHO susceptibility tests with the same active ingredient used in the candidate LN product.
- Once resistance has been detected in a study site, measuring the intensity or strength of resistance by exposing field samples to a range of doses will help to establish whether the local population contains individuals that are able to survive very high doses and may compromise the effectiveness of the candidate LN being tested.
- It is also helpful, as background information, to measure the frequency of *kdr* alleles in the local population. It is not easy to measure the population frequency of metabolic resistance mechanisms, but testing with synergists can help to establish the presence of metabolic-based resistance.
- As part of phase II studies, it is recommended that baseline information on attractiveness of experimental huts, recapture rates of known numbers of live and dead mosquitoes released in the huts, and contact bioassays on the walls to detect insecticide residues from a previous spraying be collected and reported. All mosquitoes collected during the study should be preserved using a desiccant or other medium (e.g. silica gel, ethanol) and labelled according to the location of collection in the hut, intervention in place and status of mosquitoes at the time of collection (dead or alive, blood-fed or unfed) for quality control and/or future studies of genetic markers of insecticide resistance.

- For phase III studies, the design and procedures detailed in *WHO Guidelines for monitoring durability of long-lasting insecticidal nets under operational conditions*³⁸ should be used. So far, no criteria on physical integrity of nets have been established for acceptance, as the association between the net condition (holes and insecticide content) and net performance is not known.
- Based on observations from field trials, shrinkage or compactness of some LNs, particularly of polyethylene monofilament products, has been reported but should be further documented. Measurement of changes in LN dimensions (length and width along the seams and height at the corners) should be included in phase III studies.
- A risk assessment of LNs³⁹ is performed before phase II studies. Nevertheless, any adverse effects reported by sleepers should be documented during the course of the study to provide medical care to the sleepers if necessary and to provide information to WHOPES. It should be noted that phase II studies as well as the phase III studies are not designed to evaluate the safety of the LN products in the field.
- Modifications in the protocol developed for phase I studies are required. In phase I studies of new LNs or for extending LN specifications, from each of the four nets tested for regeneration time and wash resistance, five pieces of 25 cm x 25 cm should be cut according to the WHO specification guideline for LNs. These nets should originate

³⁸ *Guidelines for monitoring the durability of long-lasting insecticidal mosquito nets under operational conditions*. Geneva, World Health Organization, 2011 (available at: <http://www.who.int/whopes/resources/en/>; accessed July 2012).

³⁹ *A generic risk assessment model for insecticide treatment and subsequent use of mosquito nets*. Geneva, World Health Organization, 2004 (available at: <http://www.who.int/whopes/guidelines/en/>; accessed July 2012).

from at least two production batches. The five pieces should be stored and their chemical content determined separately to allow accurate estimation of within-net and between-net variations. In phase I studies performed for extension of LN specifications, the same number of replicates of nets ($n = 4$) should be tested for regeneration time and wash resistance both for reference and candidate LNs. All physical criteria of the WHO specification of reference LN should be fulfilled by the candidate LN submitted for extension.

- In phase III studies, net samples of the candidate and reference LNs taken before the trial and after 6 months and 1, 2 and 3 years of use should be analysed for determination of AI content to facilitate the interpretation of bioassays results. Chemical analysis and bioassays are done on adjacent pieces from the same net.

III. Novel public health pesticides

The massive scale at which malaria control is being applied and the consequent insecticide resistance problems arising mean that the demand for new public-health pesticides (PHPs) will increase. Novel PHPs may include new active ingredients or mixed formulation insecticides for LNs and indoor residual spraying (IRS) as well as new application technologies. These may include approaches that are simple modifications of existing categories of vector control and thus may fit within existing WHOPES guidelines for evaluation. For vector control technologies that are completely new, WHO is currently considering the establishment of new assessment procedures, up to the point of establishing the proof of principle.⁴⁰ Once the proof of principle has been established, then it will be the role of WHOPES to develop new guidelines on testing methods, standards and specifications for the new technology.

⁴⁰ "Proof of Principle" in this context means that there is evidence that a new form of vector control has a useful role in public health (i.e., it is efficacious when deployed in a defined manner in a defined setting for a defined public-health purpose).

Some novel PHPs may have mechanisms of action and performance criteria that are well understood and familiar and, in this case, they may be assessed using already established WHOPES methods and criteria (e.g. IRS formulations with longer residual activity; LNs with new fabrics). In other cases, the mechanism of action may be entirely different and the conditions for effectiveness not yet known (e.g. spatial repellents for transmission control; LNs with slow-acting insecticides). In such cases, proof of principle, including epidemiological evidence, may be required. In yet other cases, there may be new PHPs within established categories that have new intended functions or purposes (e.g. LNs or IRS formulations with mixtures of insecticides to protect against resistant populations). In this case, additional test procedures and criteria will need to be established within the WHOPES scheme.

Most of the new PHPs have been brought to the market to control insecticide-resistant vector populations. WHOPES can assess the entomological efficacy of different PHPs for protection against geographically defined populations of insecticide-resistant mosquitoes and/or specific resistance mechanisms, although some modifications of existing guidelines may be required.

It should be noted that insecticide resistance management strategies are designed to prevent or delay the spread of insecticide resistance and depend on the biology, ecology and behaviour of the insect species, and on the resistance mechanisms present in field populations. This may be achieved through the use of a combination of tools and approaches. No single product can be labelled as a resistance management tool, but individual products can contribute to resistance management strategies. The development and implementation of an insecticide resistance management strategy is the responsibility of national programmes.

The recently-launched *Global plan for insecticide resistance management in malaria vectors* calls on governments of malaria-endemic countries and other stakeholders to implement a strategy to tackle the growing threat of insecticide resistance and to

facilitate the development of innovative vector control tools and strategies.⁴¹

III.I Efficacy testing of LNs with insecticides other than pyrethroids

LNs are widely used for the prevention of vector-borne diseases, particularly malaria. Currently, only pyrethroid insecticides are recommended for use on LNs. However, pyrethroid resistance is spreading in the major malaria vectors and threatens to undermine the effectiveness of these tools. Therefore, new products incorporating alternative insecticides with acceptable safety are urgently needed for use on LNs.

LNs are the only public health pesticide products for which an interim recommendation is provided by WHO. The Working Group recommended that interim recommendation be considered for future LN products with alternative insecticides as well. Some new compounds may have entirely new modes of action on mosquitoes, including some that may be non-lethal but effective in interrupting transmission. Understanding the precise mode of action of a new compound on mosquitoes, including the chemical mode of action (e.g. sodium channel blocker, acetyl-cholinesterase inhibitor) and the epidemiological mode of action (e.g. personal protection, mass effect) is essential in designing the criteria and requirements for testing and evaluation of alternative products in phase I, phase II and phase III studies. Such understanding is also essential for designing approaches to implementation.

If the primary effect of the alternative insecticide is through contact toxicity similar to pyrethroids (rapid knock-down and mortality), the existing general framework for evaluating LNs will be applicable, although some specific modifications may be required in each phase of testing. LN products acting through mortality alone, through repellency alone or through an alternative mechanism on mosquitoes, will require, as proof of principle, epidemiological

⁴¹ Available at http://www.who.int/malaria/vector_control/ivm/gpirm/en/index.html.

studies to demonstrate efficacy in reducing malaria transmission and/or disease control.

The following modifications are proposed to phase I, phase II and phase III studies of new LN products with alternative insecticides:

- Phase I testing of LNs is designed to assess efficacy, wash resistance and dynamics of the insecticide on the netting. Current guidelines recommend testing against susceptible strains of mosquitoes. As new insecticides are incorporated into LNs, cross-resistance to other insecticides should be assessed.

There is a need for establishing a series of well-characterized insecticide-resistant colony strains of mosquitoes for screening of candidate products with new active ingredients. Exchange of these colonies between laboratories is to be encouraged. Nevertheless, the establishment of such insectary colonies must take stringent care to take into account biosafety issues (i.e. the risk that genes for insecticide resistance can be accidentally introduced from a resistant colony into the wild mosquito population).

- In phase II studies, the efficacy of LNs is determined against wild, free-flying mosquitoes susceptible both to pyrethroids and to the particular insecticide on the candidate LN. With conventional insecticides, existing guidelines for phase II studies should be followed, but it is recommended that the study arms be standardized to include the following:
 - an untreated net, preferably of the same material as the candidate LN; if not, a polyester net;
 - an unwashed candidate LN;
 - a candidate LN washed 20 times;
 - an unwashed reference LN as a positive control LN (see definition above);
 - the reference LN washed 20 times.

As LNs containing novel insecticides with entirely new modes of action become available in the future, further modification of these guidelines and evaluation methods may be necessary.

A net will be considered to have met the requirements for interim recommendation if the mortality and blood-feeding inhibition of the candidate LN washed 20 times is equal to or better than the positive control washed 20 times. If the candidate LN meets these criteria when tested against a vector population that is susceptible to both pyrethroids and the novel compound, further tests should be conducted in areas with pyrethroid resistance. The vector population should also be susceptible to the novel compound used in making the candidate LN.

- Phase III studies should follow existing WHOPES guidelines, with modifications to include a positive control LN arm as recommended above. In basic design and procedures, phase III studies should follow the general guidelines provided for monitoring the durability of LNs under operational conditions.

Novel insecticides may require modification to the laboratory evaluations of these products. For example, some slow-acting insecticides may require observations on mortality at intervals beyond 24 h. As noted above, candidate LNs treated with insecticides with effects on mosquitoes that differ from the effects of pyrethroid insecticides may require proof of principle, as well as the development of new assays.

As new, non-pyrethroid insecticides are brought to the PHP market, it is important to test them against a range of mosquito strains with different resistance mechanisms. It is therefore recommended that new mosquito strains with novel resistance mechanisms be established and characterized.

If sites with pyrethroid-susceptible populations are not available for phase II testing, a reference LN should still be included in the comparison as a best practice. However, the decision to

recommend the novel product as an LN should be made based on its own performance.

III.II Efficacy testing of LNs with a mixture of insecticides

There are some circumstances in which mixtures offer benefits for insecticide resistance management, and the use of mixtures has been identified as a desirable strategy in the *Global plan for insecticide resistance management in malaria vectors*.⁴²

It is anticipated that novel LN products will have mixtures of at least two unrelated insecticides⁴³ and, at a meeting of the WHO Global Malaria Programme, the development of mixtures of insecticides for use on ITNs or in IRS was considered as a research priority. Mixtures refer to products in which at least two insecticides are co-formulated in the same product such that an insect on contact would be exposed to both insecticides at the same time.

For the purpose of resistance management strategies, the two insecticides should be of different classes. Mosquitoes that are not killed by one insecticide because of resistance will likely be killed by the other insecticide. Mixtures may also be used to capitalize on different modes of action of the two different insecticides (e.g. personal protection and direct toxicity).

There are several challenges to the development of mixtures, particularly in formulating products, such that the decay rates allow continuation of good efficacy for both insecticides and the formulated product are safe to humans. However, research in agriculture and modelling studies indicate that mixtures are one of the most effective approaches to the management of insecticide resistance.

Unless one or both of the elements in a mixture require additional

⁴² Available at

http://www.who.int/malaria/vector_control/ivm/gpirm/en/index.html.

⁴³ Mixing an insecticide with a synergist is not considered as a mixture in this context.

testing due to their different modes of action, the basic requirements for phase I studies should continue to be followed. In all cases, studies to determine efficacy, wash resistance and regeneration of the candidate LN should be done on the product as a mixture, as well as on the individual components of the product. Testing of the two (or more) components alone is necessary in order to understand and demonstrate the benefit of combining them. However, in order to minimize the burden of the testing process, it is sufficient to test products with the individual components separately in phase I (for basic information) and phase II (wild mosquitoes and taking behavioural issues into consideration) but not in phase III.

Phase I testing should be done against both susceptible mosquito strains as well as one or more pyrethroid-resistant strains. The resistant strain should be well characterized according to phenotypic susceptibility in WHO resistance assays, *kdr* genotype and metabolic enzymes. Determination of regeneration time and selection of washing interval should be based on that of the slowest regenerating compound in the mixture. Therefore, the following treatment arms are recommended for LNs in which both compounds in the mixture LN are active against mosquitoes:

- a. Candidate mixture LN with compounds A and B
- b. Candidate LN with compound A only
- c. Candidate LN with compound B only.

For phase II testing, trials should initially be conducted in an area with pyrethroid-susceptible mosquitoes and mosquitoes susceptible to compounds used in the mixture in the candidate LN. If the candidate LN product is as effective as the reference LN, it should also be tested in an area with pyrethroid-resistant mosquito populations that give reduced mortality and blood-feeding inhibition when conventional LNs with pyrethroid are used.

1. Candidate mixture LN, unwashed
2. Candidate mixture LN, washed 20 times
3. Candidate LN with compound A only, unwashed
4. Candidate LN with compound B only, unwashed
5. Candidate LN with compound A only, washed 20 times

6. Candidate LN with compound B only, washed 20 times
7. Positive control (an LN that has received a WHOPES recommendation), unwashed
8. Positive control, washed 20 times (using a regeneration time not exceeding 3 days, as discussed above)
9. Untreated net, preferably of the same material as the candidate LN; if not, a polyester net.

If one of the compounds is a synergist that causes no mortality at operational doses as determined in phase I studies, the treatment arms should include only the candidate mixture LN and the candidate LN with the insecticide only. It is not necessary to test the candidate LN with the synergist only.

The ultimate decision is based on the comparison of the candidate LN (washed 20 times) versus positive control LN washed 20 times. The candidate LN should have equal or greater efficacy in terms of mortality and blood-feeding inhibition.

As noted above, mosquitoes collected in experimental hut studies should be preserved for quality control and/or future studies of genetic markers of insecticide resistance and their relation to efficacy in the experimental huts.

III.III Efficacy testing of combination LNs

Combination LNs include two or more different nettings in their manufacture. Each netting has a different specification, which may be for different fibres and/or active ingredient(s) with or without synergists.

In phase I, each netting component of the LN must be assessed separately. In phase II, the full product should be studied. Where the netting includes mixtures of insecticides or that of insecticide plus a synergist, the principles for evaluating LNs with mixtures as described above will generally apply.

III.IV Efficacy testing of mixed formulations for IRS

Mixtures of AIs may be applied as IRS treatments to delay the selection of resistance and to provide improved control. As stated earlier and for the purpose of resistance management strategies, the two insecticides should be of different classes.

In phase I testing, the product and its components should be tested on different substrates using both susceptible and resistant strains, as recommended by WHOPES guidelines.

For phase II testing in experimental huts, the following arms are proposed:

1. Untreated hut
2. Mixture IRS
3. IRS formulation of component 1 at the same dose as in the mixture
4. IRS formulation of component 2 at the same dose as in the mixture
5. IRS formulation of component 1 at recommended application (registered manufacturer's label) rate (optional positive control)
6. IRS formulation of component 2 at recommended application (registered manufacturer's label) rate (optional positive control).

Mosquitoes collected from the experimental huts should be preserved for quality control or future studies of genetic markers of insecticide resistance.

IV. Other recommendations

The physical and chemical properties of pesticide products submitted for testing and those of any reference products should be assessed *before* starting studies to ensure that the product complies with the manufacturing or WHO specifications, where available. Products that do not meet specifications will result in causing delays in planned trials and the manufacturer will be

responsible for any costs incurred. Manufacturers should be asked to provide a certificate of analysis of their candidate product beforehand.

ANNEX 1. LIST OF PARTICIPANTS

Dr Rajendra Bhatt, National Institute of Malaria Research, Raipur, India.

Dr Daniel Boakye, Noguchi Memorial Institute for Medical Research, University of Ghana, Accra, Ghana

Dr Fabrice Chandre, Laboratoire de Lutte contre les Insectes Nuisibles (LIN/IRD), Montpellier, France.

Professor Dr Marc Coosemans, Institute of Tropical Medicine, Antwerp, Belgium.

Dr Vincent Corbel, Centre de Recherches Entomologiques de Cotonou, Benin.

Dr John Gimnig, Centers for Disease Control and Prevention , Atlanta, GA, USA.

Dr Jonathan Lines, London School of Hygiene and Tropical Medicine, London, UK.

Professor Graham Matthews, Imperial College at Silwood Park, Ascot, UK.

Dr Abraham Mnzava, Global Malaria Programme, World Health Organization, Geneva, Switzerland (unable to attend).

Dr Ralph N'Guessan, London School of Hygiene and Tropical Medicine, Cotonou, Benin.

Dr Olivier Pigeon, Walloon Agricultural Research Centre, Gembloux, Belgium.

Dr Kamaraju Raghavendra, National Institute of Malaria Research, Delhi, India.

Dr Mark Rowland, London School of Hygiene and Tropical Medicine, London, UK.

Dr Hassan Vatandoost, School of Public Health, Tehran, Iran (Islamic Republic of).

Dr Raman Velayudhan, Vector Ecology and Management, Control of Neglected Tropical Diseases, World Health Organization, Geneva, Switzerland.

Dr Rajpal Yadav, Vector Ecology and Management, Control of Neglected Tropical Diseases, World Health Organization, Geneva, Switzerland.

Dr Morteza Zaim, Vector Ecology and Management, Control of Neglected Tropical Diseases, World Health Organization, Geneva, Switzerland.

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