WHO/HTM/NTD/WHOPES/2009.1

Report of the Twelfth WHOPES Working Group Meeting

WHO/HQ, GENEVA
8—11 DECEMBER 2008

Review of:

BIOFLASH® GR
PERMANET® 2.0
PERMANET® 3.0
PERMANET® 2.5
LAMBDA-CYHALOTHRIN LN

World Health Organization

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CONTROL OF NEGLECTED TROPICAL DISEASES
WHO PESTICIDE EVALUATION SCHEME

The recommendations of the World Health Organization Pesticide Evaluation Scheme (WHOPES) are intended to facilitate the registration and use of the evaluated products by the Member States of the World Health Organization. A recommendation or interim recommendation concerning a specific product means that the World Health Organization has evaluated that product in laboratory and field trials and that the product was found to meet the criteria and requirements of the World Health Organization.

For long-lasting insecticidal mosquito nets (LNs), the World Health Organization may – pending the completion of long-term studies that may be required to fully evaluate such LNs and subject to certain

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A recommendation or interim recommendation does not imply any approval by the World Health Organization of the product in question (which is the sole prerogative of national authorities).

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CONTENTS

1. INTRODUCTION

The twelfth 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 8 to 11 December 2008. The objective of the meeting was to review the reports of testing and evaluation of BioFlash GR, a *Bacillus thuringiensis israelensis* product of Nature Biotechnology Company (Islamic Republic of Iran) for mosquito larviciding, as well as those of four long-lasting insecticidal mosquito nets (LNs) for prevention of malaria: PermaNet 2.0; PermaNet 2.5 (=PermaNet 2.0 Extra); PermaNet 3.0 (Vestergaard Frandsen, Switzerland); and Lambda-cyhalothrin LN (=ICON MAXX-Net) of Syngenta, Switzerland.

The meeting was attended by 14 scientists (see Annex I: List of participants). Professor Dr Marc Coosemans was appointed as Chairman and Dr Purushothaman Jambulingam 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plenary and group sessions, in which the reports of the WHOPES supervised trials and relevant published literature and unpublished reports were reviewed and discussed (see Annex II: References).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.

2. REVIEW OF BIOFLASH GR

BioFlash GR is a bacterial larvicide manufactured as a granular formulation by the Nature Biotechnology Company (Islamic Republic of Iran). The active ingredient (AI) is composed of viable endospores of *Bacillus thuringiensis* subspecies *israelensis* (*Bti*, serotype H-14) and delta-endotoxin crystals. The AI constitutes 10% of the formulated product, with a reported potency of 18 000 international toxic units (ITU)/mg. The manufacturer recommends a target dosage of 1.7 kg/hectare of the formulated product.

The human and environmental safety of Bt, including *Bti*, have been assessed by WHO (1999),[1] and no separate safety assessment has therefore been carried out on BioFlash GR. Application of any product formulated with *Bti* to potable water for the purposes of mosquito larviciding should be subject to a specific risk assessment, and should take into account microbial contaminants, formulants and impurities. Such an appraisal has not been part of the assessment provided hereunder.

2.1 Efficacy – WHOPES supervised trials

2.1.1 Laboratory studies

Montpellier, France

EID Méditerranée (2005) carried out bioassays with BioFlash GR (2005 batch) and VectoBac 12AS (Valent BioSciences, USA, 1200 ITU/mg) against late third-instar or early fourth-instar larvae of *Aedes aegypti* Bora-Bora strain and *Culex quinquefasciatus* S-lab strain. The potency of BioFlash GR was calculated by comparing its activity with that of the recommended international standard, IPS-82 (*Bti*, 15 000 ITU *Ae. aegypti*/mg) from the Pasteur Institute, France.

The preliminary test used eight concentrations against *Ae. aegypti* and six concentrations against *Cx. quinquefasciatus*,

[1] WHO (1999). *Microbial pest control agent, Bacillus thuringiensis*. Geneva, World Health Organization (Environmental Health Criteria 217).

with two replicates for each dosage, to arrive at dosages that would provide 0–100% mortality. Subsequently, six concentrations of BioFlash GR (0.25–4.0 mg/L), five to six concentrations of VectoBac 12AS (0.06–0.36 mg/L) and five concentrations of IPS82 powder (0.0075–0.03 mg/L) were selected for testing against each species. Each concentration was tested in five replicates and a control. The BioFlash granules were weighed, placed in osmosed water with a small drop of wetting agent (Tween 80) and homogenized for 15 minutes. The granules were separated on a filter and serial dilutions were made from the suspension. The IPS 82 powder was weighed, placed in osmosed water and homogenized in a blender; further dilutions were made from this primary suspension. The VectoBac suspension was dissolved in osmosed water and serial dilutions were made. Aliquots of aqueous dilutions were added to 99 ml of water in disposable cups containing 20 larvae.

Twenty early-third instar larvae kept in a jar containing 20–30 ml osmosed water were transferred into a cup containing 99 ml of osmosed water treated with the formulation. Temperature was maintained at 26 °C or 28 °C $\pm$ 1 °C throughout the experiment. Larval mortality was scored 24 hours after treatment and percentage mortality was corrected for control mortality using Abbott's formula. The experiments were repeated three times on three different days. Probit regression analysis was done to calculate the LC_{50} and LC_{90} values as well as their 95% fiducial limits. The potency of the test substance was calculated as follows: 15 000 ITU x LC_{50} standard IPS82/LC_{50} test substance.

The LC_{50} and LC_{90} values indicate that BioFlash GR was relatively less active against *Ae. aegypti and Cx. quinquefasciatus*, (1.0378 mg/L and 2.1871 mg/L; 1.6279 mg/L and 3.0232 mg/L) compared with VectoBac 12AS (0.1202 mg/L and 0.1977 mg/L; 0.1600 mg/L and 0.3140 mg/L) and IPS82 (0.0132 mg/L and 0.0237 mg/L; 0.0141 mg/L and 0.0278 mg/L). The potency of VectoBac 12AS against *Ae. aegypti* was 1646 ITU/mg and that of BioFlash GR was 191 ITU/mg, about 100 times lower than that reported by the manufacturer.

Montpellier, France

Bioassays were carried out in 2006 on samples of the same batch (no. 850085) of BioFlash GR sent for WHOPES field

testing and evaluation in Thailand, India and the Islamic Republic of Iran (EID Méditerranée 2006b). This included the determination of the potency and larvicidal activity of BioFlash GR against third-instar larvae of *Ae. aegypti* Bora-Bora strain (Lagneau et al., 2006). The ITU value was calculated by comparing its activity with that of the international standard, IPS-82 (Bti, 15 000 ITU *Ae. aegypti* /mg).

Initially, five concentrations were used, with two replicates for each dosage, to arrive at dosages that would provide 0–100% mortality. Subsequently, six concentrations of BioFlash GR (0.25–3.0 mg/L) and IPS-82 powder (0.005–0.03 mg/L) were tested. Each concentration was tested in five replicates and equal number of control. Procedures followed for the preparation of suspension and larval testing were identical with described above.

The LC_{50} and LC_{90} values were 0.01126 mg/L and 0.02218 mg/L for IPS 82 and 1.66639 mg/L and 3.32804 mg/L for BioFlash GR, respectively (Table 1). The potency of BioFlash GR was equivalent to 101 ITU/mg, which was much lower than that obtained in 2005 (191 ITU/mg). This variation is probably due to differences in the preparation of two batches of the product.

Montpellier, France

EID Méditerranée (2006a) carried out a simulated indoor trial in 130-L glass aquaria (1m x 0.33m: 0.33 m^2) to evaluate the larvicidal activity and persistence of BioFlash GR (2005 batch: 191 ITU/mg *Ae. aegypti* and 130 ITU/mg *Cx. quinquefasciatus)* in comparison with VectoBac® CG (Valent BioSciences, USA, 200 ITU/mg) against late third-instar or early fourth-instar larvae of *Ae. aegypti* (Bora-Bora strain) and *Cx. quinquefasciatus* (S-Lab strain). The aquaria were filled with 100 litres of tap water and placed in a room allowing a natural photoperiod. The contamination of the aquaria was tested by introducing 10 third-instar larvae of *Ae. aegypti* or *Cx. quinquefasciatus*, S-Lab strain, 72 hours after the addition of water to the aquarium and recording the mortality 24 h after the addition of mosquito larvae.

Four replicates were set up for each test species and the biocides were applied at 56.1 mg/aquarium (0.33m^2), equivalent to the recommended dosage of 1.7 kg/ha for BioFlash. One aquarium was kept as a control for each species. The efficacy

was evaluated by introducing 50 third-instar larvae of *Ae. aegypti* or *Cx. quinquefasciatus* in each aquarium, 24 hours before the treatment and recording mortalities 72 hours after the treatment. The residual activity was determined by introducing 50 third-instar larvae into each aquarium from day 3 post-treatment and recording mortalities 48 hours post-treatment. From day 3 post-treatment, 10 litres of water were removed daily for 5 days each week until termination of the experiment. The aquaria were replenished with 50 litres of water on day 5. The experiment was terminated after 94 days. The air and water temperatures were 25.06 °C +/– 0.06 °C and 23.03 °C +/– 0.08 °C, respectively. The mean water pH value was 7.75, and the mean conductivity was 577.5 mS/cm and 0_2 11.6 mg/L.

In the aquaria, BioFlash GR provided 96–100% reduction of *Ae. aegypti* larvae up to 94 days post-treatment; VectoBac CG gave 82.5–100% reduction up to 80 days post-treatment. The larvicidal efficacy of VectoBac CG was significantly lower than that of BioFlash GR (Wilcoxon's test, $p < 0.01$). The reduction of *Cx. quinquefasciatus* larvae was 86.5–100% in the aquaria treated with BioFlash GR and 67–100% in the aquaria treated with VectoBac CG for 94 days post-treatment. Until day 59 post-treatment there was no significant difference in the activity of the two formulations. Thereafter, VectoBac CG was found to be less active than BioFlash GR (Mann-Withney test).

2.1.2 Field studies

Nonthaburi, Thailand

Mulla et.al. (2007) evaluated the initial and long-term efficacy of BioFlash GR against *Ae. aegypti* larvae in Nonthaburi, Thailand, in comparison with VectoBac[1] WG (Valent BioSciences, USA, 3000 ITU/mg), which is used in mosquito control programmes. Two types of containers were used: 200-litre capacity earthen water-storage jars (used commonly by households) and 70-litre capacity plastic barrels.

Before testing, both types of containers were decontaminated by filling them fully with water and setting them open in the sun. The containers were then emptied, scrubbed, rinsed thoroughly

[1] VectoBac WDG (water dispersible granule).

with water and dried in the sun or shade for a day or two. The containers were placed on a concrete slab under a roof, where the area was open on all sides, simulating field conditions. The jars and the plastic containers were filled to capacity with 200 litres and 70 litres respectively of domestic tap water. The filled earthen jars had a water column height of 62 cm; when half-full, the water depth was 32 cm. The diameter of the jar at the water surface was 44.5 cm. The mouth of the jars was kept covered with celocrete sheets at all times except during the removal and addition of larvae. The 70-litre plastic barrels had a water column 56 cm when full; the diameter of the barrel at the water surface was 41 cm. The barrels were covered with a lid provided with a circular mesh screen of 16 cm diameter for ventilation. Into the water were placed 25 laboratory-reared third-instar larvae of *Ae. aegypti* and their survival scored 2 or 3 days later. This procedure was done for up to 6 weeks. Those jars or barrels giving 20% or greater mortality were excluded from the tests or cleaned further. The contaminated jars were washed, scrubbed and retested until they were fit for the experiments.

In a preliminary test to find the range of activity of BioFlash GR, two jars each were treated with high dosages of 100, 200 and 300 mg/L of BioFlash GR. Observation of the activity and floating capacity of the formulation was made for 3 weeks. Although all the dosages caused 100% mortality of larvae, dosages of 200 mg/L and 300 mg/L caused significant scum formation and an oily film that trapped the granules. Therefore, 100mg/L or lower dosages were used in the tests.

Each type of container and each treatment had four replicates and an equal number of controls. Two regimens of water were used in the earthen jars: jars that were full at all times and jars from which half of the water was removed and replenished weekly after the initial 3-week observation. Since some of the BioFlash GR floated for 3 weeks, the water was not emptied or replaced for 3 weeks. An electrical pump lowered to midway in each earthen jar was used for removing the water in the experiments with half-full water. Thermometers were placed in each of the control jars and barrels and the temperature recorded at each assessment interval. On average, water temperatures during the test ranged from 28 °C to 34 °C.

Before treatment, 25 third-instar laboratory-reared *Ae. aegypti* larvae were placed into a cup with a small amount of water and transferred to the jars or barrels. Ground larval food (1 g) was added to each container and 0.5 g was then added each week. The required amounts of BioFlash GR were weighed and spread over the water surface of the containers. Most of the granules floated on the surface for a week. The water surface was stirred gently with a spoon to make the granules sink to the bottom. Different dosages of the WG formulation were suspended in water and 10 ml of the suspension of a given dose was pipetted onto the water surface and gently stirred. After treatment, the jars and barrels were kept covered.

To determine the duration of efficacy, every week new cohorts of *Ae. aegypti* larvae were added to each container. For one month, larval and pupal mortality was assessed 72 hours after the addition of larvae. To assess the emergence of adult mosquitoes, 1 week after the addition of the larvae, pupal skins indicating successful emergence were removed by suction and counted in water in white trays. The reduction of emergence was calculated based on the initial number of larvae released. Correction of reduction of emergence to the control was not done as control mortality was mostly in the low range. The evaluation was carried out for about 3 months after a single treatment.

BioFlash GR was used at four dosages (10, 20, 50 and 100 mg/L) of the formulation in full jars and three dosages (20, 50 and 100 mg/L) in half-emptied jars. In full jars, BioFlash GR at 10, 20, and 50 mg/L formulation gave 80–100% reduction for 42–49 days post-treatment. The higher dosage (100 mg/L) provided 96–100% reduction up to 85 days post-treatment, i.e. until the end of the experiment. These high dosages are, however, not practically feasible because of the excessive floatage of oily film on water surface and their high cost.

The efficacy of BioFlash GR was slightly higher and it and longevity was longer in jars where half of the water was removed and replaced weekly. The low dosage 20 mg/L gave 87–100% reduction up to 49 days post-treatment, 50 mg/L gave 93–100% up to 70 days post-treatment and then declined below 80%. The higher dosage (100 mg/L) provided 100% control up to the end of the experiment (Table 2). This indicated that water removal and replenishment stir up the granules present at the

bottom of the jars, facilitating release and distribution of the toxins in the water medium.

VectoBac WG was applied at much lower rates using four dosages (0.25, 0.5, 1.0 and 2.0 mg/L). In jars without water removal, 0.25 mg/L formulation yielded 82–100% reduction for 35 days post-treatment. The next dosage (0.5 mg/L) gave 97–100% reduction for 35 days, declining to <80% 42 days post-treatment. The next higher dosage (1.0mg/L) gave >90% reduction for 35 days and >80% for 56 days. The highest dosage (2mg/L) yielded >90% reduction up to the end of the experiment i.e. at 85 days post-treatment (Table 2). The higher the dosage, the greater the efficacy and longevity.

The efficacy of VectoBac WG was lower and its longevity was shorter in half-filled earthen jars with the water removed and replenished weekly, than the constantly full jars. The lowest dosage used (0.5 mg/L) gave 92–100% reduction for 21 days, declining to 59% on day 28. The next high dosage (1.0 mg/L) gave 80–100% reduction for 35 days post-treatment. The highest dosage of 2.0 mg/L gave 85–100% reduction for 35 days post-treatment.

The plastic barrels were constantly full without water removal and replenishment. Although 20 and 50mg/L dosages gave 80–100% reduction for 42 and 56 days respectively, 10 mg/L and 100 mg/L yielded reduction only for 35 days. The short duration of efficacy of BioFlash GR in the barrels is probably a result of sunlight entering the barrels from the screened lids. Towards the end of the experiment (63 days post-treatment), the barrels were invaded by the corixid predator, *Micronecta*, through the screened lids, but the efficacy of the formulations had already declined below 80%.

In contrast to BioFlash GR, VectoBac WG showed high efficacy and longevity in the barrels. The two low dosages (0.25 and 0.5 mg/L) gave 80–100% for 49 days, while the high dosages (1.0 and 2.0 mg/L) yielded 85–100% control for up to 70–77 days post-treatment.

Another experiment was carried out in full earthen jars without water removal and replenishment using further low dosages (1, 2.5 and 5 mg/L) of BioFlash GR formulation. All three dosages gave 100% reduction or Inhibition of emergence (IE) for 6 days

post-treatment, the two lower dosages gave >80% for 20 days and the highest dosage (5 mg/L formulation) gave >85% control for 27 days post-treatment (Table 2).

The WG formulation at the three higher dosages killed larvae within 1 hour after addition. The lower dosage killed larvae within 24 hours. BioFlash GR killed larvae slowly and by 24 hours only.

The BioFlash GR formulation appears to be based on corn grit or similar inert material. However, the size of the particles is very heterogeneous with fines, some being very large and some very small. Moreover, they are flaky and have a fine texture not suitable for field application. When applied to water, the particles stick together, floating on the surface. Some were seen floating for 3–4 weeks, a feature not desirable. One month after treatment, most granules sank to the bottom and were still visible there 3 months after treatment.

Ghassreghand, Sistan and Baluchistan, Islamic Republic of Iran

The efficacy and residual activity of BioFlash 10%GR were tested against *Anopheles* and *Culex* larvae in artificial ponds in comparison with Reldan (22.5% EC), the larvicide used in a malaria control programme in the south of the Islamic Republic of Iran (Ladonni, 2006).

The ponds (100 x 100 cm; depth 50 cm) were dug at about 1 metre apart, lined with plastic sheeting and filled with soil or mud from the breeding sites of target vector species to a depth of 10 cm. The ponds were filled with water from the same habitat to a depth of 30 $\pm$ 5 cm and were allowed to remain for 2 weeks until oviposition occurred. Treatments were undertaken when adequate numberd of third-instar and fourth- instar larvae and a few pupae started appearing in the ponds. The water level was maintained throughout the trial period. In order to avoid adult emergence, mature pupae were removed daily.

BioFlash GR was tested in two phases. In the first phase, Bioflash was used at three application rates (0.85, 1.7 and 3.4 kg/ha) in order to find the efficacy range at one dosage above and one below the dosage recommended by the manufacturer. As there was no desired level of reduction of larval density at these dosages of BioFlash, three dosages much higher than

the recommended dosage (6.8, 15 and 30 kg/ha) were used. In both the phases, Reldan (22.5% EC) was used at 400 ml /ha. Each dosage and control was replicated four times. Reldan EC was applied using a hand-compression sprayer with a flat fan nozzle, while the GR formulation was broadcasted by hand (with gloves).

Larval and pupal densities were monitored before treatment using dipper sampling and thereafter at one-day intervals during the post-treatment period until the density had reached the pre-treatment level. Species composition was determined by bringing fourth-instar larvae from untreated breeding places and identifying them in the laboratory. The percentage reduction in the density of larvae and pupae was calculated using the Mulla's formula. The percentage reduction of late-instar larvae was used as the main indicator.

The following species composition was recorded: *An. culicifacies* (45.2%), *An. stephensi* (28.9%), *An. dethali* (12.2%), *An. superpictus* (9.6%) and *An. sergenti* (4.1%); *Cx. tritaeniorhynchus* (46.2%), *Cx. pipiens* (31.9%), *Cx. pseudovishnui* (15.2%) and *Cx. quinquefasciatus* (6.7%).

The percentage reduction was <50% during the post-treatment period at 0.85 kg/ha, 1.7 kg/ha & 3.4 kg/ha for both *Anopheles* and *Culex* larvae. While the reduction at 6.8 kg/ha was <50%, the maximum reduction was <60% at 15 kg/ha post-treatment. Even at 30 kg/ha, >80% mortality was obtained only on day 5 and day 7 post-treatment (Table 2), indicating that the formulation provided at this high dosage was an effective control for a short period of 3–4 days only.

Reldan EC at 400 ml of formulation/ha gave 80–100% of pupal and larval reduction up to 15 days.

Delhi, India

BioFlash 10%GR was evaluated against larvae of *An. stephensi* breeding in cement tanks with fresh water, *Cx. quinquefasciatus* in cement tanks and surface drains with organic matter and *An. subpictus* in rainwater pools in two locations around Delhi (Adak et al., 2007).

Initially, larval and pupal samples were brought from the habitats to the laboratory. Adults that emerged from the

samples were identified and the selection of habitats was then made. The surface area of cement tanks ranged from 0.62 m^2 to 3.6 m^2 and that of the drains from 50 m^2 to 100 m^2. The size of the rainwater pools varied from 9.0 m^2 to 25 m^2. Total surface area of the breeding habitats of *An. stephensi* treated with BioFlash GR was 32.4 m^2, that of *Cx. quinquefasciatus* was 518.63 m^2 and that of *An. subpictus* was 86 m^2. The total area of untreated habitats was 14.4 m^2, 200 m^2 and 29 m^2, respectively for the three mosquito species. Each of the cement tanks or drains was considered as a discrete site for replication of treatment or control. pH and other characteristics of the habitats were recorded.

The pre-treatment density of immature mosquitoes was monitored in experimental and control habitats using a standard larval dipper (300 ml volume, 9 cm diameter). A number of serial dips were made in drains. Larval instars and pupae collected in sampling dips were counted and larvae classified into early (I–II instars) and late (III and IV) instars.

Three dosages of BioFlash (1.36, 1.7 and 2.04 g/m^2, equalling 13.6, 17.0 and 20.4 kg/ha) were applied to cement tanks with fresh water. Eight incremental dosages (0.06, 0.17, 0.34, 0.68, 1.02, 1.36, 1.7, 2.04 g/m^2, equalling 0.6, 1.7, 3.4, 6.8, 10.2, 13.6, 17.0 and 20.4 kg/ha) were applied in cement tanks with water containing the organic matter. The rainwater pools were treated at 2.04g/m^2 (20.4 kg/ha). The formulation was applied to the larval habitats uniformly by hand broadcasting (wearing gloves).

Larval densities were measured in all habitats on days 1, 2, 3 and 7 post-treatment and thereafter weekly until the larval counts in the experimental sites reached the level present in the control habitats. The mean number of III–IV instar larvae collected per dip from each habitat was calculated for each day of observation. The percentage reduction in larval densities post-treatment was calculated using Mulla's formula (1971).

The highest reduction of *An. stephensi* larval density in cement tanks with fresh water was 30% 2 days after application of BioFlash GR at 1.36g/m^2 with no control in succeeding intervals. At 1.7 g/m^2, reduction of this species ranged between 37.9% and 74.1% during the 2 weeks post-treatment. At 2.04 g/m^2, the efficacy increased only to 84.7% on days 2–7 post-treatment, declining thereafter (Table 2).

In the cement tanks with water containing organic matter, the reduction of *Cx. quinquefasciatus* larval densities at the dosages up to 1.7g/m^2 was ≤66.3% during 2 weeks of follow up. With the highest dosage (2.04 g/m^2), the maximum reduction in larval counts was 86.4% on day 14 post-treatment with reductions <60% on days 1–7 post-treatment. In drains, only the highest dosage (2.04 g/m^2) was evaluated and the maximum reduction in larval densities was >80% at days 7 and 14 post-treatment after which the efficacy declined.

In rainwater pools, treatment at 2.04g/m^2 of BioFlash reduced the larval density of *An. subpictus* by 92–93%, on days 7 and 14 post-treatment. However, the reduction was <60% on days 1 and 2 post-treatment.

2.2 Conclusions and recommendations

BioFlash GR is a granular formulation containing the microbial agent, *Bacillus thuringiensis* subspecies *israelensis* (*Bti*, serotype H-14) at the concentration of 10%. The product is manufactured by the Nature Biotechnology Company (Islamic Republic of Iran). The product has been primarily developed for the control of *Anopheles* larvae that feed at the surface of the water. The formulation is capable of floating on the surface of water for a relatively long period.

The manufacturer reports that the AI constitutes 10% of the BioFlash GR formulation. The bio-potency of the AI is reported as 18 000 ITU/mg (i.e. 1800 ITU/mg for the formulated product). However, in WHOPES supervised laboratory studies, the formulation was found to have potency in the range of 101–191 ITU/mg, i.e. about 10–18 times lower than that reported by the manufacturer for the formulated product. The product is recommended by the manufacturer at the target dosage of 1.7 kg/ha.

The WHOPES supervised field trials were carried out with the samples of BioFlash GR with 101 ITU/mg. The trial in Thailand was carried out against *Ae. aegypti* larvae in earthen jars (200 L water) and plastic barrels (70 L water) for a period of 3 months. BioFlash GR at the high dosages of 20–100 mg/L provided >80% control for about 7–12 weeks, irrespective of the type of

container and the water usage pattern. The study in the Islamic Republic of Iran, carried out in artificial ponds against *Anopheles* and *Culex* mosquito populations, showed that even at very high dosages (15 and 30 kg/ha), the residual activity was much shorter, ranging from 4 to 7 days. The study in India was carried out against *Anopheles* breeding in cement tanks with fresh water, *Anopheles* and *Culex* species breeding in cement tanks and drains containing water with organic matters, and *Anopheles* species breeding in rainwater pools. BioFlash GR provided control of mosquito larvae for 7–14 days only at the high dosage (20.4 kg/ha). It is clear that BioFlash GR, having efficacy in water storage containers placed in shade and with covers, manifested only short-term efficacy in open, outdoor habitats.

During the course of evaluation, it was noted that BioFlash GR is heterogonous in terms of particle size, with a considerable amount of fine particles. While the floating feature of the particles on the water surface is important for *Anopheles* control, it is not a desirable attribute for use in water storage containers.

Noting the above, it is recommended:

- that the manufacturer develops a more cost-effective formulation and revises the product label in accordance with the product's potency and recommended application dosage. The content of the AI should be declared in ITU/mg and when determined by the method described in WHO guidelines,[1] the average bio-potency should not be less than 90% of the declared minimum content.

[1] FAO/WHO (2006). *Manual on development and use of FAO and WHO specifications for pesticides – March 2006 revision of the first edition* (available at: http://whqlibdoc.who.int/publications/2006/9251048576_eng_update2.pdf; accessed January 2009).

Table 1 **Activity of BioFlash GR, VectoBac 12AS and IPS82 against third-instar or fourth-instar mosquito larvae in the laboratory**

Country, location and year	Product	Activity (mg/L) against			
		Aedes aegypti (Bora Bora strain)		*Culex quinquefasciatus* (S-Lab strain)	
		LC_{50}	LC_{90}	LC_{50}	LC_{90}
Montpellier, France 2005	BioFlash GR	1.0378	2.1871	1.6279	3.0232
	VectoBac 12AS	0.1202	0.1977	0.1600	0.3140
	IPS 82	0.0132	0.0237	0.0141	0.0278
Montpellier, France 2006	BioFlash GR	1.66639	3.32804	–	–
	IPS 82	0.01126	0.02218	–	–

Table 2 **Efficacy of BioFlash GR and VectoBac 12AS when formulation tested against mosquito larvae in simulated field and field trials in various habitats**

Country and location	Product	Species	Habitat	Dosage	Residual activity (days)	Percentage reduction
Montpellier, France	BioFlash GR	*Aedes aegypti*	Glass aquarium	1.7 kg/ha	94	96–100
	VectoBac CG			1.7 kg/ha	80	82–100
	BioFlash GR	*Culex quinquefasciatus*		1.7 kg/ha	94	86–100
	VectoBac CG			1.7 kg/ha	66	90–100
Nonthaburi, Thailand	BioFlash GR	*Ae. aegypti*	Earthen water jars – full (200L)	1 mg/L	20	80–100
				2.5 mg/L	20	85–100
				5.0 mg/L	27	85–100
				10.0 mg/L	42	90–100
				20.0 mg/L	49	80–100
				50.0 mg/L	49	80–100
				100 mg/L	85	95–100
	VectoBac WG			0.25 mg/L	35	80–100
				0.50 mg/L	35	97–100
				1.0 mg/L	56	80–100
				2.0 mg/L	85	93–100

Table 2 (continued) **Efficacy of BioFlash GR and VectoBac 12AS when formulation tested against mosquito larvae in simulated field and field trials in various habitats**

Country and location	Product	Species	Habitat	Dosage	Residual activity (days)	Percentage reduction
Nonthaburi, Thailand	BioFlash GR	*Ae. aegypti*	Earthen water jar (200L – half exchanged)	20 mg/L	49	87–100
				50 mg/L	70	93–100
				100 mg/L	85	100
	VectoBac WG			0.5 mg/L	21	90–100
				1.0 mg/L	35	80–100
				2.0 mg/L	35	85–100
	BioFlash GR		Plastic barrels (70L-full)	10 mg/L	35	95–100
				20 mg/L	42	80–100
				50 mg/L	56	80–100
				100 mg/L	35	90–100
	VectoBac WG			0.25 mg/L	49	80–100
				0.50 mg/L	49	80–100
				1.0 mg/L	70	95–100
				2.0 mg/L	77	85–100

Table 2 (continued) **Efficacy of BioFlash GR and VectoBac 12AS when formulation tested against mosquito larvae in simulated field and field trials in various habitats**

Country and location	Product	Species	Habitat	Dosage	Residual activity (days)	Percentage reduction
Baluchistan, Islamic Republic of Iran	BioFlash GR	*Anopheles; Culex*	Artificial ponds	0.85–15 kg/ha 30 kg/ha	0 7	<80 80–96
Delhi, India	BioFlash GR	*An. stephensi*	Cement tank filled with fresh water	1.36 & 1.7 g/m^2 2.04 g/m^2	0 7	<75 >80
		Cx. quinquefasciatus	Cement tank filled with polluted water	0.065–1.7 g/m^2 2.04 g/m^2 2.04 g/m^2	0 14 7, 14	No control >60[a] >80
		An. subpictus	Drains Rainwater pool	2.04 g/m^2	7,14	>90

[a] A reduction of 86.4% was noted on day 14; this reading seems to be not valid as the control during the previous day was very low.

3. REVIEW OF PERMANET 2.0

PermaNet 2.0 is a deltamethrin-coated LN manufactured by Vestergaard Frandsen (Switzerland). The net is made of knitted poly-filament polyester fibres and is treated with deltamethrin to a target concentration of 55 mg/m^2 (= 1.4 g/kg for a 100-denier net; 1.8 g/kg for a 75-denier net). The insecticide is bound in a resin coating that reduces the amount of insecticide lost during routine washing. The PermaNet 2.0 received WHOPES interim recommendations in 2004;[1] WHO specifications for its quality control and international trade were published in 2006.[2]

PermaNet 2.0 has been widely distributed and used in many countries since 2004. However, large-scale longitudinal randomized household trials, as required by WHOPES for Phase III evaluation of LNs, is limited to one published study.[3] Published information on monitoring and evaluation of PermaNet 2.0 under operational settings is also not available. Therefore, a series of cross-sectional surveys were carried out in different sites in six African countries to generate more information on the efficacy and durability of the PermaNet 2.0. The present assessment includes a review of relevant background information as well as the results of the multi-country surveys.

3.1 Efficacy – background and supporting documents

Several studies on PermaNet 2.0 have been published since the interim recommendation given in 2004. However, only two studies in which PermaNet 2.0 had been in household use for

[1] WHO (2004). *Report of the seventh WHOPES Working Group Meeting, WHO/HQ, Geneva, 2–4 December 2003* (available at http://www.who.int/whopes/recommendations/wgm/en/; accessed January 2009).

[2] WHO (2005) *WHO specifications for pesticides used in public health* (available at: http://www.who.int/whopes/quality/newspecif/en/; accessed January 2009).

[3] Testing and evaluation of public health pesticides by WHOPES are carried out at the request of industry. *Time-limited* (4-year) *interim recommendations* were introduced by WHOPES only in 2006, giving WHO the possibility of withdrawing its interim recommendations in the absence of data or information needed to develop full recommendations.

at least three years have been published. Kroeger et al. (2004) reported on the efficacy of PermaNet 2.0 in Colombia. This study was not considered in this report because no follow-up activity on the use of the nets between the first and third years was reported, the cone assay test did not conform with the WHO standard, only four nets were assessed and no controls were included.

Kilian et al. (2008) reported on the efficacy and durability of PermaNet 2.0 in a randomized household trial in Uganda. Although initiated before the publication of WHOPES guidelines, this study largely conformed to WHOPES guidelines, with only minor differences, and hence was used in this assessment.

The PermaNet 2.0 was compared with conventionally-treated nets in a randomized double-blind study design in Kyenjojo District, west Uganda (Kilian et al., 2008). A total of 140 conventionally-treated nets and 260 PermaNet 2.0 were distributed for this study. The conventional nets were initially distributed in 2001 and were re-treated in 2002 when the PermaNet 2.0 was distributed. No specific instructions regarding use or washing were given to the net users. Nets were followed up at regular intervals to administer a questionnaire on net use, care and perception and to assess physical net condition. Seven surveys of net use and condition were done over the course of the study. A random sample of 10 nets was taken at baseline and random samples of 40 nets per treatment arm were taken five times over the course of three years.

A 30 x 30 cm sample of netting was taken from each net for bioassays, while a 10 x 10 cm sample was taken for chemical analysis. WHO cone bioassays with a 3-minute exposure time were carried out using 40 female *Anopheles gambiae s.s.* per net sample. In most cases, only one sample per net was tested. For chemical analysis, samples were extracted by heating under reflux for 60 minutes in xylene; deltamethrin content was assayed by capillary Gas Chromatography using ^{63}Ni Electron Capture Detection (GC-ECD) and external standard calibration. The analytical method used was successfully validated and showed good accuracy and precision.

At each follow-up, all net owners were visited and asked about net use, washing frequency and washing method. The majority

of nets were observed hanging over their sleeping space at the time of the surveys. Daily use was reported to be 95% throughout all seven surveys. All but two out of 2510 washes reported were done using cold water and country soap. It was estimated that nets were washed on average 1.8 times per year.

The proportion of nets with at least one hole rose rapidly after distribution to just under 50% within one year and >80% after three years. Each hole was categorized as small (the size of a coin), medium (up to the width of a person's hand) or large (larger than the width of a person's hand). A hole index was estimated by multiplying the number of holes in each category by 1 (small), 2 (medium) or 3 (large). The mean hole index was estimated at each time point for each study arm, including nets with no holes. Average hole index increased linearly up to an index of 15 after three years of use.

The median PermaNet 2.0 baseline concentration was 69.2 mg/m^2 (56.8–75.4), which is just above the acceptable range of 55 mg/m^2 $\pm$ 25%. After 36 months, average deltamethrin concentration on the PermaNet 2.0 was 28.7 mg/m^2 (41.5% of baseline dose). In contrast, deltamethrin concentrations on the conventionally re-treated nets fell from 42.8 mg/m^2 at baseline to 1.4 mg/m^2 after 12 months of routine use.

In the cone bioassays, knock-down was >95% at all time-points except for nets collected after 24 months post-distribution, where knock-down was 92.4%. Similarly, mortality was >80% at all time-points except for nets collected at 24 months post-distribution, when mortality was 73.9%. After 36 months of follow up, 90% of nets had either a knock-down rate $\geq$95% or mortality rate $\geq$80%. More than 90% of nets tested at each time-point met the WHOPES criteria for the cone test, except for nets collected at 24 months post-distribution, when only 71% of nets met the WHOPES criteria for the cone test.

The authors concluded that the PermaNet 2.0 showed excellent results after three years of routine household use and fulfilled the WHOPES criteria for an LN.

3.2 Efficacy – WHOPES supervised study

In a multi-country survey carried out by WHOPES in 2007–2008, in collaboration with the WHO Global Malaria Programme, PermaNet 2.0 net samples were taken from different sites in six African countries: Angola, Ghana, Kenya, Madagascar, Togo and Zambia. The selection of sites in each country was based on the information provided by the manufacturer as well as on information provided by WHO regional offices, and where the distribution, age and use of the nets could be verified. Collection of net samples was not limited to a specific delivery or distribution mechanism. Sites where PermaNet 2.0 nets aged between 1 and 3 years could be obtained and where nets were likely to be used year-round were selected for sampling. To achieve a sample that was as representative as possible, at least three sites were used in each country for collection of each category of net samples.

Sites were initially selected by the ministries of health (MOH), and final selection of sites was done together with the MOH and local partners. Within each selected site, all households were enumerated and randomly assigned to a specific order. Households were visited in order of selection, and owners of PermaNet 2.0 that had not been re-treated were asked to exchange their old net for a new PermaNet 2.0. This continued until the required sample size was obtained. If more than one PermaNet 2.0 was found in a selected household, one net was randomly selected. A questionnaire to assess use and washing behaviour was administered in households where PermaNet 2.0 was collected (see Annex III).

Sampled nets were returned to a central site in each country where they were hung over a frame and subjected to physical inspection, following the requirements specified in the data recording form (see Annex III). Upon completion of the inspection, subsamples were taken from each net for bioassays and chemical analyses. A unique five-digit code (first two for the country, third for the village and the last two digits for the net sample) was used to identify each net.

Bioassays

A total of 466 PermaNet 2.0 nets were collected (Table 3) and dispatched to the WHO Collaborating Centre in Montpellier[1] (LIN/IRD) (Zumbo et al., 2008). Each net was identified with its five-digit survey code. Approximately one-third of the nets from each country and each year were randomly selected by the Collaborating Centre for WHO cone bioassay. For Kenya only, one-year-old nets were available. The following number of nets were subjected to cone bioassays:

- 7 nets of one year old from each of the six countries, i.e. a total of 42 nets;
- 8 nets of two years old from each of the five countries, i.e. a total of 40 nets;
- 12 nets for three years old from each of the five countries, i.e. a total 60 nets.

A total of 142 nets were subjected to WHO cone bioassay. Four samples per net were tested. The net samples were taken from positions 2, 3, 4 and 5 (see Figure 1, Annex III). Position 1 was ignored as it may have been subjected to excessive abrasion and is the part that is supposed to be tucked under the mattress.

Two-to-five-day-old, non-blood fed females of *Anopheles gambiae s.s* (Kisumu strain) were used for bioassays. This is a standard susceptible strain originally colonized in western Kenya. It has been maintained in colony for many years at LIN/IRD and is free of any detectable insecticide resistance mechanism. The susceptibility of the strain is checked every 3 months using PCR and biochemical assays.

Per net, the pooled results of mortality and knock-down over the four samples were used to determine if a net was within the WHO requirement, i.e. $\geq$80% mortality and/or $\geq$95% knock-down. Three-year-old nets that were not within the WHO criteria were subjected to the tunnel test. Only one sample per failed net was selected for tunnel test. The sample used in the tunnel test was the one where mortality was closest to the average mortality in the cone bioassay. The WHOPES criteria for the tunnel test are $\geq$80% mortality and or $\geq$90% blood-feeding

[1] Laboratoire de Lutte contre les Insectes Nuisibles (LIN), Institut de Recherche pour le Développement (IRD), Montpellier, France.

inhibition[1]. Results of cone and tunnel tests were used together to judge on the net performance.

The mean number of *Anopheles gambiae* (Kisumu) tested per net was 25, with a minimum of 17 and a maximum of 39. Figures 1 and 2 give on overview of the variation of mortality and knock-down as observed in cone test for the different countries and years.

After one year, 98% of the nets met the WHO requirements based on the cone bioassay, decreasing to 85% and 57% in years 2 and 3 respectively (Table 4). Among the 26 three-year-old net samples (43%) that failed WHO cone bioassays, 61% were effective in tunnel tests based on mortality (≥80%) and/or blood-feeding inhibition (≥90%). Overall, 80% of three-year-old nets met the WHO requirements for either the WHO cone test or the tunnel test. Large differences were observed among countries: nets collected in Ghana and Madagascar failed to meet the WHO criteria of an LN, whereas in Angola all nets fulfilled the requirements (Table 4).

Chemical assay

Chemical assays were conducted (Pigeon 2008a and 2008b) following a method based on the CIPAC method 333/LN/M/3 for determination of deltamethrin in PermaNet, i.e. extraction by sonication and shaking with isooctane/dioxane (80/20, v/v) and chromatographic determination by gas chromatography with flame ionization detection (GC-FID). Results are given in the actual measurement (g/kg). The density of all net samples was assessed (Pigeon 2009a) to determine the denier, as this information was not available.

A total of 420 nets were analysed: 61 from Angola, 80 from Ghana, 26 from Kenya, 85 from Madagascar, 90 from Togo and 78 from Zambia (Table 3). After one year of net use, a significant loss of deltamethrin was observed in all countries compared with the baseline deltamethrin content of a 75 or 100 denier net (Figure 3). The deltamethrin content dropped further during the next two years and was at the quantification limit of

[1] WHO (2005). *Guidelines for laboratory and field testing of long-lasting insecticidal mosquito nets*. Geneva, World Health Organization (WHO/CDS/WHOPES/GCDPP/2005.11; available at http://www.who.int/whopes/guidelines/en/; accessed January 2009).

0.2 g/kg in Madagascar. In Angola, no changes in deltamethrin content were observed over the three years (Figure 3). The measured deltamethrin content was highly variable between nets and countries. Overall (all countries combined), the deltamethrin content decreased from a mean value of 0.821 g/kg (95% CI, 0.723–0.920) in year 1, >0.558 g/kg (95% CI, 0.491–0.626) in year 2 to 0.431 g/kg (95% CI, 0.367–0.495) in year 3. The overall mean deltamethrin content of a one-year-old net was 45% and 60% of the baseline concentration for a 75- and 100-denier PermaNet 2.0 respectively. A three-year-old net had a mean deltamethrin content of 24% and 31% for a 75- and 100-denier PermaNet 2.0 respectively.

The correlation between the chemical analysis and the cone bioassays on adjacent pieces of netting shows that low deltamethrin concentrations (even below the quantification limit) can still cause mortality above the threshold of 80%. Likewise, the high knock-down values were observed for nets with variable deltamethrin content (Figure 4). The mean deltamethrin content of nets causing <80% mortality was 0.274 g/kg (95% CI, 0.235–0.314) and causing <95% knock-down was 0.241 g/kg (95% CI, 0.193–0.289).

All nets that failed (cone tests and tunnel tests combined; Table 4) had a deltamethrin content <0.2 g/kg, which is the quantification limit of the analytical method used in this study. The deltamethrin content of nets that met the WHO criteria (cone and tunnel combined) was variable and ranged from 0.208 g/kg to 0.818 g/kg (Table 5).

Washing behaviour of local people

In each country, two to five people were asked to wash a new 100-denier PermaNet 2.0 one time. People were not given soap, detergent or instructions but were asked to wash the nets as they would normally do so. Samples taken before and after washing were subjected to chemical assay to evaluate how much of the insecticide was washed off in each location. A total of 19 nets were tested.

The baseline deltamethrin concentration of unwashed nets was slightly higher than the target dose of 55 mg AI/m², but was within the acceptable range of 55 mg AI/m² $\pm$ 25% for all nets with the exception of one net in Kenya. The loss of deltamethrin

after one wash was highly variable in all countries, ranging from 1% to 44% (Table 6).

Two additional three-year old PermaNet 2.0 (a white and a blue net) from Angola were sent to LIN/IRD for a wash resistance study. Previously, these nets were washed 0, 1, 5, 7, 8, 9 and 10 times in Angola according to local traditions, i.e. hand-washing with soap (in Angola, nets were washed with cold water using commercial bars or powdered soap. Nets were not rubbed against rocks or stones and were dried outside). Results show that both the knock-down effect and mortality were within WHO requirements in bioassay cones after 10 consecutive local washes for both white and blue nets.

At the time of collection, household owners were asked how many times they had washed their net. During physical inspection, the nets were assigned to one of four categories from clean to very dirty (Table 7). Nets were washed infrequently in Angola, whereas in Ghana and Madagascar they were washed >15 times during the three-year period. Despite the frequent washing of nets in Madagascar, 69% of nets were scored as dirty to very dirty in year 3 (Table 7).

Nets were classified into five different wash categories to evaluate the impact of washing on deltamethrin content. Significant differences between the classes were observed (ANOVA: $p < 0.001$, R-squared = 0.157). The deltamethrin content decreased significantly with increasing numbers of washes (Table 8). The mean deltamethrin content was 0.617 g/kg on nets washed 1–5 times, 0.454 g/kg on nets washed 6–10 times, 0.419 g/kg on nets washed 11–15 times and 0.322 g/kg on nets washed >15 times.

Physical nature of the nets (durability)

Differences in the mean number of holes were observed per country and year. The nets from Ghana had the fewest holes, whereas the nets in Madagascar were heavily torn. Most holes were small, but 25% of the holes observed on two-year-old and three-year-old nets were larger than a person's thumb. Approximately two-thirds or all holes were found on the lower half of the net (Table 9). The mean number of repairs per net ranged from 0.42 to 0.87. Few holes caused by burns were detected, with a maximum of 1.21 burns per net in Kenya. The average number of holes ranged from 2.4 to 20.1 in one-year-

old nets (66% of 75 denier nets), from 3.3 to 47.1 in two-year old nets (74% of 75 denier nets) and from 2.9 to 54.9 in three-year-old nets (57% of 75 denier nets).

3.3 Conclusions and recommendations

PermaNet 2.0 is a deltamethrin-coated LN made of knitted poly-filament polyester fibres for which WHOPES has provided interim recommendations based on Phase I laboratory tests and small-scale field testing (experimental hut studies, WHOPES Phase II) (WHO, 2004). Full WHO recommendations require further evidence of the efficacy and durability as well as acceptability of the LN under routine household use over a period of three years.

Testing and evaluation of public health pesticides by WHOPES are carried out at the request of industry. *Time-limited* (4-year) *interim recommendations* were introduced by WHOPES only in 2006, giving WHO the possibility of withdrawing its interim recommendations in the absence of data or information needed to develop the full recommendations.

Longitudinal randomized household trials to evaluate the efficacy, longevity and fabric integrity as well as the community acceptance of an LN are usually carried out in 2–3 countries over a period of three years. Published reports on performance of PermaNet 2.0 in operational settings are scarce. Only one published study followed the WHOPES guidelines, but with some modifications. Therefore, WHOPES supervised a multi-country survey that provided additional data on bio-efficacy and durability in household conditions in different sites in six countries (Angola, Ghana, Kenya, Madagascar, Togo and Zambia).

WHOPES guidelines recommend that at least 80% of nets tested after three years of routine household use meet the cut-off criteria for either the WHO cone bioassay test or the tunnel test.

In the longitudinal study in Uganda (Kilian et al., 2008), the PermaNet 2.0 showed excellent bio-efficacy after three years of routine household use. Initial deltamethrin concentrations averaged 69.2 mg/m^2, which is just above the acceptable range

of 55 mg/m^2 $\pm$ 25%. However, after three years of routine household use, the PermaNet 2.0 retained approximately 41% of the initial concentration of deltamethrin. The average hole index increased linearly with time to an average index of 15 (corresponding to 15 small holes or 5 large holes per net). Although the study did not fully conform to WHOPES guidelines for Phase III studies, it was concluded that three-year-old PermaNet 2.0 nets met the bio-efficacy criteria for Phase III evaluation of LNs in this study.

In the WHOPES supervised multi-country survey, 85% of two-year-old nets met the WHOPES criteria based on cone bioassay alone ($\geq$80% mortality or $\geq$95% knock-down). However, in two of the five countries, <80% of the PermaNet 2.0 met the criteria for the cone test.

After three years, only 57% of the nets met the criteria based on the cone test alone. In only one out of five countries did the PermaNet 2.0 meet these criteria after three years of routine household use. The addition of tunnel test carried out on three-year-old nets that failed the cone bioassay raised the proportion of nets meeting the WHOPES criteria to 80%. However, even with the inclusion of the tunnel test, the PermaNet 2.0 fulfilled the WHOPES requirements in only three of the five countries.

PermaNet 2.0 therefore has proven efficacy of three years when data are pooled from all countries. However the bio-efficacy of three-year-old PermaNet 2.0 was marginal and there was high variability among countries. Furthermore, the performance may have been overestimated as the cross-sectional study design may have under-sampled worn-out nets that may have been discarded by householders or where the age of the nets could not be verified due to washed-out or faded labels.

The durability of the sampled nets as measured by inspection of the number and size of holes was highly variable between countries. The average number of holes per net was 12 after one year, increasing to 20 after two years. Some 25–30% of these holes were larger than a person's thumb. Two-thirds of the holes were situated on the lower half of the side panels. These percentages remained constant between years 2 and 3. The average number of openings along the seams was 0.4 per net. The durability of PermaNet 2.0 in this survey may have

been overestimated as the cross-sectional study design may have under-sampled worn-out nets.

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 PermaNet 2.0 in a multi-country study, which is borderline, and the published literature, full recommendation is granted;

- noting the borderline overall bio-efficacy and its high variability among countries, the poor fabric integrity in all study sites and the lack of criteria for durability, national programmes are strongly encouraged to monitor and evaluate the performance of the PermaNet 2.0 under local conditions when selecting the most suitable LN for their local setting.

Note: WHO recommendations on the use of pesticides in public health are valid ONLY if linked to WHO specifications for their quality control.[1]

[1] WHO specifications for pesticides used in public health are available at http://www.who.int/whopes/quality/newspecif/en/; accessed January 2009.

Table 3 **Number of PermaNet 2.0 nets sampled and the number of chemical assays, cone tests and tunnel tests conducted by country**

Country	Number of nets collected	Chemical test	Cone test	Tunnel test
Angola	77	61	27	1
Ghana	87	80	27	6
Kenya	30	26	7	0
Madagascar	90	85	27	10
Togo	90	90	27	3
Zambia	92	78	27	6
Total	466	420	142	26

Table 4 **Percentage of PermaNet 2.0 net samples exceeding WHO efficacy criteria[a] in cone tests and tunnel tests, and their combined results (number of nets tested shown in parentheses)**

	Cone bioassay				Cone test and tunnel test
Country	Year 1	Year 2	Year 3	Tunnel test[b]	combined
Angola	100 (7)	88 (8)	92 (12)	100 (1)	100
Ghana	100 (7)	75 (8)	50 (12)	50 (6)	75
Kenya	100 (7)	No nets	No nets	–	–
Madagascar	86 (7)	75 (8)	17 (12)	40 (10)	50
Togo	100 (7)	88 (8)	75 (12)	33 (3)	83
Zambia	100 (7)	100 (8)	50 (12)	83 (6)	92
All countries	98 (42)	85 (40)	57 (60)	–	80

[a] WHO criteria: cone test: ≥80% mortality and/or ≥95% knock-down; tunnel test: ≥80% mortality and/or ≥90% blood-feeding inhibition.
[b] Tunnel tests were carried out on three-year-old nets that did not meet the WHO criteria in the cone test.

Table 5 **Mean deltamethrin content and 95% confidence interval (CI) of PermaNet 2.0 net samples meeting WHOPES efficacy criteria for long-lasting insecticidal mosquito nets (cone tests and tunnel tests combined)**

Country	Number of nets	Content (g/kg)	95%CI
Angola	12	0.818	0.415–1.220
Ghana	9	0.509	0.243–0.775
Madagascar	5	0.208	0.191–0.225
Togo	10	0.355	0.279–0.431
Zambia	11	0.399	0.201–0.597

Table 6 **Mean deltamethrin concentration of new PermaNet 2.0 samples before and after washing, and the range of insecticidal loss as a percentage of its initial concentration**

Country	Number of nets	Mean concentration before washing mg AI/m^2 (95% CI)[b]	Mean concentration after one wash mg AI/m^2 (95% CI)[b]	Range of percentage deltamethrin loss[a]
Angola[c]	4	62.0 (55.0–67.0)	49.5 (43.9–55.0)	6–26%
Ghana	4	69.6 (66.7–72.5)	Not available	Not available
Kenya	2	72.9	56.0	21–28%
Madagascar	2	62.7	55.7	1–20%
Togo	5	66.9 (63.5–70.4)	47.0 (40.0–54.0)	18–44%
Zambia	2	67.3	43.3	34–40%

[a] The percentage of deltamethrin loss was calculated on the actual measurement, i.e. g/kg.
[b] The 95% confidence interval (CI) is given only if more than two nets were tested.
[c] One sample contained more deltamethrin after washing than before washing; this measurement was removed from the mean.

Table 7 **PermaNet 2.0: denier, number of times washed and general aspect of the net (Category: 1 = clean; 2 = a bit dirty; 3 = dirty; 4 = very dirty)**

Country	Year	Number of nets	Denier (percentage)		Mean number of washes	General aspect of nets (% of nets per category)			
			75	100		1	2	3	4
Angola	1	10	80	20	0.00	50	20	0	30
	2	43	70	30	0.00	19	49	19	14
	3	24	NA	NA	0.04	8	71	17	4
Ghana	1	26	100	0	5.88	27	42	19	12
	2	34	100	0	7.71	38	44	15	3
	3	27	16	84	16.37	33	33	22	11
Kenya	1	30	100	0	3.50	17	47	23	13
Madagascar	1	30	7	93	8.97	10	60	30	0
	2	28	11	89	10.29	0	29	57	14
	3	32	3	97	17.66	13	19	53	16
Togo	1	20	0	100	2.55	60	30	10	0
	2	26	100	0	4.92	31	38	27	4
	3	44	100	0	6.48	16	41	32	11
Zambia	1	33	100	0	2.88	27	30	30	12
	2	34	93	7	3.09	26	24	32	18
	3	25	92	8	3.76	28	24	32	16

NA = not available

Table 8 **Mean deltamethrin content of PermaNet 2.0 nets by different classes of wash**

Class	Description of class	Number of nets	Mean deltamethrin content (g/kg)	95% CI
1	never washed	80	0.944	0.811–1.077
2	1–5 washes	169	0.617	0.551–0.683
3	6–10 washes	81	0.454	0.361–0.548
4	11–15 washes	47	0.419	0.302–0.536
5	>15 washes	40	0.322	0.251–0.394

Table 9 **Physical status of PermaNet 2.0 by country and age (in years) of net**

Country	Year	No. of nets	Mean no. of holes per net	Percentage of holes per size[a]			Percentage of holes per position[b]			Mean no. of open seams
				small	medium	large	lower	upper	roof	
Angola	1	10	5.6	98	2	0	67	27	7	0.2
	2	43	14.5	88	8	4	65	26	9	0.2
	3	24	10.7	81	14	5	68	28	4	0.5
Ghana	1	26	3.2	77	10	13	81	18	1	0.3
	2	34	3.3	66	20	14	74	23	3	0.1
	3	27	2.9	73	23	4	57	37	5	0.2
Kenya	1	30	13.7	50	29	21	49	44	7	0.5
Madagascar	1	30	19.7	84	12	4	80	9	10	0.2
	2	28	47.1	75	20	5	82	9	9	0.3
	3	32	54.9	80	15	4	87	7	6	0.0
Togo	1	20	2.4	91	6	2	72	23	4	0.0
	2	26	5.9	68	25	7	68	27	5	0.1
	3	44	10.8	71	22	8	78	18	4	0.1
Zambia	1	33	20.1	68	24	8	61	25	13	0.8
	2	34	34.0	69	23	9	50	30	20	1.1
	3	25	23.8	50	36	14	64	24	12	0.9

[a] small = a hole smaller than will allow a thumb to pass through; medium = a larger hole that will not allow a closed fist to pass through; large = a hole bigger than a closed fist.
[b] lower = the lower half of the net; upper = the upper half of the net; roof .

Table 9 (continued) **Physical status of PermaNet 2.0 by country and age (in years) of net**

Country	Year	Number of nets	Mean number of holes per net	Percentage of holes per size[a]			Percentage of holes per position[b]			Mean no. of open seams
				small	medium	large	lower	upper	roof	
All countries	1	149	12.4	71	20	9	66	24	10	0.4
	2	165	20.4	75	19	6	67	21	12	0.4
	3	152	20.8	73	20	7	79	15	7	0.3

[a] small = a hole smaller than will allow a thumb to pass through; medium = a larger hole that will not allow a closed fist to pass through; large = a hole bigger than a closed fist.
[b] lower = the lower half of the net; upper = the upper half of the net; roof .

Figure 1 **Mortality in WHO cone tests by country and year (plotted values are the pooled results of mortality for the four samples per PermaNet 2.0)**

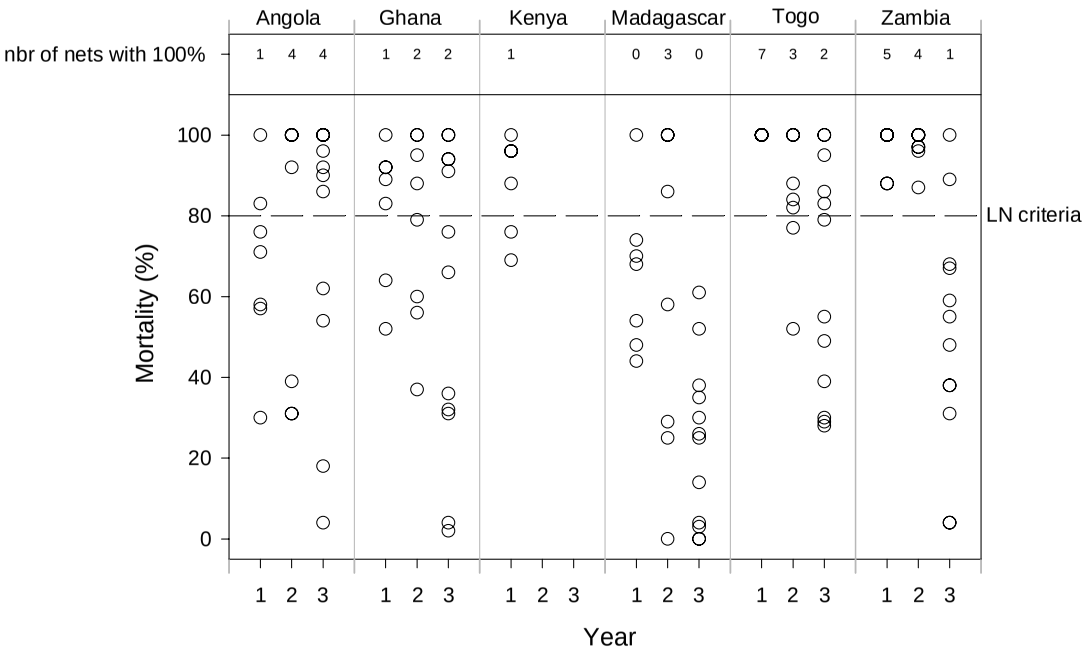


Figure 2 **Knock-down in WHO cone tests by country and year (plotted values are the pooled results of knock-down for the four samples per PermaNet 2.0)**

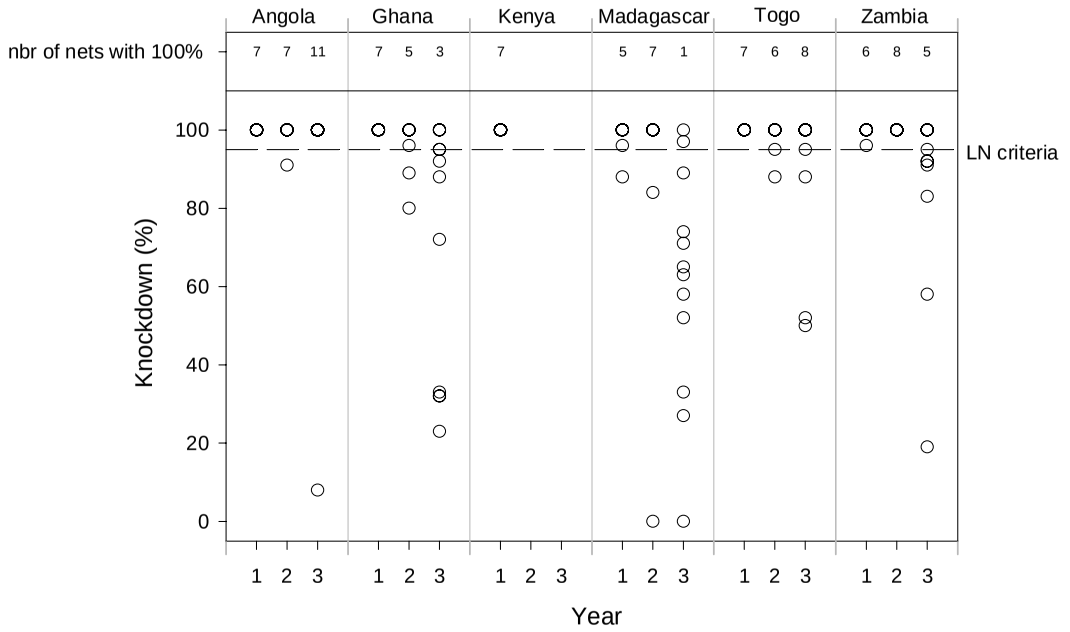


Figure 3 **Mean deltamethrin content and 95% confidence interval of PermaNet 2.0 samples, as measured by gas chromatography with flame ionization detection, per country and year**

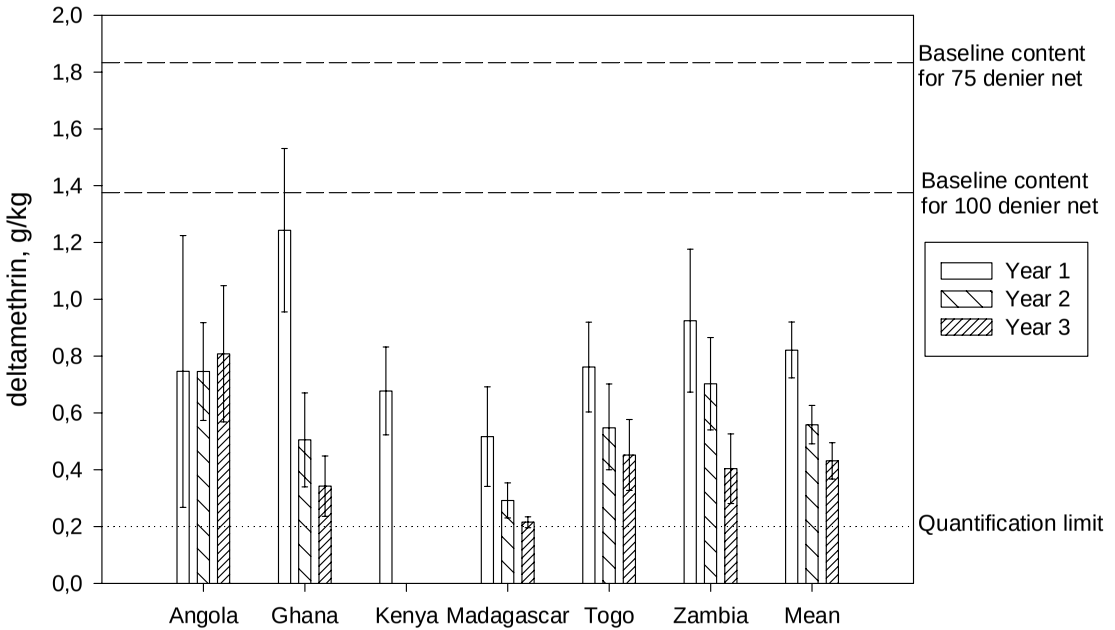
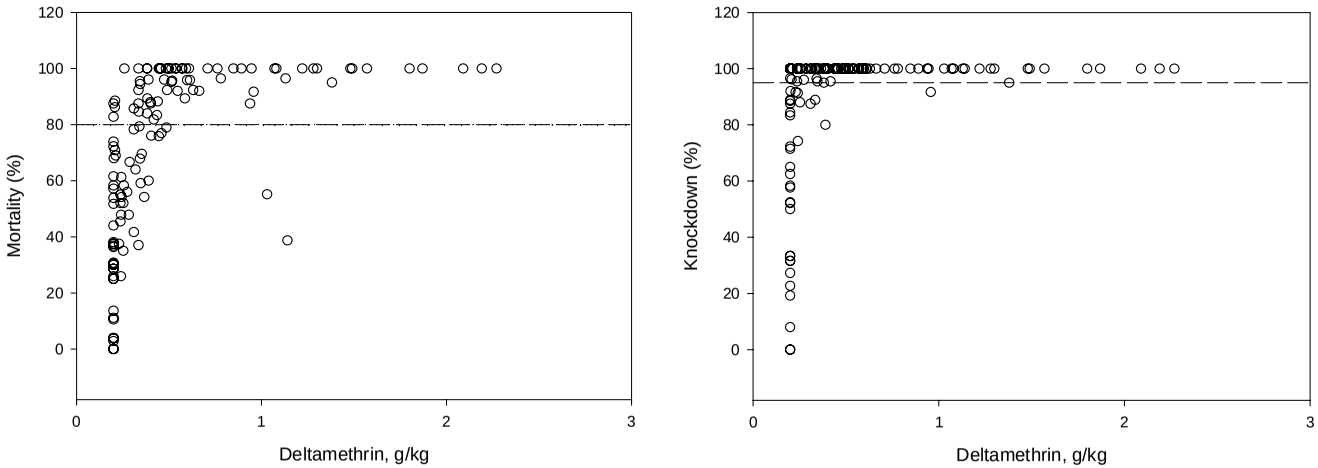


Figure 4 **Percentage mortality (A) and knock-down (B) in WHO cone tests, by deltamethrin content (samples for the chemical analysis and cone bioassays were taken on adjacent pieces of PermaNet 2.0 netting)**



4. REVIEW OF PERMANET 3.0

PermaNet 3.0 is manufactured by Vestergaard Frandsen (Switzerland). The product is a combination of different LN technologies and is designed for the control of insecticide-resistant mosquito populations. The roofing of PermaNet 3.0 utilizes deltamethrin and a synergist, piperonyl butoxide, incorporated into monofilament polyethylene yarn of 100 denier (warp-knitted fabric, with weight of 40 ± 15% g/m^2) at the target dosage of 4.0 g AI/kg and 25 g AI/kg of netting material respectively. The side panels of PermaNet 3.0 are made of multi-filament polyester fibres, treated with deltamethrin in a resin coating (75 ± 5% denier, warp-knitted fabric, atlas construction). The side netting has two parts: a strengthened lower part, so-called border (70 cm) by using 75 ± 5% denier yarn (weight 40 ± 10% g/m^2) and a side panel made of 75 ± 5% denier (weight of 30 ± 10% g/m^2). The target dosage of deltamethrin in the side panels is 2.8 g AI/kg of netting material, i.e. 115 mg AI/m^2 of the border and 85 mg AI/m^2 of the remaining of the side panels.

4.1 Safety assessment

The assessment of the risk to humans of washing and sleeping under the LN, provided by the manufacturer, was assessed by the Finnish Institute of Occupational Health (FIOH, 2007b) on behalf of the WHO Programme on Chemical Safety. The WHO *Generic risk assessment model for insecticide treatment and subsequent use of mosquito nets*[1] was used as a guiding document. The following assumptions were used by the proposer in drafting the assessment:

- the hazards of the active components, deltamethrin and piperonyl butoxide, are adequately characterized in the most recent JMPR (FAO/WHO Joint Meeting on Pesticide Residues) assessments;

[1] WHO (2004). *A generic risk assessment model for insecticide treatment and subsequent use of mosquito nets*. Geneva, World Health Organization (WHO/CDS/WHOPES/GCDPP/2004.6; available at http://whqlibdoc.who.int/hq/2004/WHO_PCS_04.1.pdf; accessed January 2009).

- for inhalation exposure, skin contact and hand-to-mouth transfer from sleeping under the treated nets, the WHO generic model and its default values are applied;
- for direct mouth contact with the net, rather than the default assumptions of the generic model (which would indicate unacceptable exposures for newborns and 15-kg infants), experimental data on the release of deltamethrin and piperonyl butoxide from the WHO washing procedure are assumed to reflect the availability for absorption of the AI in the net. Thus it is assumed that 4% (average of 10 successive washings) of the deltamethrin in the side net is translodgeable; for piperonyl butoxide (in the roof net), the equivalent average removal by washing is 1.8%;
- for the assessment of the health risks from the washing of the treated nets, it is assumed that the net is washed in 10 litres of water, rather than the 2 litres often used in WHOPES assessments to date. However, it is assumed that the transfer of the AI to the washing liquid is similar to that of the default value of the generic model, i.e. 30%, while the experimental data demonstrate that a more accurate figure would be 5–10%. As the exposure thus estimated represents <0.1% of the dermal acceptable exposure level, these minor differences have little impact on the risk estimates.

FIOH concluded that:

- the assessment of risk to health of the maintenance and use of PermaNet 3.0 by the manufacturer is generally performed in compliance with the WHO generic model;
- when the generic model assumptions or default values are not adopted, they are replaced by data derived experimentally and are appropriately justified;
- the conclusions of the safety assessment are justified;
- when the instructions for use are followed, washing or sleeping under the LN does not pose undue risk to users (adults, children or newborns).

4.2 Efficacy – background and supporting documents

PermaNet 3.0 nets were evaluated in experimental huts in three field stations in central and west Africa: in Burkina Faso against

natural Kdr-resistant populations of *An. gambiae s.s.*, in northern Cameroon against pyrethroid-resistant *An. gambiae s.l.* showing metabolic resistance and in Togo against free-flying pyrethroid-resistant *Cx. quinquefasciatus.*

The west African-type experimental huts were used in these three settings. Efficacy was evaluated in terms of blood-feeding inhibition, deterrence, induced exophily and mortality.

In each locality, six trap huts were used and six treatment arms were tested, following the WHO guidelines. Every week in Burkina Faso, northern Cameroon and Togo, after cleaning and ventilating the huts, treatment arms were rotated through the huts according to a Latin-square scheme. Sleepers were rotated randomly among huts each night of the study. Each net was deliberately holed with six holes (4 cm x 4 cm) to simulate a torn net. One additional net per treatment arm was used for chemical analyses and bioassays. Standard WHO procedures[1] for Phase II were used for washing the nets. For analysing the data of hut trials, rank tests for numeric data and logistic regression for proportional data were used.

According to WHOPES supervised Phase I experiments (Duchon et al., 2008b), no regeneration time was required after washing, so that one day interval between successive washes was applied.

The following arms were tested: (i) untreated net (same fabric as PermaNet 3.0); (ii) PermaNet 2.0 unwashed; (iii) PermaNet 2.0 washed 20 times; (iv) PermaNet 3.0 unwashed; (v) PermaNet 3.0 washed 20 times; and (vi) a conventionally treated net (CTN) of 75-denier polyester net treated with deltamethrin at 25 mg AI/m² (0.83 g AI /kg) and washed to just before exhaustion, defined as the last wash that provides mortality >80% or knock-down >95%.

The fully susceptible reference Kisumu strain of *An. gambiae s.s*. (2–5-day-old unfed females) was used for the cone bioassays.

[1] WHO (2005). *Guidelines for laboratory and field testing of long-lasting insecticidal mosquito nets*. Geneva, World Health Organization (WHO/CDS/WHOPES/GCDPP/2005.11; available at http://www.who.int/whopes/guidelines/en/; accessed January 2009).

Kou Valley, Burkina Faso

The efficacy of PermaNet 3.0 was investigated against natural Kdr-resistant populations of *An. gambiae* in experimental huts in the Kou Valley in Burkina Faso (Dabire et al., 2008b). The Kou Valley is a rice cultivation area surrounded by wooded savannah situated 30 km north of Bobo Dioulasso. Both molecular forms of *An. gambiae s.s.* occur in sympatry but the S form increases in frequency (up to 85%) at the end of the rainy season (October–November). Knock-down (*kdr*) mutation occurs in both forms but at different frequencies (>90% in the S form and at around 20% for the M form).[1,2]

All treated nets were fully effective before any washing, indicating the full bio-availability of deltamethrin regardless of treatment (knock-down and mortality 100%). The bio-efficacy of PermaNet 3.0 washed 20 times before and after the trial was very high (knock-down and mortality 100%) and satisfactory with PermaNet 2.0 (knock-down 100% and/or mortality >93%). The number of washes to just before exhaustion was two for the CTN (before field testing knock-down: 98%, mortality 90%).

The AI content for unwashed PermaNet 2.0 (deltamethrin) and 3.0 (deltamethrin + PBO) complied with the target doses (± 25%) (Tables 12–14). Overall deltamethrin retention for PermaNet 2.0 after 20 washes was 19%. Overall AI retention for PermaNet 3.0 after 20 washes was 31% and 80% for deltamethrin on the side panels and roof respectively and 74% for PBO on the roof (Pigeon 2008c).

The CTNs washed to just before exhaustion (two washes) contained 3.2 mg/m² (0.10 g/kg) deltamethrin, corresponding to a retention rate of about 15%.

During a 6-week period (October–November 2007), 908 *An. gambiae s.l.* were collected in the control huts. The unwashed PermaNet 2.0 and 3.0 induced a significant deterrent effect (64% and 49% respectively), while this was not significant for

[1] Dabiré KR et al (2008). Dynamics of multiple insecticide resistance in the malaria vector *Anopheles gambiae* in a rice growing area in South-Western Burkina Faso. *Malaria Journal*, 2008, 7:188.

[2] Pennetier C et al (2008). Mixture for controlling insecticide-resistant malaria vectors. *Emerging Infectious Disease*, 14(11):1707–1714.

the other arms. Insecticide induced exophily was significant in all treated arms (42–72%).

PermaNet 2.0 washed 20 times showed efficacy equal to CTNs washed to just before exhaustion, while PermaNet 3.0 washed 20 times showed significantly higher efficacy in terms of blood-feeding inhibition and mortality (Tables 10–11).

Blood-feeding inhibition and mortality demonstrated the better efficacy of PermaNet 3.0 over PermaNet 2.0 when comparing unwashed nets or nets washed 20 times (Tables 10–11). No adverse effects were reported during the study.

Pitoa, northern Cameroon

The efficacy of PermaNet 3.0 against natural populations of *An. gambiae* was studied in experimental huts in northern Cameroon (Etang et al., 2008). The study site is located in a Soudanian area where *An. gambiae s.l*. (95% *An. arabiensis*, 5% *An. gambiae* S form) and *An. funestus* are the major malaria vectors. Insecticide susceptibility tests performed during the field trial showed that *An. gambiae s.l.* is moderately resistant to permethrin 0.75% (84% mortality) and deltamethrin 0.05% (70% mortality) but not to DDT 4% (93% mortality). Increased oxidase and esterase activities have been observed in both *An. gambiae* and *An. Arabiensis*.[1,2] The *kdr* mutation is very low (<5%) and only present in *An. gambiae s.s.*

All treated nets were fully effective before any washing, indicating the full bio-availability of deltamethrin regardless of treatment (knock-down and mortality 100%). After 20 washes of PermaNet 3.0 and 2.0, KD and mortality remain above the threshold for both indicators (KD>95%, mortality >80%) and this before and after the trial. The number of washes to just before exhaustion was three for the CTN (before field testing KD: 92%, mortality 83%).

[1] Etang J et al (2007). Spectrum of metabolic-based resistance to DDT and pyrethroids in *Anopheles gambiae* s.l. populations from Cameroon. *Journal of Vector Ecology*, 32 (1):123–133.

[2] Muller P et al (2008). Pyrethroid tolerance is associated with elevated expression of antioxidants and agricultural practice in *Anopheles arabiensis* sampled from an area of cotton fields in Northern Cameroon. *Molecular Ecology*, 17(4):1145–1155.

The AI content for unwashed PermaNet 3.0 (deltamethrin + PBO) complied with the target doses (± 25%) (Tables 12-14). However for unwashed PermaNet 2.0, deltamethrin content (2.61 g/kg on average) was higher (+ 45%) than the target dose (1.8 g/kg). Overall deltamethrin retention for PermaNet 2.0 after 20 washes was 25%. Overall AI retention for PermaNet 3.0 after 20 washes was 13% and 74% for deltamethrin on the side panels and roof respectively and 92% for PBO on the roof (Pigeon 2008f). The CTNs washed to just before exhaustion (3 washes) contained 2.7 mg/m² (0.08 g/kg) deltamethrin, corresponding to a retention rate of about 10%.

The results of experimental huts are reported for *Anopheles* without distinction of species (Etang et al., 2008).

During a 6-week period (14 July to 23 August 2008), 401 *Anopheles* (11.1 per night) were collected in the control huts. The PermaNet 2.0 washed 20 times and the CTN washed to before exhaustion did not reduce significantly the entry of *Anopheles*. Induced exophily (24–40%) increased significantly in all treated arms, but no difference was observed between these arms.

The efficacy of PermaNet 2.0 and PermaNet 3.0 washed 20 times on blood-feeding inhibition and mortality was equal to or higher than for a CTN washed to before exhaustion.

PermaNet 3.0 induced a higher mortality than PermaNet 2.0, compared with both washed and unwashed nets. PermaNet 2.0 induced a higher blood-feeding inhibition, although this was only significant for unwashed nets (Tables 10–11). No adverse effects were reported during the study.

Akodésséwa, Lomé, Togo

The efficacy of PermaNet 3.0 was investigated against wild populations of resistant *Cx. quinquefasciatus* in experimental huts in Togo (Ketoh et al., 2008). These huts are located in Akodésséwa, an urban district of Lomé where *Cx. quinquefasciatus* is abundant. A high level of resistance to DDT 4% (mortality 7%), carbosulfan 0.4% (mortality 11%), permethrin 1% (mortality 10%), deltamethrin 0.05% (mortality 6%) and, to a lesser extent, to organophosphates (chlorpyrifos-methyl 0.4%; mortality 50%) was observed by WHO susceptibility tests performed in March 2008.

All treated nets were fully effective before any washing (knock-down and mortality 100%), indicating the full bio-availability of deltamethrin regardless of treatment. The bioefficacy of PermaNet 3.0 washed 20 times before and after the trial was maximum (knock-down and mortality 100%). The mortality with PermaNet 2.0 washed 20 times was >80%, while knock-down was just below the cut off point (94%). The number of washes to just before exhaustion was three for the CTN (before field testing KD: 98%, mortality 86%).

The AI content for unwashed PermaNet 2.0 (deltamethrin) and PermaNet 3.0 (deltamethrin + PBO) complied with the target doses (± 25%) (Pigeon 2008e). Overall deltamethrin retention for PermaNet 2.0 after 20 washes was 16%. Overall AI retention for PermaNet 3.0 after 20 washes was 23% and 85% for deltamethrin on the side panels and roof respectively and 58% for PBO on the roof (Tables 12–14).

The CTNs washed to just before exhaustion (three washes) contained 4.6 mg/m² (0.14 g/kg), deltamethrin corresponding to a retention rate of about 19%.

During a 6-week period corresponding to one full Latin square (18 February to 29 March 2008), 190 *Cx. quinquefasciatus* (5.3 per night) were collected in the control arm. No deterrent effect was observed among the treated arms. Only LN arms induced a significant higher exophily than the control (22–50%). CTNs washed to just before exhaustion did not induce more blood-feeding inhibition and mortality than the untreated nets, confirming the high level of pyrethroid resistance in *Cx. quinquefasciatus*. Blood-feeding inhibition induced by both PermaNet (before and after washing) was higher compared with the CTN arm. Moreover, PermaNet 3.0 induced significantly higher blood-feeding inhibition (69%) than PermaNet 2.0 washed and unwashed. However, both PermaNet 2.0 and PermaNet 3.0 did not induce significantly higher mortality than the untreated nets. No adverse effects were reported during the study (Tables 10–11).

4.3 Efficacy – WHOPES supervised trials

4.3.1 Laboratory studies

The regeneration time, wash resistance and efficacy of PermaNet 3.0 provided by the manufacturer, were determined in laboratory (Phase I) studies according to WHOPES guidelines [1] against susceptible and pyrethroid-resistant An*. gambiae s.s.* (Duchon et al., 2008b).

Chemical analyses were performed on net samples washed 0, 1, 3, 5, 10, 15, 18, 20 and 25 times (Pigeon 2008d). Per wash regimen, 12 pieces from 4 nets taken on each part on the net (upper sides, lower border and roof) were analysed for determination of deltamethrin and piperonyl butoxide content. The analytical method used was based on CIPAC methods and involved extraction by heating under reflux with xylene and chromatographic determination by GC-FID using the internal standard calibration.

Results of analysis for AI content and retention (wash curve) are presented in Figures 5–6. The between-net variation is expressed as the relative standard deviation (RSD) of the content found on the four pieces. Retention is calculated according to Annex 1 of the Report of the eleventh WHOPES Working Group Meeting, [2] assuming a free migration stage behaviour.

The deltamethrin content in the sides (upper sides = 2.82 g/kg and strengthened border = 3.27 g/kg) and in the roof (4.66 g/kg) of the unwashed net complied with the target dose of 2.8 g/kg (± 25%) and 4 g/kg (± 25%) respectively. The piperonyl butoxide content (26.8 g/kg) in the roof of the unwashed net complied with the target dose of 25 g/kg (± 25%). For the

[1] WHO (2005). *Guidelines for laboratory and field testing of long-lasting insecticidal mosquito nets*. Geneva, World Health Organization (WHO/CDS/WHOPES/GCDPP/2005.11; available at: http://www.who.int/whopes/guidelines/en/; accessed January 2009).
[2] WHO (2008). *Report of the eleventh WHOPES Working Group Meeting, WHO/HQ, Geneva 10–13 December 2007* (WHO/HTM/NTD/WHOPES/2008.1; available at http://www.who.int/whopes/recommendations/wgm/en/; accessed January 2009).

unwashed net, the between-net RSD ranged from 3.4% to 5.6% for deltamethrin and was 3.8% for piperonyl butoxide.

After 20 washes, for the sides of PermaNet 3.0, the average deltamethrin content was 1.30 g/kg (upper sides) and 1.57 g/kg (strengthened border). The overall deltamethrin retention after 20 washes was 46% (upper sides) and 48% (strengthened border), corresponding to an average retention per wash of 93% and 94% respectively.

For the roof of PermaNet 3.0, the average AI content after 20 washes was 4.17 g/kg for deltamethrin and 20.4 g/kg for piperonyl butoxide. The overall retention after 20 washes was 90% for deltamethrin and 76% for piperonyl butoxide, corresponding to an average retention per wash of 99% and 96% respectively.

After three consecutive washes, the regeneration-time study carried out on susceptible *An. gambiae* Kisumu strain showed that biological activity was maximal (knock-down 100%, mortality 100%) after 1 day for the three different parts (side, border, roof) of the net. This means that no regeneration time is needed after washing.

Applying the same method on the roof material using the pyrethroid resistant *An. gambiae* strain (VKPR), 5 days were required to reach the plateau of knock-down. However, mortality sharply decreased after three consecutive washes (3%) and no regain of efficacy occurred after 15 days of storage at 30 °C.

The wash-resistance study performed on fully susceptible mosquitoes showed that the efficacy of all parts of PermaNet fulfil the WHO requirements (mortality >80% and/or knock-down >95%) after 20 washes.

However, despite a higher concentration of deltamethrin and the presence of PBO, the roof caused after 10 washes lower mortality (68%) on susceptible *An. gambiae* than the side panels (97%).

Unwashed roof samples treated with PBO only (average 20.16 mg/kg) have the capacity to kill 100% of the susceptible

mosquitoes. However, this killing effect was lost after washing (after 10 washes: 2% mortality, 3% knock-down).

Roof samples were also bio-assayed using a *kdr* homozygote strain of *An. gambiae* s.s. (strain VKPER). Despite the combination of a high dose of deltamethrin with the PBO, the efficacy of the roof samples against kdr-resistant mosquitoes was much lower than that recorded with fully susceptible vectors. Mortality fell to 57% after 1 wash and knock-down of 89% after five washes. It should be noted that besides the target kdr resistance, no other biochemical resistance mechanism was described for the pyrethroid-resistant colony strain VKPER.

4.3.2 Experimental hut studies

Three field studies were implemented under WHOPES supervision: one in the Mekong Delta (Viet Nam) on a pyrethroid-resistant *An. epiroticus* population (Chinh et al., 2008), one in Muheza district (United Republic of Tanzania) on a susceptible *An. gambiae s.s.* population (Tungu et al., 2008), and one in Malanville (Benin) on a partially pyrethroid-susceptible *An. gambiae s.l.* population (Chabi et al., 2008). Experimental hut trials included also efficacy studies on wild *Culex* populations in Viet Nam and the United Republic of Tanzania.

In Benin and the United Republic of Tanzania, the west and east African experimental huts respectively were used to evaluate the efficacy of PermaNet 3.0 in terms of blood-feeding inhibition, deterrence, induced exophily and mortality.[1]

In Viet Nam, an adapted version of the west African experimental huts was used where mosquitoes can only escape outside to a single veranda trap. The entry side of the experimental huts faces a large brackish water swamp. Two entry slits (0.75 m) are foreseen at each side of the door and one large slit above the door over the entire width of the front

[1] WHO (2005). *Guidelines for laboratory and field testing of long-lasting insecticidal mosquito nets*. Geneva, World Health Organization (WHO/CDS/WHOPES/GCDPP/2005.11; available at: http://www.who.int/whopes/guidelines/en/; accessed January 2009).

side (3 m). These entry traps are covered by a curtain from 05:00 to 18:00. The back side of the house is foreseen with a full screened veranda. The houses are built on a concrete floor and have a wooden structure. The walls are covered outside with nipa leaves and inside with plastic hessian sheeting. The roof is of corrugated iron covered outside with palm tree leaves and inside with the plastic sheeting.

Recapture rates for mosquitoes artificially released into these huts was 88%, indicating a satisfactorily low escape rate. The huts were free of scavengers (95% of the dead released mosquitoes were recaptured). Before the trial, no significant difference was observed between the entry rates of the different houses.

In each locality, six trap huts were used and six treatment arms were tested, following the WHO guidelines. Each week after cleaning and ventilating the huts, treatment arms were rotated through the huts according to a Latin square scheme. Sleepers were rotated randomly among huts each night of the study. Each net was deliberately holed with six holes (4 cm x 4 cm) to simulate a torn net. One additional net per treatment arm was used for chemical analyses and bioassays. Standard WHO procedures[1] for Phase II were used for washing the nets. For analysing the data of hut trials rank tests (Benin, United Republic of Tanzania) or binomial negative regression (Viet Nam) for numeric data and logistic regression for proportional data were used.

According to WHOPES supervised Phase I experiments (Duchon et al., 2008b) no regeneration time was required after washing, so that one day interval between successive washes was applied.

In each locality, six trap huts were used and six treatment arms were tested, following the above-mentioned WHO guidelines.

The following arms were tested: (i) untreated polyester net; (ii) PermaNet 2.0 unwashed; (iii) PermaNet 2.0 washed 20 times;

[1] WHO (2005). *Guidelines for laboratory and field testing of long-lasting insecticidal mosquito nets*. Geneva, World Health Organization (WHO/CDS/WHOPES/GCDPP/2005.11; available at: http://www.who.int/whopes/guidelines/en/; accessed January 2009).

(iv) PermaNet 3.0 unwashed; (v) PermaNet 3.0 washed 20 times; and (vi) polyester net conventionally treated with deltamethrin at 25 mg/m², and washed just before exhaustion defined as the last wash that provide mortality >80% or knock-down >95%. Cone bioassays were carried out using pyrethroid-susceptible colony strains of *An. gambiae* (Kisumu strain) in Benin and the United Republic of Tanzania and of *An. dirus s.s*. in Viet Nam.

Muheza District, United Republic of Tanzania

In the study area, the wild *An. gambiae s.s* (S form) was fully susceptible and the *Cx. quinquefasciatus* population was highly resistant to deltamethrin (Tungu et al., 2008). Mortalities in WHO susceptibility tests were respectively 100% and 52% when exposed to deltamethrin discriminative dose of 0.05%. Earlier work on *Culex* in Meheza suggests the involvement of oxidases (Khayrandish & Wood 1993).[1] Recent work with synergists indicates additional involvement of esterases and a non-metabolic mechanisms in pyrethroid resistance (Rowland, unpublished).

Before washing, all treatment arms showed 100% knock-down and mortality, indicating the full bio-availability of deltamethrin regardless of treatment. Bioassay tests on the 20 washed PermaNet 2.0. and 3.0 before and after the trial fulfilled the WHO requirements in terms of knock-down and mortality. The number of washes to just before exhaustion was three for the CTN (before testing knock-down: 100%, mortality 100%). At the end of the trial, knock-down and mortality decreased to 88% and 90% respectively.

The AI content for unwashed PermaNet 2.0 (deltamethrin) and PermaNet 3.0 (deltamethrin + PBO) complied with the target doses (± 25%) (Tables 12–14). Overall deltamethrin retention for PermaNet 2.0 after 20 washes was 32%. Overall AI retention for PermaNet 3.0 after 20 washes was 47% and 80% for deltamethrin on the side panels and roof respectively and 80% for PBO on the roof. The conventionally treated nets

[1] Khayandish A, Wood RJ (1993) A multiple basis for insecticide resistance in a strain of *Culex quinquefasciatus* from Muheza Tanzania, studied as resistance declined. *Bulletin of Entomological Research*, 83:75–86.

washed to just before exhaustion (three washes) contained after the trial 3.8 mg/m² (0.11 g/kg) deltamethrin (Pigeon 2008g).

During a 9-week period (7 July to 4 October 2008), 723 *An. gambiae* (13.4 per night) and 81 *Culex* (1.5 per night) were collected in the control huts. No significant deterrent effect was induced by any of the treatments for either species.

For *Culex*, induced exophily (58–74%) was significant for all treatment arms. For *An. gambiae* owing to the high natural exiting rate (86%) no significant induced exophily was observed in the treated arms except for the CTN (7%).

The mortality of susceptible wild *An. gambiae* with unwashed PermaNet 2.0 (96%) and unwashed PermaNet 3.0 (96%), or with these LN both washed 20 times (87% or 95% respectively) exceeded that of the CTN washed to just before exhaustion (73%)(Table 11). However PermaNet 2.0 and PermaNet 3.0 washed 20 times did not induce significantly higher blood-feeding inhibition than a CTN washed to just before exhaustion (Table 10).

For *Cx. quinquefasciatus*, blood-feeding inhibition was very high (>70%) in all treatment arms compared to control and reached 100% with both PermaNets washed 20 times. Both PermaNet 2.0 and PermaNet 3.0 performed equal to or better than the CTN just before exhaustion in terms of blood-feeding inhibition and mortality. Mortality was higher with PermaNet 3.0 (51%) than with PermaNet 2.0 (36%) when unwashed but this difference was no longer evident after 20 washes (about 35% mortality) (Table 11).

Additional cone bioassays tests and tunnel tests using also a pyrethroid-resistant *Cx. quinquefasciatus* strain (Masimbani strain resistant to permethrin) produced trends consistent with the experimental hut trial. A higher mortality rate was observed with the roof panel over the side panel against pyrethroid resistant *Culex* (Masimbani strain) but this effect was lost after washing.

Bac Lieu, Viet Nam

In this study area, *An. epiroticus* (formally *An. sundaicus*) is the dominant mosquito species (Chinh et al., 2008).

An. epiroticus was found to be resistant to permethrin, deltamethrin, alpha-cypermethrin and lambda-cyhalothrin but fully susceptible to DDT[1]. No kdr mutation has been observed for this species and biochemical assays suggest an esterase mediated pyrethroid detoxification (Verhaeghen *et al.*, 2008, unpublished report). Tests performed in 2005 shows also a full susceptibility against propoxur and malathion. The wild population was retested in October 2008 and resistance to deltamethrin was reconfirmed (deltamethrin 0.05%: mortality 75%). The *Culex* population has not been tested for insecticide susceptibility.

Before any wash, all treated nets were effective (knock-down >95% and mortality 100%) indicating the bio-availability of deltamethrin regardless to the treatments. Resistance to 20 washes of both PermaNet 2.0. and 3.0 was within the WHO requirements (knock-down >95% and/or mortality >100%) before and after the trial.

The number of washes to just before exhaustion was five for the CTN (knock-down 94%, mortality 90%). At the end of the trial, knock-down and mortality decreased to 38% and 24% respectively.

The AI content for unwashed PermaNet 3.0 (deltamethrin + PBO) and PermaNet 2.0 (deltamethrin) complied with the target doses (± 25%) (Pigeon 2009b). However for one unwashed net of PermaNet 3.0, the average deltamethrin content in the sides panel (2.05 g/kg) was just below the lower limit (2.1 g/kg) of the target dose. For this same unwashed PermaNet 3.0, the within-side RSD was very high. The overall AI retention for PermaNet 3.0 after 20 washes was 55% and 100% for deltamethrin on the side panels and roof respectively and 72% for piperonyl butoxide on the roof. The overall deltamethrin retention for PermaNet 2.0 after 20 washes was 50%.

The within-side RSD of deltamethrin content was 4.4–10.4% and 1.6% for the side panels and roof respectively in one unwashed PermaNet 3.0, and 29.8–42.4% and 1.1% for the side panels and roof respectively for another unwashed PermaNet 3.0. The within-side and roof RSD of deltamethrin

[1] Van Bortel W et al. (2008). The insecticide resistance status of malaria vectors in the Mekong region. *Malaria Journal*, 7:102.

content in unwashed PermaNet 2.0 was 0.7-6.2%. The within-net RSD of deltamethrin content in the side panels was 1.9-12.0% and 2.1-4.3% respectively for unwashed PermaNet 3.0 and 2.0.

The conventionally treated net washed to just before exhaustion (5 washes) contained 5.5 mg/m² (0.17 g/kg) deltamethrin corresponding to a retention rate of 22%.

During a 6-week period (22 September to 2 November 2008), 4,114 *An. epiroticus* (114.3 per night) and 1,141 *Culex* (31.7 per night) were collected in the control huts.

For *An. epiroticus* only, PermaNet 3.0 washed 20 times induced a significant deterrent effect (35%) while only PermaNet 2.0 unwashed deterred *Culex* mosquitoes (50%). All treatments increased the exophily of *Culex* where no clear trend was observed with *An. epiroticus.*

All PermaNet arms are performing almost equally to or slightly better than CTNs washed to just before exhaustion, and we can conclude that PermaNet 3.0 fulfils the criteria of an LN (Tables 10–11).

For *Anopheles epiroticus,* PermaNet 3.0 washed 20 times did not induce a significantly higher blood-feeding inhibition than PermaNet 2.0 washed 20 times (73% versus 68%) (Table 10). The significant difference in proportion of blood fed mosquitoes between PermaNet 2.0 and PermaNet 3.0 unwashed was biologically not relevant (6.6% and 4.6% respectively).

A high *Anopheles* mortality was observed in the control hut (34%) which was mainly attributable to the unfed females (mortality of 42%) while the mortality of fed females was around 10%. The mortality with PermaNet 2.0 and PermaNet 3.0, unwashed or washed 20 times, exceeded that of the CTN washed before exhaustion (70%). The overall mortality with PermaNet 3.0 washed 20 times was significantly higher than with PermaNet 2.0 washed 20 times (92% and 82% respectively), but this difference disappears when considering only blood-fed females (94% and 92% respectively) (Table 11).

The blood-feeding rate in *Culex* was significantly reduced in the PermaNet treated arms (1.6–4.8%) compared with the

untreated nets (15%) and CTNs washed to just before exhaustion (9%) (Table 10). For *Culex* mosquitoes, only immediate mortality (1.2% in the control arm) was observed. The mortality for Culex in all treated arms was rather low (<20%). The mortality was not different between the two PermaNet washed 20 times and the CTN washed to just before exhaustion (about 7%) (Table 11).

Malanville, Benin

In this irrigated area *An. gambiae s.l.* is the main malaria vector with 95% *An. gambiae* s.s. (M form) and 5% *An. arabiensis*. Both species when exposed to discriminative doses are almost susceptible to permethrin 0.75% (mortality 93%), deltamethrin 0.05% (mortality 85%) and lamda-cyhalothrin 0.05% (mortality 92%).

Before any washing, all treated nets were fully effective indicating the full bio-availability of deltamethrin regardless of the treatments (knock-down and mortality 100%) (Chabi et al., 2008). Resistance to 20 washes of both PermaNet 2.0. and 3.0 met the WHO requirements before and after the trial. The number of washes to just before exhaustion was three for the CTN (before field testing knock-down: 95%, mortality 82%). At the end of the trial, knock-down and mortality were 85% and 82% respectively.

The AI content for unwashed PermaNet 3.0 (deltamethrin + PBO) and PermaNet 2.0 (deltamethrin) complied with the target doses (± 25%) (Pigeon 2009c). However for one unwashed net of PermaNet 2.0, the average deltamethrin content in the sides panel and roof was higher (2.41 and 2.87 g/kg respectively) than the upper limit of the target dose (2.25 g/kg).

The overall AI retention for PermaNet 3.0 after 20 washes was 26% and 91% for deltamethrin on the side panels and roof respectively and 70% for piperonyl butoxide on the roof. The overall deltamethrin retention for PermaNet 2.0 after 20 washes was 23%.

The within-net RSD of deltamethrin content in the side panels is 4.2-7.3% and 1.1-7.3% for unwashed PermaNet 3.0 and 2.0 respectively.

The conventionally treated net washed to just before exhaustion contains 2.0 mg/m² (0.06 g/kg) deltamethrin corresponding to a retention rate of 11%.

During a 12-week period (21 July to 11 October, 72 nights) corresponding to two full Latin square schemes, 285 *An. gambiae s.l.* (3.9 per night) were collected in the control huts. No deterrent effect was observed with neither of the treatments, however all treatments induced significantly higher exophily (23–57%).

This study demonstrated that the global efficacy (blood-feeding inhibition and mortality) of 20 times washed PermaNet 2.0 and 3.0 was equal to or higher than that of a CTN washed to just before exhaustion (Tables 10–11).

The blood-feeding inhibition of PermaNet 3.0 washed 20 times was significantly lower (66%) than for PermaNet 2.0 unwashed or washed 20 times (respectively 90% and 84%). For 20 times washed nets, mortality (69%) was similar between PermaNet 3.0 and 2.0.

4.4 Conclusions and recommendations

PermaNet 3.0, an LN manufactured by Vestergaard Frandsen (Switzerland), is intended to control pyrethroid-resistant mosquitoes. The net is a combination of two fabrics: the roof with a knitted 100 denier monofilament polyethylene fibre blended with deltamethrin 4 g/kg + piperonyl butoxide (PBO) 25 g/kg and side panels with knitted multifilament polyester (75 denier) fibres coated with deltamethrin at the target dose of 2.8 g/kg. The fabric of the lower part of the side panels is more densely knitted (40± 10% g/m²) than the upper part (30± 10% g/m²).

The WHO assessment of the manufacturer's compliance with the assessment of exposure to and risks of washing and sleeping under a PermaNet 3.0 was in line with the WHO generic risk assessment model and their conclusions were accepted.

Laboratory studies on fully susceptible mosquitoes revealed that PermaNet 3 meets WHOPES Phase I requirements

(mortality ≥80% or knock-down ≥95%) after 20 washes for each of the three parts of the net (side, border, roof). No regeneration time is needed after washing any of the three different parts of the net. Despite a higher concentration of deltamethrin and the presence of PBO, the roof after washing induces lower mortalities than the side panels.

The unwashed roof panel is fully effective against a homozygte kdr resistant strain of *An.gambiae*. However biological activity (mortality and knock-down) declines after only a few washes.

Chemical analyses of unwashed PermaNet 3.0 in Phase I testing showed a compliance of the AI and synergist content with the target doses and a good between-net homogeneity. The deltamethrin retention after washing was much higher in the roof than in the sides. There was no difference observed between upper and lower part of the sides (Figures 5 & 6). The PBO retention after washing was slightly lower than that of the deltamethrin in the roof. PBO retention increases with the number of washes and after 15 washes no release of PBO seems to occur.

In all the Phase II trials excepted for some samples, the AI and synergist content in unwashed PermaNet 3.0 complied with the target doses (Tables 12-14), PermaNet 3.0 showed also a good within-net homogeneity. The deltamethrin and PBO retention in the roof was around 2.5 times higher than that of deltamethrin in the sides panels.

Field studies demonstrated a better or equal impact of PermaNet 3.0 LNs washed 20 times on mortality and blood-feeding inhibition of prominent malaria vectors compared with that of the conventionally treated polyester nets (25 mg/m² AI) washed until just before exhaustion. This confirms that the PermaNet 3.0 fulfils the WHOPES efficacy criteria of Phase II studies for LN.

In most studies, an unwashed PermaNet 3.0 performed better than an unwashed PermaNet 2.0 on *Anopheles* populations. After washing 20 times, PermaNet 3.0 performed better for both mortality and blood-feeding inhibition only in Burkina Faso compared to a PermaNet 2.0 but still 50% of the resistant anopheles survived after the exposure to PermaNet 3.0. In the

other four sites (Viet Nam, Benin, Cameroon, United Republic of Tanzania) little or no additional benefit over PermaNet 2.0 was observed (Tables 10 and 11).

PermaNet 3.0 washed or unwashed did not kill more resistant *Culex* mosquitoes than PermaNet 2.0 and the mortality rates were low. In Togo, blood-feeding inhibition with PermaNet 3.0 (washed and unwashed) was higher than that induced by Permanent 2.0 (Table 11).

Considering the safety, efficacy and wash-resistance of PermaNet 3.0 in laboratory studies and small-scale field studies, its is recommended:

- that a time limited interim recommendation be given for the use of PermaNet 3.0 in the control and prevention of malaria;

- that WHOPES coordinates large-scale studies (WHOPES Phase III studies) of PermaNet 3.0 to confirm its long-lasting efficacy, fabric integrity and community acceptability as a requirement for developing full recommendations on the use of the product.

Following a review of the available evidence, the meeting concluded

- that the PermaNet 3.0 cannot be considered as a tool to control mosquito populations resistant to pyrethroids or to prevent the spread of pyrethroid resistance. However, the meeting commended the manufacturer for its initiative in developing tools to control pyrethroid-resistant mosquitoes and encourages it to conduct further research and development in this area.

Note: WHO recommendations on the use of pesticides in public health are valid ONLY if linked to WHO specifications for their quality control.[1]

[1] WHO specifications for pesticides used in public health are available at http://www.who.int/whopes/quality/newspecif/en/; accessed January 2009.

Table 10 **Overview of the blood-feeding (%) and blood-feeding inhibition (% shown in bold type) induced by PermaNet 2.0 and PermaNet 3.0 compared with conventionally-treated nets (CTN) washed to just before exhaustion (values in the same row sharing the same superscript letter do not differ significantly (P>0.05))**

Study sites (number of and mosquito species collected in control hut)	Status of pyrethroid resistance	Untreated net	PermaNet 2.0 unwashed	PermaNet 3.0 unwashed	CTN washed to just before exhaustion	PermaNet 2.0 washed 20 times	PermaNet 3.0 washed 20 times
Anopheles sp.							
Malanville, Benin	Susceptible	37.5^{a}	3.7^{e}	0.4^{d}	$10.4^{b,c}$	$6.1^{b,e}$	12.6^{c}
(285 *An. gambiae s.s.*)			**90.1**	**98.7**	**72.2**	**83.6**	**65.7**
Muheza, UR Tanzania	Susceptible	27.9^{a}	10.3^{c}	2.6^{b}	10.5^{c}	9.2^{c}	10.4^{c}
(723 *An.gambiae s.l.*)			**63.2**	**90.7**	**62.3**	**67.0**	**62.8**
Pitoa, northern Cameroon	Oxidase+ esterase	52.1^{a}	$15.2^{e,f}$	$28.1^{b,d}$	$28.7^{c,d}$	$20.3^{b,f}$	$24.5^{b,c,f}$
(401 *An.gambiae s.l.*)			**70.8**	**46.1**	**45.0**	**61.0**	**52.9**
Kou Valley, Burkina Faso	*kdr* mutation	75.7^{a}	35.3^{c}	20.7^{b}	49.4^{d}	48.5^{d}	36.3^{c}
(908 *An.gambiae s.l.)*			**53.4**	**72.6**	**34.7**	**35.9**	**52.0**
Bac Lieu, Viet Nam	Esterase	24.3^{a}	6.6^{b}	4.6^{c}	8.7^{d}	$7.8^{b,d}$	6.7^{b}
(4114 *An.epiroticus*)			**72.7**	**81.2**	**64.0**	**68.0**	**72.6**
Culex sp.							
Muheza, UR Tanzania)	Oxidase -esterase-*kdr*	50.6^{a}	5.8^{b}	5.7^{b}	14.7^{b}	0.0^{c}	0.0^{c}
(81 *C. quinquefasciatus)*			**88.6**	**88.7**	**70.9**	**100.0**	**100.0**
Bac Lieu, Viet Nam	Not available	15.3^{a}	2.7^{b}	1.8^{b}	9.0^{d}	4.8^{c}	1.6^{b}
(1141 *Culex)*			**82.7**	**88.4**	**41.6**	**69.0**	**89.8**

Table 10 (continued) **Overview of the blood-feeding (%) and blood-feeding inhibition (% shown in bold type) induced by PermaNet 2.0 and PermaNet 3.0 compared with conventionally-treated nets (CTN) washed to just before exhaustion (values in the same row sharing the same superscript letter do not differ significantly (P>0.05))**

Study sites (number of and mosquito species collected in control hut)	Status of pyrethroid resistance	Untreated net	PermaNet 2.0 unwashed	PermaNet 3.0 unwashed	CTN washed to just before exhaustion	PermaNet 2.0 washed 20 times	PermaNet 3.0 washed 20 times
Akodésséwa, Togo	Resistant	45.8[a]	24.9[b]	13.3[c]	38.9[a]	28.2[b]	15.1[c]
(190 *C. quinquefasciatus)*			**45.7**	**71.0**	**–**	**38.4**	**67.1**

Table 11 **Overview of mortality (%) and corrected mortality (% shown in bold type) induced by PermaNet 2.0 and PermaNet 3.0 compared with conventionally-treated nets (CTN) washed to just before exhaustion (values in the same row sharing the same superscript letter do not differ significantly (P>0.05))**

Study sites (number of and mosquito species collected in control hut)	Status of pyrethroid resistance	Untreated Net	PermaNet 2.0 unwashed	PermaNet 3.0 unwashed	CTN washed to just before exhaustion	PermaNet 2.0 washed 20 times	PermaNet 3.0 washed 20 times
Anopheles sp.							
Malanville, Benin	Susceptible	4.2^{a}	88.8^{e}	96.7^{f}	61.2^{d}	$70.7^{b,c}$	70.0^{b}
(285 *An. gambiae s.s.*)			**88.4**	**96.5**	**59.5**	**69.4**	**68.6**
Muheza, UR Tanzania	Susceptible	14.9^{a}	95.5b	95.8^{b}	73.4^{e}	87.0^{d}	95.2^{b}
(723 *An.gambiae s.l.*)			**94.7**	**95.0**	**68.7**	**84.8**	**94.3**
Pitoa, northern Cameroon	Oxidase+ esterase	13.0^{a}	82.9^{c}	93.8^{e}	41.9^{d}	56.5^{b}	77.9^{c}
(401 *An.gambiae s.l.*)			**80.3**	**92.9**	**33.2**	**50.0**	**74.6**
Kou Valley, Burkina Faso	*kdr* mutation	4.9a	44.4^{c}	78.2^{b}	28.6^{d}	30.2^{d}	49.3^{c}
(908 *An.gambiae s.l.)*			**41.5**	**77.1**	**25.0**	**26.7**	**46.7**
Bac Lieu, Viet Nam	Esterase	34.1^{a}	92.2^{b}	96.4^{c}	69.7^{e}	82.3^{d}	91.6^{b}
(4114 *An.epiroticus*)			**88.2**	**94.5**	**53.9**	**73.1**	**87.2**
Bac Lieu, Viet Nam		10.1^{a}	89.9^{b}	94.7^{b}	81.2^{c}	93.6^{b}	92.0^{b}
(**blood fed** *An.epiroticus*			88.8	94.1	**79.1**	**92.8**	**91.2**
Culex sp.							
Muheza, UR Tanzania)	Oxidase-esterase-*kdr*	6.2^{a}	36.5^{c}	51.4^{b}	$38.2^{b,c}$	34.5^{c}	36.5^{c}
(81 *C. quinquefasciatus)*			**32.4**	**48.2**	**34.2**	**30.2**	**32.3**

Table 11 (continued) **Overview of mortality (%) and corrected mortality (% shown in bold type) induced by PermaNet 2.0 and PermaNet 3.0 compared with conventionally-treated nets washed to just before exhaustion (values in the same row sharing the same superscript letter do not differ significantly (P>0.05))**

Study sites (number of and mosquito species collected in control hut)	Status of pyrethroid resistance	Untreated Net	PermaNet 2.0 unwashed	PermaNet 3.0 unwashed	ITN washed to before exhaustion	PermaNet 2.0 washed × 20	PermaNet 3.0 washed × 20
Bac Lieu, Viet Nam	Not available	1.2[a]	18.6[b]	11.1[c]	7.7[d]	9.1[c,d]	8.0[d]
(1141 *Culex)*			**17.5**	**10.0**	**6.6**	**7.9**	**6.9**
Akodésséwa, Togo	Resistant	10.5[a,b]	13.0[a,b]	20.3[b]	9.7[a]	12.5[a,b]	17.3[b]
(190 *Cx. quinquefasciatus)*			**2.8**	**10.9**	**–**	**2.2**	**7.6**

Figure 5 **Deltamethrin content and retention (wash curve) of side panels (upper side and strengthened border) and roof of PermaNet 3.0 (WHOPES Phase I)**

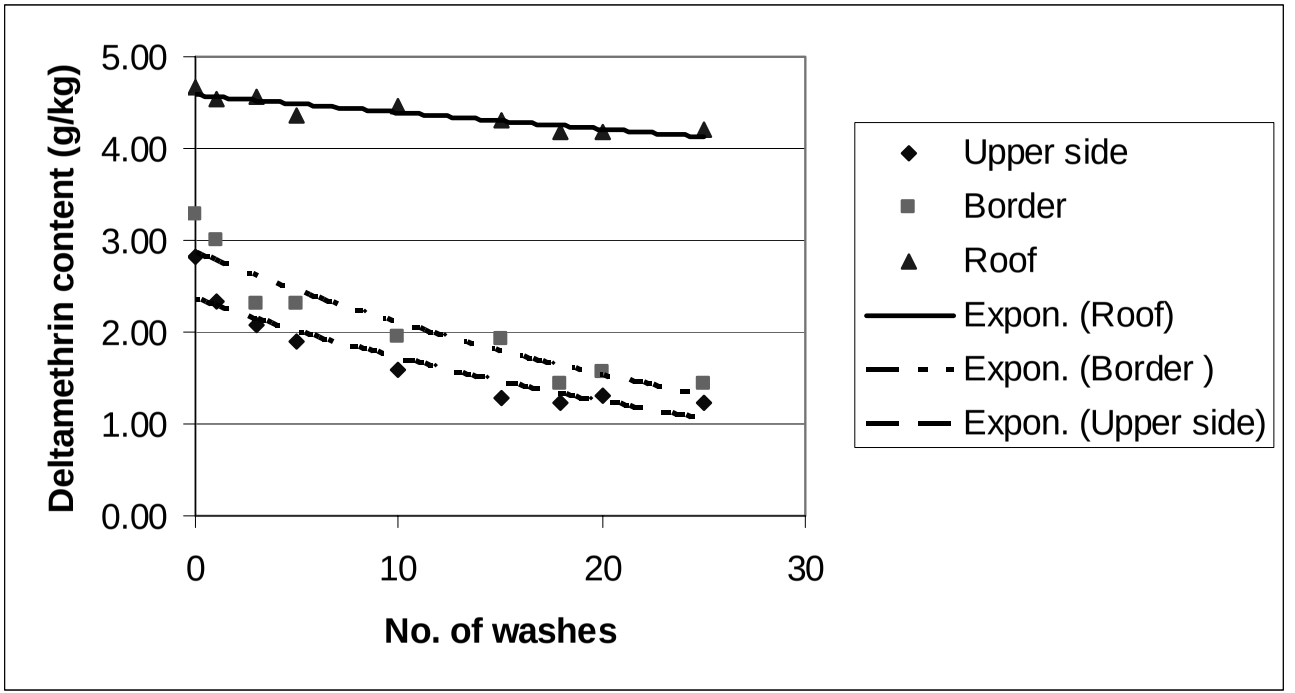


Figure 6 **Piperonyl butoxide content in the roof and retention (wash curve) of PermaNet 3.0 (WHOPES Phase I)**

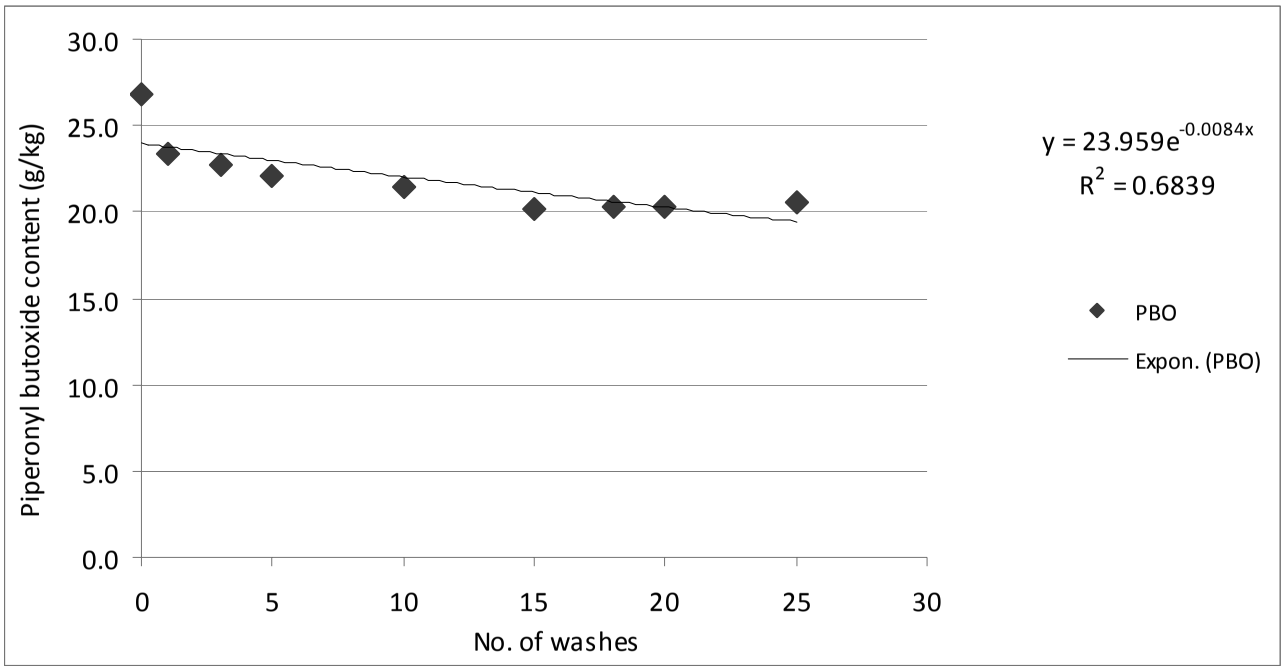


Table 12 **Deltamethrin content in the roof of different treated nets (target dose and tolerance limits for deltamethrin in PermaNet 3.0 (100 denier for all countries) = 4 g/kg ± 25% [3–5 g/kg]; target dose and tolerance limits for deltamethrin in PermaNet 2.0 (75 denier for Benin, Cameroon, Togo, the United Republic of Tanzania and Viet Nam = 1.8 g/kg ± 25% [1.35–2.25 g/kg]; and target dose and tolerance limits for deltamethrin in PermaNet 2.0 (100 denier for Burkina Faso) = 1.4 g/kg ± 25% [1.05–1.75 g/kg])**

a. Before washing

Treatment	Deltamethrin content (g/kg)					
	Togo	Burkina Faso	Cameroon	UR Tanzania	Viet Nam	Benin
PermaNet 3.0 0 wash	3.97	4.33	3.21	3.14	3.74	3.69
PermaNet 3.0 20 washes	4.02	4.18	3.38	2.99	3.12	3.65
PermaNet 2.0 0 wash	2.10	1.21	2.99	1.97	2.13	2.14
PermaNet 2.0 20 washes	2.14	1.26	3.16	2.08	2.24	2.87
Conventionally-treated net exhausted	0.76	0.67	0.83	0.70	0.82	0.47
Untreated net	<0.01	<0.01	<0.01	–	<0.01	<0.01

Table 12 (continued) **Deltamethrin content (DMC, in g/kg) and deltamethrin retention (DMR, in percentage of wash zero) in the roof of different treated nets**

b. After washing, before testing

Treatment	Togo		Burkina Faso		Cameroon		UR Tanzania		Viet Nam		Benin	
	DMC	DMR	DMC	DMR	DMC	DMR	DMC	DMR	DMC	DMR	DMC	DMR
PermaNet 3.0 0 wash	3.94		4.27		3.84		3.21		–		3.64	–
PermaNet 3.0 20 washes	3.33	85	3.42	80	2.86	74	2.57	80	3.12	100	3.33	91
PermaNet 2.0 0 wash	0.26[a]		1.30		2.24		1.92		–		2.16	
PermaNet 2.0 20 washes	0.37	17	0.25	19	0.79	35	0.66	34	1.14	51	0.49	23
Conventionally-treated net exhausted	0.15	20	0.10	15	0.08	10	<0.01[a]	–	0.16	20	0.06	13
Untreated net	<0.01		<0.01		<0.01		–		–		<0.01	

[a] These values are outliers and were not considered.

Table 12 (continued) **Deltamethrin content in the roof of different treated nets**

c. After testing

Treatment	Deltamethrin content (g/kg)					
	Togo	Burkina Faso	Cameroon	UR Tanzania	Viet Nam	Benin
PermaNet 3.0 0 wash	3.82	4.07	3.22	2.96	3.59	3.00
PermaNet 3.0 20 washes	3.37	3.36	2.57	2.58	2.28	3.16
PermaNet 2.0 0 wash	1.96	1.04	2.92	1.93	2.24	1.84
PermaNet 2.0 20 washes	0.38	0.27	0.75	0.74	1.08	0.72
Conventionally-treated net exhausted	0.09	0.12	0.08	0.10	0.22	0.08
Untreated net	<0.01	<0.01	<0.01	–	<0.01	<0.01

Table 13 **Deltamethrin content (DMC, in g/kg) and within-net relative standard deviation (RSD, in percentage) in the side panels of different treated nets (target dose and tolerance limits for deltamethrin in PermaNet 3.0 (75 denier for all countries) = 2.8 g/kg ± 25% [2.1–3.5 g/kg]; target dose and tolerance limits for deltamethrin in PermaNet 2.0 (75 denier for Benin, Cameroon, Togo, the United Republic of Tanzania and Viet Nam) = 1.8 g/kg ± 25% [1.35–2.25 g/kg]; and target dose and tolerance limits for deltamethrin in PermaNet 2.0 (100 denier for Burkina Faso) = 1.4 g/kg ± 25% [1.05–1.75 g/kg])**

a. Before washing

Treatment	Togo		Burkina Faso		Cameroon		UR Tanzania		Viet Nam		Benin	
	DMC	RSD	DMC	RSD	DMC	RSD	DMC	RSD	DMC	RSD	DMC	RSD
PermaNet 3.0 0 wash	2.74	8.6	2.89	10.0	2.44	11.7	2.45	0.5	2.72	1.9	2.61	4.2
PermaNet 3.0 20 washes	2.94	6.1	3.10	5.5	2.48	13.4	2.75	1.0	2.05	12.0	2.58	7.1
PermaNet 2.0 0 wash	1.99	2.7	1.31	5.5	2.53	7.6	2.21	1.5	2.13	2.1	2.09	2.2
PermaNet 2.0 20 washes	1.99	4.6	1.30	1.4	2.65	7.3	1.85	18.0	2.20	4.3	2.41	7.3
Conventionally-treated net exhausted	0.76	4.4	0.72	12.7	0.88	7.0	0.71	5.7	0.74	13.0	0.59	13.8
Untreated net	<0.01	–	<0.01	–	<0.01	–	–	–	<0.01	–	<0.01	–

Table 13 (continued) **Deltamethrin content (DMC, in g/kg), within-net relative standard deviation (RSD, in percentage) and retention (DMR, in percentage of wash zero) in the side panels of different treated nets**

b. After washing, before testing

Treatment	Togo			Burkina Faso			Cameroon		
	DMC	RSD	DMR	DMC	RSD	DMR	DMC	RSD	DMR
PermaNet 3.0 0 wash	2.88	8.9		3.06	9.4		2.75	3.2	
PermaNet 3.0 20 washes	0.67	42.8	23	0.96	19.7	31	0.36	50.9	13
PermaNet 2.0 0 wash	0.25[a]	1.7		1.29	3.0		2.11	1.1	
PermaNet 2.0 20 washes	0.30	5.2	15	0.24	7.7	19	0.40	21.8	19
Conventionally-treated net exhausted	0.13	13.0	17	0.10	8.6	14	0.08	9.1	9
Untreated net	<0.01	–		<0.01	–		<0.01	–	

[a] This value is an outlier and was not considered.

Table 13 (continued) **Deltamethrin content (DMC, in g/kg), within-net relative standard deviation (RSD, in percentage) and retention (DMR, in percentage of wash zero) in the side panels of different treated nets**

b. After washing, before testing

Treatment	UR Tanzania			Viet Nam			Benin		
	DMC	RSD	DMR	DMC	RSD	DMR	DMC	RSD	DMR
PermaNet 3.0 0 wash	2.36	8.0		–	–	–	2.58	7.3	
PermaNet 3.0 20 washes	1.11	1.1	47	1.12	15.0	55	0.66	39.1	26
PermaNet 2.0 0 wash	2.21	0.4		–	–	–	2.08	1.1	
PermaNet 2.0 20 washes	0.71	5.4	32	1.05	2.5	48	0.47	4.9	23
Conventionally-treated net exhausted	<0.01[a]	–	–	0.18	11.6	24	0.06	8.2	10
Untreated net	–	–		–	–		<0.01	–	

[a] This value is an outlier and was not considered.

Table 13 (continued) **Deltamethrin content (DMC, in g/kg) and within-net relative standard deviation (RSD, in percentage) in the side panels of different treated nets**

c. After testing

Treatment	Togo		Burkina Faso		Cameroon		UR Tanzania		Viet Nam		Benin	
	DMC	RSD	DMC	RSD	DMC	RSD	DMC	RSD	DMC	RSD	DMC	RSD
PermaNet 3.0 0 wash	2.52	13.0	2.94	7.8	2.50	7.2	1.95	1.0	2.75	1.8	2.32	9.6
PermaNet 3.0 20 washes	0.26	47.8	0.90	51.6	0.33	72.6	0.81	8.7	1.03	14.1	0.53	40.4
PermaNet 2.0 0 wash	2.12	16.8	1.32	3.8	2.51	8.5	1.81	4.4	2.08	1.3	1.74	6.2
PermaNet 2.0 20 washes	0.16	13.3	0.27	6.2	0.41	26.0	0.72	5.3	1.06	1.8	0.99	4.2
Conventionally-treated net exhausted	0.07	15.2	0.11	7.6	0.08	6.2	0.12	17.7	0.19	10.5	0.09	2.0
Untreated net	<0.01	–	<0.01	–	<0.01	–	–	–	<0.01	–	<0.01	–

Table 14 **Piperonyl butoxide (PBO) content in the roof of PermaNet 3.0 (target dose and tolerance limits for PBO in PermaNet 3.0 (100 denier for all countries) = 25 g/kg ± 25% [18.75 g/kg - 31.25 g/kg])**

a. Before washing

	PBO content (g/kg)					
Treatment	Togo	Burkina Faso	Cameroon	UR Tanzania	Viet Nam	Benin
PermaNet 3.0 0 wash	17.1	23.1	26.0	24.8	22.3	20.8
PermaNet 3.0 20 washes	18.2	22.9	28.0	25.1	18.9	20.7

Table 14 (continued) **Piperonyl butoxide content (PBOC, in g/kg) and retention (RET, in percentage of wash zero) in the roof of PermaNet 3.0 (target dose and tolerance limits for PBO in PermaNet 3.0 (100 denier for all countries) = 25 g/kg ± 25% [18.75 g/kg - 31.25 g/kg])**

b. After washing, before testing

Treatment	Togo		Burkina Faso		Cameroon		UR Tanzania		Viet Nam		Benin	
	PBOC	RET	PBOC	RET	PBOC	RET	PBOC	RET	PBOC	RET	PBOC	RET
PermaNet 3.0 0 wash	20.3		22.7		21.9		24.2		–		20.7	
PermaNet 3.0 20 washes	11.7	58	16.9	74	20.1	92	19.3	80	13.7	72	14.5	70

Table 14 (continued) **Piperonyl butoxide (PBO) content in the roof of PermaNet 3.0 (target dose and tolerance limits for PBO in PermaNet 3.0 (100 denier for all countries) = 25 g/kg ± 25% [18.75 g/kg - 31.25 g/kg])**

c. After testing

	PBO content (g/kg)					
Treatment	Togo	Burkina Faso	Cameroon	UR Tanzania	Viet Nam	Benin
PermaNet 3.0 0 wash	17.3	20.4	29.8	20.8	19.3	23.1
PermaNet 3.0 20 washes	12.2	15.1	17.7	19.4	10.0	12.4

5. REVIEW OF PERMANET 2.5

PermaNet 2.5 (= PermaNet 2.0 Extra) is a deltamethrin-coated) LN manufactured by Vestergaard Frandsen (Switzerland). The net is made of warp-knitted multi-filament polyester fibres. The side netting has two parts: a strengthened lower panel, the so-called border (70 cm) made of 75 ± 5% denier yarn (weight 40 ± 10% g/m^2) and the upper side panel made of 75 ± 5% denier (weight of 30 ± 10% g/m^2). The target dosage of deltamethrin in the side panels is 2.8 g AI/kg of netting material, i.e. 115 mg AI/m^2 of the border and 85 mg AI/m^2 of the remaining of the side panels and the roof.

5.1 Safety assessment

Noting the specifications of PermaNet 2.5 and that netting used in making the product is of the same specifications as that of the side panels of PermaNet 3.0, the WHO risk assessment of the latter has been used and no separate assessment carried out (see sections 4 and 4.1). It has been concluded that when the instructions for use are followed, washing or sleeping under the LN do not pose undue risk to the users (adults, children or newborns).

5.2 Efficacy – WHOPES supervised trials

5.2.1 Laboratory studies

The regeneration time, wash resistance and efficacy of netting used in making PermaNet 2.5, were determined in laboratory (Phase I) according to WHOPES guidelines[1] against susceptible An*. gambiae s.s.* (Duchon et al., 2008b) and as part of WHOPES testing and evaluation of PermaNet 3.0.

Chemical analyses were performed (Pigeon 2008d) on net samples washed 0, 1, 3, 5, 10, 15, 18, 20 and 25 times. For

[1] WHO (2005). *Guidelines for laboratory and field testing of long-lasting insecticidal mosquito nets*. Geneva, World Health Organization (WHO/CDS/WHOPES/GCDPP/2005.11; available at: http://www.who.int/whopes/guidelines/en/; accessed January 2009).

each wash interval, 8 pieces from 4 nets taken from upper side panel and lower border panel were analysed for determination of deltamethrin content. The analytical method used was based on CIPAC method and involved extraction by heating under reflux with xylene and chromatographic determination by GC-FID using the internal standard calibration.

Results of analysis for AI content and retention (wash curve) are presented in Figure 5. The between-net variation is expressed as the RSD of the content found on the 4 pieces. Retention is calculated according to the Annex 1 of the Report of the eleventh WHOPES Working Group Meeting, assuming a free migration stage behaviour.

The deltamethrin content in the side panel (upper section = 2.82 g/kg and lower strengthened border = 3.27 g/kg) of the unwashed net complied with the target dose of 2.8 g/kg (± 25%). The between-net variation as expressed by RSD ranged from 3.4% to 5.6%.

After 20 washes, the average deltamethrin content was 1.30 g/kg (upper side panel) and 1.57 g/kg (lower strengthened border). The overall deltamethrin retention after 20 washes was 46% (upper side panel) and 48% (strengthened border) corresponding to an average retention per wash of 93% and 94% respectively.

The regeneration time after 3 consecutive washes as determined by cone bioassay using susceptible *An. gambiae* Kisumu strain showed that bio-efficacy was fully restored after 1 day (i.e. knock-down: 100%, mortality 100%). This means that no regeneration time is needed after washing.

The wash resistance study performed on fully susceptible mosquitoes showed that the efficacy of the two parts of PermaNet fulfil the WHO requirements (mortality >80% and/or knock-down >95%) after 20 washes.

5.2.2 Experimental hut studies

The experimental hut trials were set out to demonstrate whether (i) PermaNet 2.5 meets the criterion of an LN by comparison with conventionally treated net (CTN) washed to just before exhaustion and (ii) whether PermaNet 2.5 shows superiority or equivalence to its predecessor PermaNet 2.0.

Malanville, North Benin

PermaNet 2.5 LNs were evaluated in experimental huts located in irrigated rice field area, Malanville North Benin (Chabi *et al.* 2008). This evaluation was built into a larger study of PermaNet 3.0 conducted at the same time. However, only the treatment arms pertinent to the evaluation of PermaNet 2.5 are described in this section. *An. gambiae* s.l. is the main malaria vector in Malanville with 95% *An. gambiae* s.s. (M form) and 5% *An. arabiensis*. Both species when exposed to discriminative doses are susceptible to permethrin (93% mortality), deltamethrin (85% mortality) and lamda-cyhalothrin (92.2% mortality). The west African experimental huts were used in this setting and the procedure followed the above-mentioned WHOPES Guidelines. Efficacy was evaluated in terms of blood-feeding inhibition, deterrence, induced exophily and mortality.

Six treatment arms were tested: (i) untreated net (same fabric as PermaNet 2.5); (ii) PermaNet 2.0 unwashed; (iii) PermaNet 2.0 washed 20 times; (iv) PermaNet 2.5 washed 20 times; (v) Polyester net conventionally treated with deltamethrin at 25 mg/m² AI; and (vi) Polyester net conventionally treated with deltamethrin at 25 mg/m² AI and washed 3 times i.e., to just before exhaustion using cone tests with *An. gambiae* Kisumu strain. An unwashed PermaNet 2.5 treatment arm could not be included in this trial owing to a shortage of huts. However, its absence did not affect the conclusions of the study.

Six nets per treatment arm were tested during each weekly rotation, one net on each of the six nights. On night 7, the huts were cleaned and ventilated. Two complete Latin square rotations were necessary to obtain sufficient numbers of mosquitoes for statistical power.

The fully susceptible Kisumu strain of *An. gambiae* was used for the cone bioassays. Prior to washing treated nets in each treatment group gave 100% KD and 100% mortality. After field

testing, the 20 times washed PermaNet 2.5 induced 100% KD and 100% mortality. The PermaNet 2.0 washed 20 times gave 97% mortality and the conventional treated net washed just before exhaustion gave 89% mortality.

Overall, 285 *An.gambiae s.l.* (3.9 per night) were collected in the control huts during the trial period (21 July to 11 October). No deterrent effect was observed with any of the treatments. All insecticide treatments induced significantly higher exophily (33 to 50%).

This study demonstrated that blood-feeding inhibition and mortality of 20 times washed PermaNet 2.0 (84% and 71% respectively) and PermaNet 2.5 (91% and 78%) were not significantly different from one another. Each was significantly higher than that of a conventionally deltamethrin treated net washed to just before exhaustion (72% BFI and 61% mortality).

After Phase II testing, the PermaNet 2.5 washed 20 times still contained 1.38 g/kg and 0.72 g/kg deltamethrin in the side panels and roof respectively, corresponding to a retention rate of 49% and 26% respectively (Pigeon 2009c).

Kilimanjaro district, United Republic of Tanzania

PermaNet 2.5 was tested in the Lower Moshi rice irrigation scheme (Kilimanjaro district, United Republic of Tanzania) where *An. arabiensis* is a dominant mosquito species from October to November (Oxborough et al., 2008). The east African experimental hut type was used.[1]

The following arms were tested: (i) untreated net; (ii) PermaNet 2.0 unwashed; (iii) PermaNet 2.0 washed 20 times; (iv) PermaNet 2.5 unwashed; (v) PermaNet 2.5 washed 20 times; and (vi) polyester net conventionally treated with deltamethrin at 25 mg/m² and washed three times, i.e. to just before exhaustion in cone tests using the pyrethroid susceptible *An. gambiae* Kisumu strain.

[1] WHO (2005). *Guidelines for laboratory and field testing of long-lasting insecticidal mosquito nets*. Geneva, World Health Organization (WHO/CDS/WHOPES/GCDPP/2005.11; available at: http://www.who.int/whopes/guidelines/en/; accessed January 2009).

Each treatment was rotated through each of the six huts. Owing to time constraints each rotation took only 3 days and the entire trial was completed in 24 days. Three nets per treatment arm were tested during each rotation, one net on each of the 3 nights. On night 4, the huts were cleaned and ventilated. Only one Latin square rotation was necessary to obtain sufficient numbers of mosquitoes for statistical analysis owing to the high densities of *An. arabiensis* present during the trial period.

The fully susceptible Kisumu strain of *An. gambiae* was used for cone bioassays. Prior to washing all treated nets gave 100% KD and 100% mortality regardless of treatment. After field testing, the PermaNet 2.5 washed 20 times induced 95% KD and 81% mortality. The PermaNet 2.0 washed 20 times gave 97% knock-down and 81% mortality. The conventional treated net washed just before exhaustion gave 59% knock-down and 33% mortality.

Not considering an outlier value, the deltamethrin content in unwashed PermaNet 2.5 and 2.0 complied with the target doses (± 25%) (Pigeon 2009d). The overall deltamethrin retention in PermaNet 2.5 and 2.0 after 20 washes was 27% and 30% respectively.

The within-net RSD of deltamethrin content in unwashed PermaNet 2.5 and 2.0 was 5.4-14.5% and 5.8-8.5% respectively.

The conventionally treated net washed to just before exhaustion contained 2.7 mg/m² (0.09 g/kg) deltamethrin corresponding to a retention rate of 12%.

During the collecting period (20 October to 12 November 2008), 197 *An. arabiensis* (11 per night) were collected in the control huts. No deterrent effect was observed with any of the treatments. *An. arabiensis* is inherently exophilic and 75% of mosquitoes were collected from the veranda traps in the mornings. Insecticide induced exophily was observed for all types of insecticide treated nets tested.

The blood-feeding rate with the untreated net was 43%. A slight but significant reduction in blood-feeding was observed with all types of treated net. Blood-feeding inhibition of the 20 times washed PermaNet 2.0 (17%) and PermaNet 2.5 (21%) were not significantly different from one another or from a

conventionally deltamethrin treated net washed to just before exhaustion (24%). Mortality with the unwashed PermanNet 2.5 (50%) did not differ significantly from that of the unwashed PermaNet 2.0 (46%). Mortality was significantly lower in the PermaNet washed 20 times, but again there was no significant difference between PermaNet 2.5 (35% mortality) and PermaNet 2.0 (36%). Each was significantly higher than that of a conventionally deltamethrin treated net washed to just before exhaustion (23% mortality). The relative low mortality as compared to trials with *An. gambiae* can be explained by the natural behavioural trait of *An. arabiensis* towards zoophily and exophily.

5.3 Conclusions and recommendations

PermaNet 2.5 (= PermaNet 2.0 Extra) is a deltamethrin-coated LN manufactured by Vestergaard Frandsen (Switzerland). The net is made of warp-knitted multi-filament polyester fibres. The side netting has two parts: a lower, so-called border (70 cm) made of 75 ± 5% denier yarn with strengthened durability (weight 40 ± 10% g/m^2) and the upper side panel made of 75 ± 5% denier (weight of 30 ± 10% g/m^2). The target dosage of deltamethrin in the side panels is 2.8 g AI/kg of netting material, i.e. 115 mg AI/m^2 on the border and 85 mg AI/m^2 on the upper side panels.

The WHO assessment of the compliance of the manufacturer's assessment of exposure to and risks of washing and sleeping under a PermaNet 2.5 LN was generally in line with the WHO generic risk assessment model. When the generic model assumptions or default values were not adopted, they were replaced by data derived experimentally and appropriately justified. The assessment concluded that washing or sleeping under the LN do not pose undue risk to adults, children or newborns.

The deltamethrin content in the side panel (upper section = 2.82 g/kg; lower strengthened border = 3.27 g/kg) of the unwashed net was consistent with the target dose of 2.8 g/kg (± 25%). The between-net variation as expressed by RSD ranged from 3.4% to 5.6%. After 20 washes, the average deltamethrin content was 1.30 g/kg (upper side panel) and 1.57 g/kg (lower strengthened border). The overall deltamethrin retention after

20 washes was 46% (upper side panel) and 48% (strengthened border), corresponding to an average retention per wash of 93% and 94% respectively.

The regeneration time after three consecutive washes as determined by cone bioassay using susceptible *An.gambiae* Kisumu strain showed that bioefficacy was fully restored after 1 day (i.e. KD: 100%, mortality 100%). This means that no regeneration time is needed after washing. The wash resistance study performed on fully susceptible mosquitoes showed that the efficacy of the two parts of PermaNet 2.5 fulfil the WHO requirements (mortality >80% and/or knock-down >95%) after 20 washes.

The experimental hut trials in Benin and the United Republic of Tanzania set out to demonstrate whether (i) PermaNet 2.5 meets the criterion of a long lasing insecticidal net by comparison with CTN washed to just before exhaustion and (ii) whether PermaNet 2.5 shows superiority or equivalence to its predecessor PermaNet 2.0.

Both experimental hut studies demonstrated that the PermaNet 2.5 washed 20 times performed equal to or better than a conventionally treated net washed until exhaustion in terms of blood-feeding inhibition and mortality. However, there was no difference in efficacy of a PermaNet 2.5 washed 20 times compared to a PermaNet 2.0 washed 20 times in terms of blood-feeding inhibition and mortality. A loss of efficacy was observed in a PermaNet 2.5 over 20 washes.

In the Phase II trials, deltamethrin content in unwashed PermaNet 2.5 complied with the target doses and was uniformly distributed within the net.

Given the safety, efficacy and resistance to washing of the PermaNet 2.5 in laboratory studies and small-scale field studies, it is recommended:

> that a time-limited interim recommendation be given for the use of PermaNet 2.5 in the prevention and control of malaria;
>
> that WHOPES coordinates large-scale field studies (WHOPES Phase III studies) of PermaNet 2.5 to

confirm its long-lasting efficacy and fabric integrity, as well as community acceptability, as a requirement for developing full recommendations on the use of the product.

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

6. REVIEW OF LAMBDA-CYHALOTHRIN LN OF SYNGENTA

The lambda-cyhalothrin LN of Syngenta, Switzerland (= ICON MAXX-Net) is a factory-produced polyester LN treated with the slow-release capsule suspension of lambda-cyhalothrin, ICON 10 CS. The insecticide is coated on polyester netting at the target dose of 50 mg AI/m^2 using a polymer as a binder. Lambda-cyhalothrin CS has been previously evaluated by WHOPES and recommended for treatment of mosquito nets.[1]

The manufacturer has disclosed the nature of the binder used in coating the LN and has confirmed that it is the same as the binder used in making ICON MAXX mosquito net treatment kit[2] already subject to the WHO safety assessment.

6.1 Safety assessment

The assessment of the risk to humans of washing and sleeping under the LN, provided by the manufacturer was assessed by FIOH (FIOH, 2007a) on behalf of the WHO Programme on Chemical Safety. The WHO *Generic risk assessment model for insecticide treatment and subsequent use of mosquito nets*[3] was used as a guiding document.

The following assumptions/methodologies were used by the proposer in drafting the assessment:

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[1] *Report of the fourth WHOPES Working Group Meeting, WHO/HQ, Geneva, 4-5 December 2000*. Geneva, World Health Organization, 2001 (WHO/CDS/WHOPES/2001.4; available at: http://whqlibdoc.who.int/hq/2001/WHO_CDS_WHOPES_2001.4.pdf; accessed January 2009).

[2] *Report of the eleventh WHOPES Working Group Meeting, WHO/HQ, Geneva, 10–13 December 2007*. Geneva, World Health Organization, 2008 (WHO/HTM/NTD/WHOPES/2008.1; available at: http://whqlibdoc.who.int/hq/2008/WHO_HTM_NTD_WHOPES_2008.1_eng.pdf; accessed January 2009).

[3] *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; available at http://whqlibdoc.who.int/hq/2004/WHO_PCS_04.1.pdf; accessed January 2009).

1. inhalation exposure during sleeping under the net is negligible given the low vapour pressure of lambda-cyhalothrin;
2. adopts 0.0038 and 0.0025 mg/kg body weight as AELs for "acute" and "chronic" systemic exposure (values identical to those set by JECFA for long-term exposure to cyhalothrin[1] and to that of the European Union for short-term and long-term exposures to lambda-cyhalothrin[2]);
3. uses 0.1% and 1% as the dermal absorption rates for the concentrated preparations and the dilutions of the insecticide. The values differ greatly from the default assumptions of the generic model, but the proposer justifies this by experimental data in vivo and in vitro from studies in animals and in humans.

FIOH concluded that the characterization of the risks performed by the proposer closely follows the WHO generic model; where default assumptions are not accepted, justification mostly in the form of actual experimental data, are presented. The conclusion, in line with the generic model, is that no unacceptable exposures were found in maintenance and use of the nets, and that washing or sleeping under the LN does not pose undue risk to adults, children or newborns.

6.2 Efficacy – WHOPES supervised trials

6.2.1 Laboratory studies

Montpellier, France

Laboratory studies were conducted to determine the efficacy and wash resistance of the Lambda-cyhalothrin LN and to study the dynamics of the insecticide on the fibre following WHOPES guidelines.[3] The evaluation included a determination of the time

[1] *WHO Food Additives Series 53: Cyhalothrin (addendum) 2004* (available at http://www.inchem.org/documents/jecfa/jecmono/v53je04.htm.

[2] http://ec.europa.eu/food/plant/protection/evaluation/existactive/list1-24_en.pdf).

[3] WHO (2005). *Guidelines for laboratory and field testing of long-lasting insecticidal mosquito nets*. Geneva, World Health Organization (WHO/CDS/WHOPES/GCDPP/2005.11; available at: http://www.who.int/whopes/guidelines/en/; accessed January 2009).

required for full regeneration following washing and a determination of the wash resistance and efficacy of the Lambda-cyhalothrin LN against susceptible *Anopheles gambiae* mosquitoes (Duchon et al., 2008a).

Four LNs were provided by the manufacturer for the evaluation. Two pieces of netting (25 cm x 25 cm) were cut from each net. Of these, four were used for a regeneration study and four were used in a wash resistance study.

The procedures for net washing and bioassays were the same for assessing both regeneration time and wash resistance. Net samples were washed by placing them in 1-litre beakers containing a soap solution of 0.5 L of deionized water with 2 g/L of soap ("Savon de Marseille", pH 10–11). The beakers were shaken for 10 minutes at 155 movements per minute and at 30^{o}C. After 10 minutes, the samples were rinsed by placing them in clean deionized water (0.5 L) and shaking them for 10 minutes in the same shaking conditions as above. The rinsing procedure was repeated a second time and then the net samples were dried at room temperature for 2 hours before being stored in aluminum foil in the dark at 30 ^{o}C until their next wash.

Bioassays were conducted using the WHO cone test method. Four WHO cones were attached to each piece of netting and 5 *Anopheles gambiae* females (Kisumu strain, non-blood fed, 2–5 days old) were introduced into each cone. Mosquitoes were exposed for 3 minutes and then transferred to separate cages with access to sugar solution. The process was repeated until a total of 50 mosquitoes had been exposed to each net sample. Knock-down was measured 60 minutes post-exposure while mortality was measured 24 hours post-exposure. Results were pooled for analysis so that a total of 200 mosquitoes had been exposed at each time point. Control bioassays were conducted on untreated nets (negative control) as well as on unwashed lambda-cyhalothrin treated nets (15 mg/m^2).

Regeneration time was estimated by washing 4 net samples 3 times on consecutive days. Bioassays were done on the nets before washing and then at 1, 3, and 5 days following the third wash. Mortality was low, even on an unwashed sample (24%). After 3 washes, mortality was 28% at day 1 post-washing, 43% at day 3 and 41% at day 5. Knock-down was 100% for an

unwashed LN and 98% at 1 day after the net samples had been washed 3 times. At day 3 and day 5 post-washing, knock-down was 100%. Based on these data, it was concluded that the regeneration time was 1 day.

Wash resistance was done by washing four net samples as described above. WHO cone bioassays were conducted after 0, 1, 5, 10, 15 and 20 washes. Washing was done five times per week and each bioassay was done just after the regeneration time and just before the next wash. Mortality of mosquitoes exposed to the lambda-cyhalothrin LN samples ranged from 24% to 64%. Lowest mortality was observed at 0 wash while the highest mortality was observed after 10 washes. By 20 washes, mortality had fallen to 26%. Knock-down was 100% through the experiment, regardless of the number of washes. Based upon the high knock-down in the WHO cone test after 20 washes, the Lambda-cyhalothrin LN met the WHOPES criteria for Phase I testing (>80% mortality or >95% knock-down after 20 washes) and further studies using the tunnel test were not required.

Montpellier, France

Since chemical analyses of net samples were not available in the previous study, a second set of laboratory studies was conducted on a new batch to determine the efficacy and wash resistance of the Lambda-cyhalothrin LN as well as to study the dynamics of the insecticide on the fibres (Finot et al., 2008). The manufacturer provided four LNs for the study. Two pieces of netting (25cm x 25 cm) were cut from each net. Four samples were used for the regeneration study and four were retained for the wash resistance study. An additional 24 samples were retained for chemical analysis.

The net washing procedure followed that recommended by WHOPES guidelines. Washing and bioassay were done as reported above.

To estimate regeneration time, four net samples were washed 3 times on consecutive days. Bioassays were conducted on the nets before washing and then at 1, 3, 5, 7, and 10 days following the third wash. Mortality was low, even on an unwashed sample (27%). For net samples that had been washed three times, mortality in bioassays conducted 1, 3, 5, 7, and 10 days after washing ranged from 17% to 31%. There

was no significant difference in mortality at different time points after washing and no evidence of increasing mortality with increasing time since washing. Knock-down was 100% for an unwashed LN. After 3 washes, knock-down was 100% at 1, 3, 5, and 7 days post-washing. Knock-down was 98% 10 days after the net samples had been washed 3 times. Based on these data, it was concluded that the regeneration time was 1 day.

Wash resistance was done by washing four net samples as described above. WHO cone bioassays were conducted after 0, 1, 5, 10, 15 and 20 washes. Washing was done 5 times per week and each bioassay was done just after the regeneration time and just before the next wash. Mortality of mosquitoes exposed to the lambda-cyhalothrin LN samples was 85% at 0 wash. Mortality fell to 53% after 1 wash. Mortality continued to decline to 11% at 15 washes and 12% at 20 washes. Knock-down was >95% through 10 washes but fell to 86% at 15 washes and 48% at 20 washes. Unlike the previous study, the Lambda-cyhalothrin LN in this study did not meet the WHO efficacy criteria for Phase I testing by the cone test (>80% mortality or >95% knock-down after 20 washes) and additional tunnel tests were therefore performed.

The tunnel test was conducted using a glass tunnel that was 60 cm in length and had a cross-sectional area of 25 cm x 25 cm. At each end of the tunnel, a 25 cm square cage was fitted to the tunnel and covered with polyester netting to prevent the escape of mosquitoes. At one third the length of the tunnel, a disposable cardboard frame was fitted with a net sample that had been washed 20 times. The surface available to the mosquitoes was 20 cm x 20 cm with 9 holes, 1 cm in diameter cut into the netting to allow passage of mosquitoes. In the shorter section of the tunnel, a guinea pig was restrained as a bait. In the longer section of the tunnel, 100 female mosquitoes (Kisumu strain, non-blood fed, 5–8 days old) were released at 18:00. Females were free to move about the tunnel but had to land on the netting and locate the holes to pass through and feed upon the guinea pig. At 09:00 the following morning, the mosquitoes were removed from each section of the tunnel and mortality and blood-feeding rates were recorded. Two cages were run each night, one with test netting and one with untreated control netting. Mortality was estimated as the total number of mosquitoes in the tunnel divided by the total number

released. Blood-feeding inhibition was estimated as the proportion fed in the treated tunnel relative to the proportion fed in the control tunnel. During the test, cages were held at 27±2°C and 80±10% RH.

After 20 washes, mortality of mosquitoes in the tunnel with the Lambda-cyhalothrin LN was 95%. In the control tunnel, mortality was 11%. In the tunnel with the Lambda-cyhalothrin LN, 47% of mosquitoes passed through the netting to the side with the guinea pig, compared to 99% of all mosquitoes released in the control tunnel. Relative to the control tunnel, blood-feeding rates were reduced by 96% in the tunnel with the Lambda-cyhalothrin LN washed 20 times. Based upon the tunnel test results, the Lambda-cyhalothrin LN washed 20 times met the criteria to continue to Phase II testing (mortality >80% and blood-feeding inhibition >95%).

Chemical analysis (Pigeon 2008h) were performed on net samples washed 0, 1, 5, 10, 15 and 20 times. Per wash cycle, four pieces from 4 nets were analysed for determination of lambda-cyhalothrin content. The analytical method used involved extraction by heating under reflux with xylene and chromatographic determination by Gas Chromatography with ^{63}Ni Electron Capture Detection (GC-ECD) using the external standard calibration. The analytical method was successfully validated.

Results of analysis for lambda-cyhalothrin content and retention (wash cure) are presented in Table 15 and figure 7. The between-net variation is expressed as the RSD of the content found on four pieces from different nets. Retention is calculated according to the Annex 1 of the report of the eleventh WHOPES Working Group Meeting, assuming a free migration stage behaviour (see Annex 1 of the Report of the eleventh WHOPES Working Group Meeting [1]).

[1] WHO (2008). *Report of the eleventh WHOPES Working Group Meeting, WHO/HQ, Geneva 10–13 December 2007* (WHO/HTM/NTD/WHOPES/2008.1; available at http://www.who.int/whopes/recommendations/wgm/en/; accessed January 2009).

The lambda-cyhalothrin content (1.33 g/kg) in the unwashed net complied with the target dose of 1.25 g/kg (± 25%) but the between-net RSD was high (24.5%).

After 20 washes, the average lambda-cyhalothrin content was 0.39 g/kg. The overall lambda-cyhalothrin retention after 20 washes was 29% corresponding to an average retention per wash of 93%.

6.2.2 Experimental hut studies

Kou Valley, Burkina Faso

Experimental hut studies were conducted in the Kou Valley of Burkina Faso to determine the efficacy of washed and unwashed Lambda-cyhalothrin LN in comparison with a net conventionally treated with lambda-cyhalothrin and to assess their impact on the behaviour of *An. gambiae* populations (Dabire et al., 2008a).

The study area was situated in the Kou Valley, 30 km north of Bobo-Dioulasso. The Kou Valley is a rice cultivation area surrounded by wooded savannah. Both molecular forms of *An. gambiae s.s.* occur in sympatry but the S form increased in frequency (up to 85%), at the end of the rainy season (October-November). Knock-down (*kdr*) mutation occurs in both forms but at different frequencies (>90% in S form, around 20% for the M form) (Dabiré *et al.* 2008b; Pennetier et al., 2008). The trial was run from 9th of August till 12th of September 2007.

Five treatment arms were included in the experiment: (I) untreated polyester net; (ii) lambda-cyhalothrin LN, unwashed; (iii) lambda-cyhalothrin LN, washed 20 times; (iv) polyester net, conventionally treated with lambda-cyhalothrin at 15 mg/m^2, unwashed; and (v) polyester net, conventionally treated with lambda-cyhalothrin at 15 mg/ m^2, washed until just before exhaustion (1 wash). All lambda-cyhalothrin LNs used in this study were of 100 denier.

Nets were washed by study staff according to a standard protocol. Nets were washed in aluminium bowls containing 10 L of well water with 2 g/L of soap (Savon de Marseille). Nets were agitated with a pole for 3 minutes at 20 rotations per minute, left to soak for 4 minutes and then agitated for an

additional 3 minutes. After washing, the nets were rinsed twice in 10 L of clean water using the same procedure then dried horizontally in the shade.

To determine the point of exhaustion of the conventionally treated nets, nets were washed as described above and bioassays were conducted after every wash. Mortality fell below the threshold (80%) after just 2 washes, though knock-down did not fall below the threshold (95%) until after 3 washes. However, knock-down had declined to just above the threshold after just 1 wash. The point of exhaustion, defined as the maximum number of washes before a conventional net falls below the threshold, was therefore set at 1 wash.

Five nets per treatment were used for the study. The nets were bio-assayed on the day before washing, just after washing and at the conclusion of the study. Bioassays were conducted with *Anopheles gambiae*, Kisumu strain. Five WHO cones were fixed to each side of the net (top and 4 sides) and 5 female mosquitoes were introduced into each cone for 3 minutes. A total of 50 mosquitoes were tested on each net. Before washing, knock-down and mortality on the untreated polyester net were 0%. Mortality was 94.7% and 91.8% on the conventionally treated nets and 76.7% and 77.6% on the lambda-cyhalothrin LNs. Knock-down was 100% on all treated nets. After washing, mortality fell to 82.4% and 80.3% on the unwashed and washed conventional nets. Mortality was 74.0% and 20.6% on the unwashed and washed lambda-cyhalothrin LNs. Knock-down was 100% on the treated nets, with the exception of the lambda-cyhalothrin LN where knock-down fell to 92.1% after washing 20 times.

A sixth net in each treatment arm was used for chemical analysis. Five pieces of netting (100 cm^2) were taken from each side and roof of the nets for chemical analysis. After washing, another 5 pieces were taken from each side and roof of the nets. At the end of the study, 5 pieces of netting were taken from each side and roof of the nets used in the study for chemical analysis (Table 16). The lambda-cyhalothrin content of nets before washing was 1.72 g/kg and 1.47 g/kg for the lambda-cyhalothrin LN (Pigeon 2008i). The target dose of a 100 denier lambda-cyhalothrin LN is 1.25 g/kg and acceptable tolerance limits are 0.94 to 1.56 g/kg. For the conventionally treated nets, the lambda-cyhalothrin content was 0.36 g/kg.

After completion of the washing, the lambda-cyhalothrin content of the unwashed lambda-cyhalothrin LN was 1.75 g/kg while that of the lambda-cyhalothrin LN washed 20 times was 0.83 g/kg corresponding to a retention of 47%. The lambda-cyhalothrin content was 0.33 g/kg for the unwashed conventional net and 0.10 g/kg for the conventional net washed 1 time corresponding to a retention of 30%. At the completion of the study, the lambda-cyhalothrin content was 0.85 g/kg (an outlier value) for the unwashed Lambda-cyhalothrin LN, 0.99 for the lambda-cyhalothrin LN washed 20 times, 0.28 g/kg for the unwashed conventional net, and 0.15 g/kg for the conventional net washed 1 time. The RSD of lambda-cyhalothrin content before washing was 17.8% and 17.2% on the lambda-cyhalothrin LNs. The RSD for the conventionally treated nets before washing was 21.5% and 43.1%. After the washings were completed but before the trial had started, the RSD was 24.9% for the unwashed lambda-cyhalothrin LN and 7.9% for the washed lambda-cyhalothrin LN. For the conventionally treated nets, the RSD was 14.3% for the unwashed net and 21.0% for the net washed 1 time. At the end of the study, the RSD was 83.1% (an outlier value) for the unwashed Lambda-cyhalothrin LN, 33.0% for the washed lambda-cyhalothrin LN, 16.7% for the unwashed conventional net and 11.2% for the conventional net washed 1 time.

The experimental huts were constructed from concrete blocks with a corrugated iron roof, a ceiling of thick polyethylene sheeting, and a concrete base surrounded by a water filled channel to prevent the entry of ants. Mosquitoes could enter the huts through 4 window slits constructed from pieces of metal and fixed at an angle to create a funnel with a 1 cm wide gap. A veranda trap made of polyethylene sheeting and screening mesh, measuring 2 m long, 1.5 m wide and 1.5 m high, was located at the back of the hut to capture exiting mosquitoes.

Nets used in the experimental hut study were deliberately holed to simulate a torn net. Six holes 4 cm in diameter were made in each test net. Adult volunteers slept under the nets and mosquitoes were collected each morning. Mosquitoes were scored by location as well as whether they were dead or alive and fed or unfed. Live mosquitoes were placed in cages and held for 24 hours to assess delayed mortality. Sleepers and

nets were rotated randomly among the huts each night in a Latin square design.

The primary outcomes measured in the experimental huts were: (i) deterrence (reduction in hut entry relative to the control huts); (ii) induced exophily (proportion of mosquitoes exiting early and found in the veranda traps); (iii) blood-feeding inhibition (reduction in blood-feeding compared with the control huts); and (iv) immediate and delayed mortality (proportion of dead mosquitoes).

The experimental hut trial was run on 35 consecutive nights. A total of 365 *Anopheles gambiae* were recorded in the control huts, of which 34.8% had exited early, 65.2% had blood fed and 1.1% died. There was no difference in the number of mosquitoes entering the control huts, the huts with an unwashed lambda-cyhalothrin LN or the huts with a polyester net washed to exhaustion. Compared to the control huts, there were significantly fewer mosquitoes in the huts with the unwashed conventional net (deterrence=61.9%) and significantly more mosquitoes in the huts with a lambda-cyhalothrin LN washed 20 times. There were significantly more *Anopheles gambiae* in huts with the lambda-cyhalothrin LN washed 20 times compared to huts with a conventional net washed to exhaustion.

The proportion of mosquitoes which exited huts with treated nets ranged from 60.9% to 72.7%. For all treated nets, the rate of exit from huts was significantly higher than that observed in the control huts. The rate of exit from huts with the conventional net washed to exhaustion was significantly lower than that observed for the other treated nets, including the lambda-cyhalothrin LN washed 20 times.

The proportion of female mosquitoes which were blood fed was 65.2% in the control huts. In the huts with treated nets, the proportion blood fed ranged from 35.9% to 39.3%, corresponding to blood-feeding inhibition rates of 39.8% to 45.0%. The proportion blood fed in the control huts was significantly lower than the proportion fed in all huts with treated nets. There were no differences in the proportion fed among huts with treated nets. The proportion fed in the huts with the lambda-cyhalothrin LN washed 20 times was 39.2% which was

not significantly different from huts with the conventional net washed to exhaustion (proportion fed=36.9%).

The mortality in huts with treated nets ranged from 16.0% to 29.5%. Mortality in all the huts with treated nets was significantly higher compared to the huts with untreated nets. Mortality in the huts with the lambda-cyhalothrin LN washed 20 times was 19.5% and was not significantly different from the mortality observed in the hut with a conventional net washed to exhaustion (mortality=16.0%).

Since the lambda-cyhalothrin LN washed 20 times performed as well as or better than a conventional net washed to exhaustion in terms of blood-feeding inhibition and mortality, it was concluded that the lambda-cyhalothrin LN met the criteria for Phase II testing of long-lasting insecticidal nets.

Muheza, United Republic of Tanzania

The efficacy of the lambda-cyhalothrin LN was evaluated in veranda trap experimental huts in Muheza (United Republic of Tanzania) against wild, free-flying *Anopheles gambiae* and *Anopheles funestus* (Tungu et al., 2008). Six treatment arms were included in the study: (iv) lambda-cyhalothrin LN, unwashed; (ii) lambda-cyhalothrin LN, washed 20 times; (iii) lambda-cyhalothrin LN, washed to cut off (26 washes); (iv) polyester net, conventionally treated with lambda-cyhalothrin at 15 mg/^{2}, washed until just before exhaustion (2 washes); (v) polyester net, conventionally treated with lambda-cyhalothrin at 15 mg/m^2, washed 20 times; and (vi) untreated net. All lambda-cyhalothrin LNs used in this study were of 100 denier.

Three nets were used for each treatment arm. Nets were washed according to a standard protocol. Nets were placed in 10 L of water with 2 g/L of soap (Savon de Marseille) and washed for a total of 10 minutes. During the washing, the nets were agitated for 3 minutes, allowed to soak for 4 minutes and then agitated again for 3 minutes. Agitation was done by stirring the net with a long pole at 20 rotations per minute. The nets were then rinsed twice in 10 L of water and dried between washings.

The point of exhaustion was determined by conducting bioassays after each wash. The point of exhaustion was defined as the maximum number of washes a net could

withstand before mortality of mosquitoes exposed in standard WHO cone assays fell below 80% and knock-down fell below 95%. Cone bioassays using *Anopheles gambiae* (Kisumu strain) showed that knock-down on the conventionally treated net fell below 95% after the first wash. Mortality fell below 80% after 3 washes. The point of exhaustion for the conventionally treated net was therefore set at 2 washes. The point of exhaustion was also determined for the lambda-cyhalothrin LN. For this net, knock-down fell below 95% after 5 washes while mortality fell below 80% after 26 washes. The point of exhaustion for the lambda-cyhalothrin LN was therefore set at 26 washes.

Cone bioassays were conducted on all nets that were used in the experimental hut study. The bioassays were done just before the washings, just after the washings and at the end of the trial. Just before the washings, knock-down and mortality were 100% on all treatment arms except the untreated net, where knock-down and mortality were 0%. After the washing of nets was completed, knock-down fell to 92% on the conventional net washed 2 times, 14% on the conventional net washed 20 times, 86% on the lambda-cyhalothrin LN washed 20 times, and 68% on the lambda-cyhalothrin LN washed 26 times. Mortality on these same nets was 95% for the conventional net washed 2 times, 32% for the conventional net washed 20 times, 92% for the lambda-cyhalothrin LN washed 20 times and 78% for the lambda-cyhalothrin LN washed 26 times. At the end of the experimental hut study, knock-down fell to 46% on the conventional net washed 2 times, 2% for the conventional net washed 20 times, 90% on the lambda-cyhalothrin LN washed 20 times and 76% for the lambda-cyhalothrin LN washed 26 times. Mortality on these nets was 24% for the conventional net washed 2 times, 10% for the conventional net washed 20 times, 80% for the lambda-cyhalothrin LN washed 20 times and 52% for the lambda-cyhalothrin LN washed 26 times.

One net in each treatment arm was not tested in the huts but used for chemical analysis by gas chromatography. Eighteen pieces were cut from each net (3 or 4 pieces taken from each side and roof of the net) before any washing was done. After washing was completed, an additional 18 pieces were cut from each net. At the end of the trial, 18 pieces were cut from one net in each arm of the study (Table 16). Before washing was

conducted, lambda-cyhalothrin content ranged from 1.42 to 1.60 g/kg (57.2 to 68.9 mg/m^2) for the lambda-cyhalothrin LNs and from 0.16 to 0.19 g/kg (6.7 to 7.8 mg/m^2) for the conventionally treated nets (Pigeon 2008j). One of the three unwashed nets was outside acceptable tolerance limits (0.94 to 1.56 g/kg). After washing, lambda-cyhalothrin content on the lambda-cyhalothrin LN washed 20 times fell to 0.63 g/kg (28.2 mg/m^2) while that on the lambda-cyhalothrin LN washed 26 times fell to 0.47 g/kg (19.6 mg/m^2). For the conventionally treated nets washed 2 or 20 times, lambda-cyhalothrin content after washing was 0.02 g/kg (0.7 mg/m^2) and <0.01 g/kg ($\leq$0.4 mg/m^2), respectively. At the conclusion of the study, the lambda-cyhalothrin content on the unwashed Lambda-cyhalothrin LN remained unchanged at 1.43 g/kg (62.0 mg/m^2). Regular use of the lambda-cyhalothrin LN did not affect the washed nets either. At the end of the study, the lambda-cyhalothrin content on nets washed 20 or 26 times was 0.60 g/kg (25.4 mg/m^2) and 0.44 g/kg (17.9 mg/m^2), respectively. Lambda-cyhalothrin content on the conventionally treated nets washed 2 or 20 times was 0.01 g/kg (0.5 mg/m^2) and <0.01 g/kg (<0.4 mg/m^2), respectively. Before the washings were completed, the within-net RSD in lambda-cyhalothin content of the unwashed lambda-cyhalothrin LN was 21.5%. The RSD of the lambda-cyhalothrin LN to be washed 20 times was 16.9% and the RSD of the lambda-cyhalothrin LN to be washed 26 times was 21.9%. The RSD of the conventional net to be washed 2 times was 44.1% and the RSD of the conventional net to be washed 20 times was 61.3%. After the washings were completed, the RSD of the unwashed lambda-cyhalothrin LN was 29.4%, the RSD of the lambda-cyhalothrin LN washed 20 times was 38.6% and the RSD of the lambda-cyhalothrin LN washed 26 times was 19.6%. The RSD of lambda-cyhalothrin content on the conventionally treated nets after washing 2 times was 5.3%. After washing 20 times, the lambda-cyhalothrin was below the quantification limits so no RSD was given. At the end of the hut trial, the RSDs of the lambda-cyhalothrin LNs that were washed 0, 20 or 26 times were 13.4%, 22.1% and 20.3%, respectively. The RSD of the lambda-cyhalothrin content on the conventionally treated net washed 2 times was 11.1%.

Six experimental huts were used in the study. The huts were the traditional east African veranda trap design. The huts were made of concrete walls smeared with mud and an iron roof with a wooden ceiling lined with hessian cloth. The eaves were

open on all sides to allow passage of mosquitoes. Two verandas on opposite sides were screened to capture mosquitoes that exited through the eaves or windows while the verandas on the remaining two sides were left open to allow for mosquito entry. The screens on the verandas were rotated periodically to reduce any biases introduced by the position of the veranda screens. The huts were built on concrete plinths and surrounded by a water filled moat to prevent the entry of ants or other scavengers.

Volunteers slept under nets in the huts on 36 nights over 6 weeks. The nets were deliberately holed to simulate a torn net. Six holes, 4 cm x 4 cm were cut in each net, with 2 holes on each long side of the net and 1 hole at each end. The three nets per treatment arm and the sleepers were rotated through the huts in a Latin square design. Each net was tested in each hut on at least 2 nights. Mosquitoes were collected each morning from the floors, walls, exit traps and inside the nets and scored as dead or alive, fed or unfed. Live mosquitoes were held for 24 hours to assess delayed mortality. For data analysis, the number of mosquitoes captured in the veranda traps was doubled to account for mosquitoes escaping through the open verandas.

The trial took place between 31 March and 10 May 2008. An average of 24.7 *An. gambiae* females were captured each night in the huts with untreated nets. For huts with treated nets, the number of mosquitoes entering each night ranged from 14.5 to 22.3. There were significantly fewer *Anopheles gambiae* in the huts with treated nets compared to the huts with untreated nets. There was no difference in the number of *Anopheles gambiae* entering huts with a lambda-cyhalothrin LN washed 20 times (16.6 females per night) compared to huts with a conventional net washed to the point of exhaustion (17 females per night). An average of 4.2 female *An. funestus* were captured in the huts with untreated nets during the study. This was significantly higher than the number of *Anopheles funestus* captured in huts with all other treatments except for huts with conventional nets washed until exhaustion (3.7 *An. funestus* per night). The number of female *An. funestus* captured in the huts with conventional nets treated to the point of exhaustion was significantly higher than the number captured in huts with the lambda-cyhalothrin LN washed 20 times (2.3 *An. funestus* per night).

There was a high rate of exophily among all treatment groups for both *An. gambiae* and *An. funestus*. For *An. gambiae*, 77% of all mosquitoes were captured in the exit traps in huts with the untreated nets. This was significantly lower than the proportion that were captured in the veranda traps in the other treatment arms where exophily ranged from 88.5% to 94.9%. There was no difference in the rate of exophily in huts with the lambda-cyhalothrin LN washed 20 times (91.8%) compared to the conventionally treated net washed to exhaustion (93.6%). For *An. funestus*, 79.6% of mosquitoes collected in huts with untreated nets were captured in the veranda traps. This was significantly lower compared to all other treatment groups except the lambda-cyhalothrin LN washed 20 times (86.6%). The rate of exophily in the huts with the lambda-cyhalothrin LN washed 20 times was significantly lower than that observed in huts with a conventional net washed to the point of exhaustion (95.5%).

In huts with untreated nets, 32.1% of *An. gambiae* were blood fed. There were significantly more blood fed *An. gambiae* in huts with untreated nets compared to all other treatment groups where the percent of mosquitoes that were fed ranged from 8.7% to 19.7%. Blood-feeding inhibition ranged from 38.8% to 72.9%. The proportion of *An. gambiae* that were fed in huts with the lambda-cyhalothrin LN washed 20 times (13.7%) was not significantly different from the proportion that were fed in huts with conventional nets washed to exhaustion (13.9%). For *An. funestus*, 24.3% of females were fed in the huts with untreated nets. The proportion fed in the control huts was significantly higher than all other treatments except for the huts with the 20 times washed lambda-cyhalothrin LN where 18.3% of female *An. funestus* were blood fed. Blood-feeding inhibition ranged from 24.7% to 63.2%. The proportion of blood fed *An. funestus* in the huts with the lambda-cyhalothrin LN washed 20 times was not significantly different from the proportion blood fed in huts with the conventional net washed to the point of exhaustion (9.0%).

Mortality of *An. gambiae* was 11.8% in the huts with untreated nets. This was significantly lower than the mortality observed in all other treatments. The overall killing effect for the treated nets ranged from 30.9% to 47.2%. For huts with the lambda-cyhalothrin LN washed 20 times, mortality was 87.5% and was

significantly higher than the mortality rate in huts with the conventional net washed to exhaustion (65.9%). For *Anopheles funestus*, mortality in the huts with untreated nets was 15.1%, which was significantly lower than the mortality observed in all other treatment arms. The overall killing effect for *Anopheles funestus* ranged from 26.3% to 46.1%. The mortality of *Anopheles funestus* in huts with the lambda-cyhalothrin LN washed 20 times was 84.1% which was significantly greater than the mortality of *Anopheles funestus* in huts with the conventional net washed to the point of exhaustion (56.7%).

The lambda-cyhalothrin LN washed 20 times showed similar efficacy in terms of blood-feeding inhibition for both *Anopheles gambiae* and *Anopheles funestus* compared to a conventional net washed to the point of exhaustion. In terms of mortality and killing effect, the lambda-cyhalothrin LN washed 20 times showed greater efficacy against both *Anopheles gambiae* and *Anopheles funestus* compared to a conventional net washed to exhaustion. It was therefore concluded that the lambda-cyhalothrin LN met the criteria for Phase II testing.

6.3 Conclusions and recommendations

The lambda-cyhalothrin LN of Syngenta, Switzerland (= ICON MAXX-Net), is a factory-produced polyester mosquito net treated with the slow-release capsule suspension of lambda-cyhalothrin, ICON 10 CS. The insecticide is coated on polyester netting at the target dose of 50 mg AI/m^2, using a polymer as a binder. Lambda-cyhalothrin CS has previously been evaluated by WHOPES and has been recommended for treatment of mosquito nets.[1]

The manufacturer has disclosed the nature of the binder used in coating the LN and has confirmed that it is the same as the

[1] *Report of the fourth WHOPES Working Group Meeting, WHO/HQ, Geneva, 4–5 December 2000*. Geneva, World Health Organization, 2001 (WHO/CDS/WHOPES/2001.4; available at: http://whqlibdoc.who.int/hq/2001/WHO_CDS_WHOPES_2001.4.pdf; accessed January 2009).

binder used in making ICON MAXX mosquito net treatment kits[1] already subject to the WHO safety assessment.

The WHO assessment of the compliance of the manufacturer's assessment of exposure to and risks of washing and sleeping under a lambda-cyhalothrin LN was in line with the WHO generic risk assessment model although some default values of the guideline were not used but appropriately justified. The assessment concluded that washing or sleeping under the LN does not pose undue risk to adults, children or newborns.

The performance of two batches of lambda-cyhalothrin LN in standard laboratory testing in the same WHO collaborating centre was different. In one study the mortality in WHO cone bioassays was consistently below the efficacy criteria while knock-down was 100% through 20 washes. In the second study mortality fell below the criteria after just one wash and knock-down fell below the criteria after 15 washes. However in the second study the tunnel test performed on nets washed 20 times met the WHOPES criteria.

Field studies demonstrated a better or equal impact of the lambda-cyhalothrin LN washed 20 times on mortality and blood-feeding inhibition of prominent malaria vectors compared with that of conventionally treated polyester nets (15 mg/m² lambda-cyhalothrin) washed until just before exhaustion. This confirms that the lambda-cyhalothrin LN fulfils the WHOPES main efficacy criteria of Phase II studies.

Chemical analysis of the lambda-cyhalothrin LN, however, indicated high within and between-net variability. The within-net RSD of the lambda-cyhalothrin content of an unwashed lambda-cyhalothrin LN ranged from 16.9% to 29.4% in 2 separate studies (Table 16). The between-net RSD of the lambda-cyhalothrin content of the unwashed lambda-cyhalothrin LN was 24.5% in one study. Furthermore, the lambda-cyhalothrin content of 3 of 6 nets tested exceeded the target dose by >25% in WHOPES Phase II trials. According to

[1] *Report of the eleventh WHOPES Working Group Meeting, WHO/HQ, Geneva, 10–13 December 2007*. Geneva, World Health Organization, 2008 (WHO/HTM/NTD/WHOPES/2008.1; available at: http://whqlibdoc.who.int/hq/2008/WHO_HTM_NTD_WHOPES_2008.1_eng.pdf; accessed January 2009).

the WHO guidelines, the acceptable within-net variability of the AI should not exceed 5% RSD when five pieces of 25 cm x 25 cm are analyzed as a single sample. Moreover, the average AI content between nets should not exceed ± 25% of the declared AI content[1].

Noting the above, the meeting concluded:

> that the high within-net and between-net variability in the content of lambda-cyhalothrin may affect its performance under routine domestic use over the lifetime of the net. This will also impact the development of standards and methods for quality control of this product. The manufacturer is therefore urged to reduce the variability of lambda-cyhalothrin content in conformity with limits recommended by WHO.

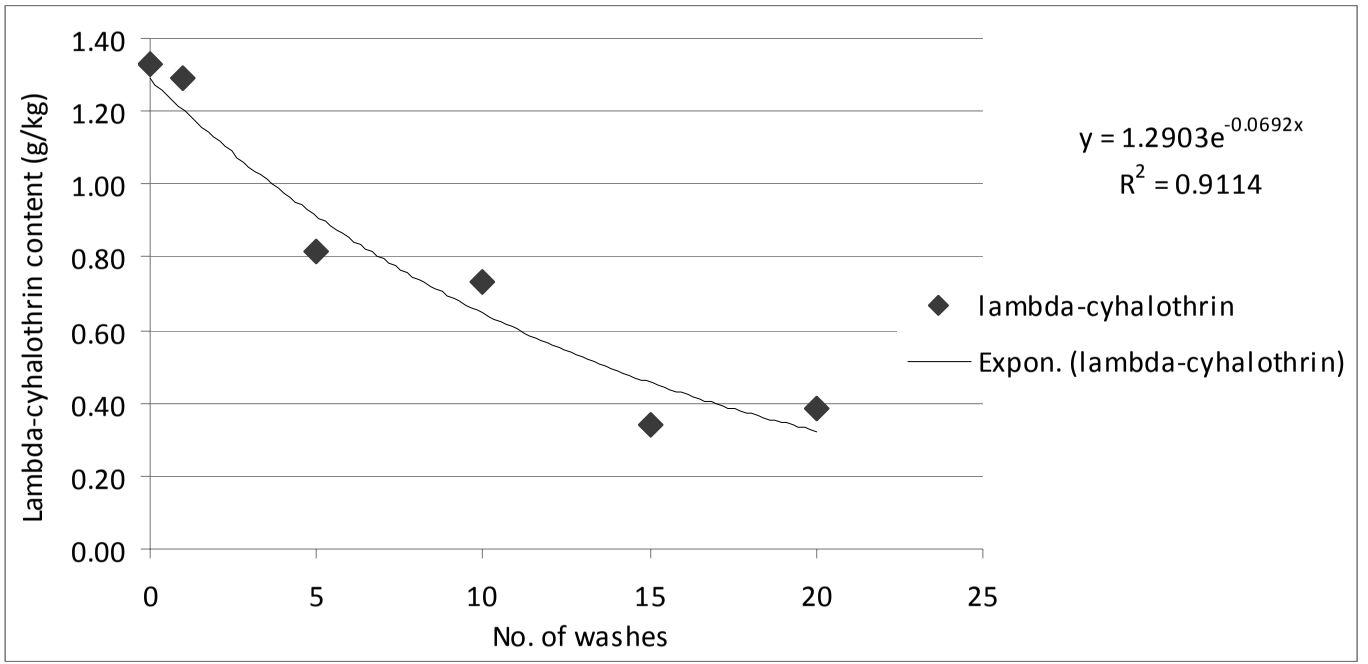
[1] FAO/WHO (2006). *Manual on development and use of FAO and WHO specifications for pesticides*. Revised first edition, Rome, Food and Agriculture Organization of the United Nations (available at http://www.who.int/whopes/quality/en/; accessed January 2009).

Table 15 **Lambda-cyhalothrin (LCH) content and retention (wash curve) of LCH long-lasting insecticidal mosquito net in a laboratory wash resistance study[a] (target dose and tolerance limits for lambda-cyhalothrin in lambda-cyhalothrin LN (100 denier) = 1.25 g/kg ± 25% [0.94 g/kg - 1.56 g/kg])**

Wash	LCH content (g/kg)	Between-net RSD (%)	LCH retention (% of wash 0)	Average LCH retention (% at each wash)
0	1.33	24.5		
1	1.29	32.0	96.8	96.8
5	0.81	27.5	61.1	90.6
10	0.73	52.7	54.7	94.2
15	0.34	21.6	25.8	91.4
20	0.39	37.3	29.1	94.0

[a] Finot et al., 2008; Pigeon 2008h

Figure 7 **Lambda-cyhalothrin content and retention (wash curve) for lambda-cyhalothrin long-lasting insecticidal mosquito net (WHOPES Phase I)[a]**



[a] Target dose and tolerance limits for lambda-cyhalothrin in lambda-cyhalothrin LN (100 denier) = 1.25 g/kg ± 25% [0.94 g/kg - 1.56 g/kg].

Table 16 **Lambda-cyhalothrin (LCH) content and retention in lambda-cyhalothrin long-lasting insecticidal mosquito net (LN) in WHOPES Phase II studies in Burkina Faso and the United Republic of Tanzania (target dose and tolerance limits for LCH in LCH LN (100 denier) = 1.25 g/kg ± 25% [0.94 g/kg - 1.56 g/kg])**

a. Before washing

Treatment	Burkina Faso		UR Tanzania	
	LCH content (g/kg)	Within-net RSD (%)	LCH content (g/kg)	Within-net RSD (%)
LN 0 wash	1.72	17.8	1.42	21.5
LN 20 wash	1.47	17.2	1.60	16.9
LN exhausted	–	–	1.56	21.9
Conventionally-treated net 0 wash	0.36	21.5	–	–
Conventionally-treated net 20 washes	–	–	0.16	61.3
Conventionally-treated net exhausted	0.22	43.1	0.19	44.1
Untreated net	<0.01	–	–	–

Table 16 (continued) **Lambda-cyhalothrin (LCH) content and retention in lambda-cyhalothrin long-lasting insecticidal mosquito net (LN) (WHOPES Phase II)**

b. After washing, before testing

Treatment	Burkina Faso			UR Tanzania		
	LCH content (g/kg)	Within-net RSD (%)	LCH retention (% of wash 0)	LCH content (g/kg)	Within-net RSD (%)	LCH retention (% of wash 0)
LN 0 wash	1.75	24.9		1.53	29.4	
LN 20 washes	0.83	7.9	47	0.63	38.6	41
LN exhausted	–	–		0.47	19.6	31
Conventionally-treated net 0 wash	0.33	14.3		–	–	
Conventionally-treated net 20 washes	–	–		<0.01	–	
Conventionally-treated net exhausted	0.10	21.0	30	0.02	5.3	
Untreated net	<0.01	–		–	–	

Table 16 (continued) **Lambda-cyhalothrin (LCH) content and retention in lambda-cyhalothrin long-lasting insecticidal mosquito net (LN) (WHOPES Phase II)**

c. After testing

Treatment	Burkina Faso		UR Tanzania	
	LCH content (g/kg)	Within-net RSD (%)	LCH content (g/kg)	Within-net RSD (%)
LN 0 wash	0.85[a]	83.1[a]	1.43	13.4
LN 20 washes	0.99	33.0	0.60	22.1
LN exhausted	–	–	0.44	20.3
Conventionally-treated net 0 wash	0.28	16.7	–	–
Conventionally-treated net 20 washes	–	–	<0.01	–
Conventionally-treated net exhausted	0.15	11.2	0.01	11.1
Untreated net	<0.01	–	–	–

[a] These values are outliers and were not considered.

7. GENERAL RECOMMENDATIONS

The twelfth WHOPES working group meeting made the following general recommendations:

- that as part of the Phase III evaluation of all types of LNs, WHOPES should commission cross-sectional surveys from multiple countries in addition to supervised longitudinal studies recommended in WHOPES guidelines;

- that the research community should conduct Phase II experimental hut studies of nets that have been used under routine household use for several years with varying levels of bio-efficacy and varying numbers of holes to better understand the long-term effectiveness of LNs;

- that WHOPES should develop guidelines to evaluate durability as part of Phase III supervised longitudinal studies and cross-sectional surveys of LNs that are in household use for three years;

- that LN manufacturers should provide all LNs with wash-resistant labels (tags) indicating the date of manufacture, denier and other information as recommended by WHO;

- that the research community and other stakeholders should develop methods and criteria for the evaluation of resistance-breaking tools.

ANNEX I: LIST OF PARTICIPANTS

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ANNEX III. QUESTIONNAIRE - WHOPES LARGE-SCALE TESTING AND EVALUATION OF PERMANET 2.0

Five digit survey code (first two digits country; one digit village; two digits for sample: ----------

Country …….
Province ……
District …… …
Village ……..
nearest town …….

Date of survey (DD/MM/YY) ……

Specify exact location of household in the village …….
……

Batch number of the net sampled (if readable)…….
(attach the LN label to the form)

Date of production of the net (if readable) ……

Information on net usage provided by:

1) User of this net
2) Caretaker of those using the net
3) Head of household
4) Other (specify) ……

Date of receipt/purchase of the net: ---------- (months)

Information on net usage:

1) Year-round and every night.
2) Year-round but occasionally.

3) Seasonally but every night.
4) Seasonally and occasionally

How is the net used?

1) Hanging over the bed
2) Hanging over sleeping mat/mattress on the ground
3) Other (specify) ……..

How did you get the net ?

1) Paid or purchased yourself?
2) Given free
3) Specify

How the net was last washed? ……

When was the last time you washed the net? ……(month)

How frequently you wash the net? ……(month)

How many times a net is washed in a year time? ……. times

How was the net last washed?

Water:

1) cold …. 2) warm …. 3) hot ….

Soap:

1) village (local)-made soap
2) Commercial bar
3) Commercial powder
4) mix of soap and powder

Soaking:

1) yes …. 2) No ….
If yes, how long? ……. (Hour)

Rinsing:
1) yes …. 2) No ….

Rubbing against rocks/stone:
1) yes …. 2) No ….

Where net was dried:
1) inside … 2) outside

Physical Inspection of the net

Net is found to have holes
1) Yes … 2) No …

If yes, use the following code for sizes of holes
1) hole smaller than will allow a thumb to pass through
2) a larger hole, but will not allow a closed fist to pass through
3) hole bigger than a closed fist

Total number of holes:
…….. total size 1
…….. total size 2
…….. total size 3

Total number of holes:
…….. lower half of the net
…….. upper half of the net
…….. roof

Total number of open/failed seams using the size coding provided above:
…….. total size 1
…….. total size 2
…….. total size 3

Total number of repairs:

….. with stitches
….. with knots
….. with patches

Total number of holes due to burns? # …….

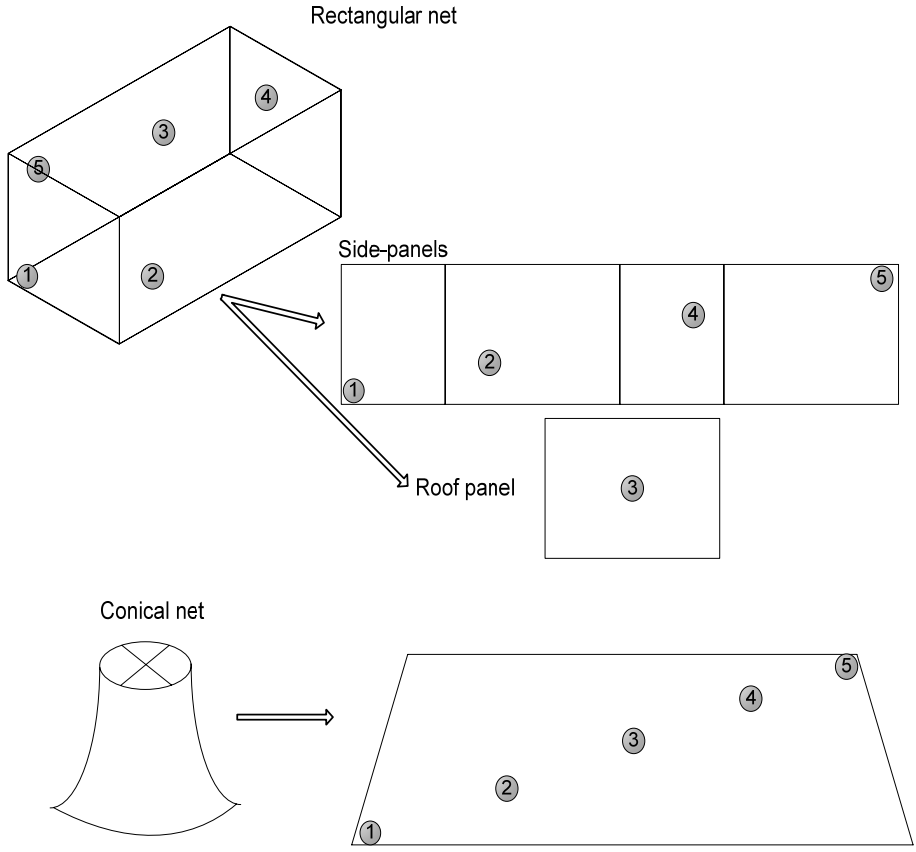
Presence of patches, nods or apparent repairs on the net

Aspect of net:

1) clean
2) a bit dirty
3) dirty
4) very dirty

Name and signature of
investigator ……

Figure 1 **Sample preparation for determination of deltamethrin content and biological assays (position 1 was ignored as it may have been subjected to excessive abrasion and is the part that is supposed to be tucked under the mattress)**



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