Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Comparing surface and total insecticide content as predictors of bioefficacy in PBO-pyrethroid nets across durability trials

Emma Reid
emma.reid@lstmed.ac.uk
Liverpool School of Tropical Medicine
Frank Mechan
Innovative Vector Control Consortium
Gemma Harvey
Liverpool School of Tropical Medicine
Janet Hemingway
Infection Innovation Consortium
Victoire Nsabatien
Liverpool School of Tropical Medicine
Nono Mvuama
University of Kinshasa
Fiacre Agossa
Abt Associates
Josue Zanga
University of Kinshasa
David Weetman
Liverpool School of Tropical Medicine

Research Article

Keywords: Insecticide treated bed nets, ITN, Surface availability, HPLC, Mosquitoes, PBO

Posted Date: November 24th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11693787/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Comparing surface and total insecticide content as predictors of bioefficacy in PBO-pyrethroid nets across durability trials.

Emma Reid[1], Frank Mechan[2], Gemma Harvey[1], Janet Hemingway[1,3], Victoire Nsabatien[1,5], Nono Mvuama[5], Fiacre Agossa[4,5], Josue Zanga[5], David Weetman[1].

Author Affiliations:

[1]Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, L3 5QA, UK
[2]Innovative Vector Control Consortium, Pembroke Place, Liverpool, L3 5QA, UK
[3]Infection Innovation Consortium, Liverpool Life Sciences Accelerator, 1 Daulby Street, Liverpool L7 8XZ, UK
[4]Abt Associates, Kinshasa, Democratic Republic of Congo.
[5]Kinshasa School of Public Health, University of Kinshasa, Kinshasa, Democratic Republic of Congo.

Corresponding Author: Emma Reid emma.reid@lstmed.ac.uk

Abstract

Insecticide treated bed nets (ITNs) are the key malaria vector control tool across sub-Saharan Africa with well-proven impact in reducing malaria cases and deaths since the advent of mass distributions. Durability of ITNs is a persistent concern however raising concerns over current distribution cycle lengths. Methods to test ITN durability over time use physical examination for holes and mosquito bioassays to assess bioefficacy, supplemented by chemical assessment of the total active ingredient (A.I) present in net fibres. The latter represents a potential mismatch if A.I in the total fibre is not representative of that contacted by mosquitoes exposed to the net surface.

We test the hypothesis that measuring A.I. on the surface could be a more informative approach for chemical durability assessment. To investigate this, we analysed the bioefficacy and chemical integrity of ITNs collected at intervals across 3 years as part of 3 field trials. Surface and total A.I. content was measured using HPLC and bioefficacy assessed with mosquito colonies of known resistance profiles using WHO cone assays. Chemical and bioassay data from the same nets were compared to assess which of surface or total A.I is the better predictor of mosquito knockdown and mortality.

The unused nets continued to regenerate surface A.I over the study period. In contrast, whilst used ITNs regenerated surface A.I, the concentration was insufficient to replace that lost during use, resulting in reducing surface content over time. Surface content of both pyrethroid insecticides and the synergist PBO were better predictors of knockdown and mortality than complete A.I for each net type tested.

In conclusion, our data support the prediction that surface content is a more accurate measure of bioefficacy of ITNs than total A.I content, as well as validating the solvent stripping method used here for surface chemical analysis. The use of this method should improve durability

analysis and give a more informative estimation of net longevity, rather than relying on bioassays with mosquito colonies which are known to show high heterogeneity, especially among testing locations.

Keywords: *Insecticide treated bed nets, ITN, Surface availability, HPLC, Mosquitoes, PBO*

1. Background

Malaria remains a major public health problem with an estimated 282 million cases and 610 thousand deaths reported in 2024, with progress toward malaria elimination having stalled in the last decade. Sub-Saharan Africa accounts for 94% of cases worldwide and 95% of malaria deaths, with Nigeria, the Democratic Republic of Congo (DRC), Uganda, Ethiopia and Mozambique accounting for over half of all cases (1). Increasing vector resistance to insecticides, *Plasmodium* drug resistance and the effects of regional instability can all increase the transmission of malaria. In addition, recent reductions in international funding, have limited vector control intervention coverage potentially contributing to increased transmission (1).

Indoor residual spraying (IRS) and insecticide treated nets (ITNs) are proven insecticide-based vector control methods to reduce malaria, with broad-scale coverage of ITNs being the most common intervention. Treated nets protect the user by providing a physical barrier from blood-seeking females and killing those that come into contact with the net, thus providing both personal and community protection (2). Across Sub-Saharan Africa approximately 181 million nets were distributed in 2024, which were estimated to avert over 100 million clinical cases of malaria (Malaria Atlas Group manuscript in prep) For many communities this is the only programmatic source of vector management, with only 3.2% of the at-risk population in Africa protected by IRS in 2024 (1). The WHO recommends mass ITN distribution campaigns should be repeated approximately every 3 years (3), however previous studies have indicated the ITNs

77 can lose insecticide content well before their replacement (4), with some studies showing nets
78 lost bioefficacy against pyrethroid resistant mosquitoes after 18 months (4).

79 For many years all ITNs were treated with a pyrethroid insecticide. As pyrethroid resistance
80 increased, new net types were developed. For several years, the most common 'second
81 generation' ITN type has been a pyrethroid with addition of a synergist, piperonyl butoxide
82 (PBO). Within resistant mosquito populations, P450 enzymes, which can metabolise
83 pyrethroids before they reach their target site, are often found to be upregulated. PBO binds to
84 P450s and inhibits their action and can restore mosquito susceptibility to pyrethroids (5).

85 At present, ITNs are treated with active ingredients (A.I) by one of two methods: incorporation or
86 impregnation (also known as coating). Incorporated nets are currently made from polyethylene
87 and are produced by mixing the A.I into the plastic before it is extruded into fibres. This
88 produces a reserve in the centre of the fibre enabling the insecticide to regenerate to the
89 surface over time, with the aim of maintaining a near constant level of insecticide at the surface
90 (6). Coated nets are presently made from polyester and are treated by binding the A.Is to the
91 fibres after their production, resulting in all insecticide being bound within the surface coating.
92 These different treatment methods may result in variation in how much insecticide is
93 bioavailable (i.e. presented at the surface) to mosquitoes, as well as potentially requiring
94 different laboratory techniques to measure chemical durability.

95 Currently, durability studies of ITNs focus on the total insecticide rather than bioavailable
96 insecticide (4, 7-9). Whilst a large percentage of the target dose of insecticide may remain on
97 the net, it may not be accessible to landing mosquitoes. Durability studies that use mosquito
98 bioassays, most commonly the WHO cone assay, to assess net efficacy exhibit high
99 heterogeneity and poor reproducibility resulting in comparability issues (10). The production of
100 a standardised chemical ITN testing method would enable a more direct estimation of net

efficacy and durability, better facilitating reproducibility among locations to compliment bioassay test results.

As part of three field trials, conducted in Sud-Ubangi province DRC, Tanganika province DRC, and Busia, Uganda, each involving different ITN-types, net samples were collected at different, increasing, time points. The aims of this study were to investigate the durability of each ITN, comparing insecticide loss from incorporated and coated ITNs to evaluate whether treatment methods affect insecticide retention. Furthermore, the study aimed to test whether surface active ingredient content is a stronger predictor of mortality than total on incorporated nets.

2. Materials and Methods

2.1. Study site

Net samples were collected from 3 different field trials across Democratic Republic of Congo (DRC) and Uganda.

In trial 1, net samples were collected from Sud-Ubangi province, northern DRC across 6 health zones: Bominenge, Bogosenubea, Gemena, Tandala, Bulu, and Bwamanda. Nets were collected at 7, 14, 24 and 36 months after distribution. This trial also contained a selection of nets that were stored in ambient conditions in a central warehouse (unused); samples were collected from the stored nets at the same time intervals as field samples to investigate regeneration without use.

In trial 2, net samples were collected from Tanganika province, south-western DRC. Nets were collected at the time of distribution (baseline; unused), 12 months and 24 months after distribution.

In trial 3, net samples were collected from Busia, eastern Uganda as part of the LINEUP trial (4, 8). Nets were collected prior to the trial starting (baseline; unused), 12 months and 25 months after distribution.

2.2. Net types

Overall, four different types of ITNs were assessed, which were treated with different pyrethroids, some with the addition of PBO. Trial 1: PermaNet 3.0 and PermaNet 2.0 (Data shown in Figure S1 & Table S2); trial 2 Veeralin and trial 3 Olyset Plus. Full details of each net type used can be found in Table 1.

137 Table 1 - Net types from each trial with target doses, treatment methods and material.

Trial	Net Type	Manufacturer	Target dose (accepted range)	Denier	Material	Method of Treatment
1	PermaNet 2.0	Vestergaard Sarl	1.4 g/kg (±0.35 g/kg) of deltamethrin	100	Polyester	Coated
1	PermaNet 3.0	Vestergaard Sarl	Roof - 4.0 g/kg (± 1.0 g/kg) deltamethrin and 25 g/kg (± 2.5 g/kg) PBO. Side - 2.1 (± 1.0 g/kg) deltamethrin.	Roof-100 Side -100	Roof - Polyethylene Sides - Polyester	Roof - Incorporated Side - Coated
2	Veeralin	V.K.A. Polymers Pvt. Ltd	6 g/kg of Alpha-cypermethrin and 2.2 g/kg of PBO	130	Polyethylene	Incorporated
3	Olyset Plus	Sumitomo Chemical Ltd	20 g/kg (± 5.0 g/kg) permethrin and 10 g/kg (± 2.5 g/kg) PBO	150	Polyethylene	Incorporated

138

2.3 Cone Assays

Following the WHO cone assay procedure (11) bioefficacy was assessed for all nets. All mosquitoes were unfed adult females, 3-5 days old, reared and tested in temperature- and humidity-controlled insectaries (25 ± 2 °C, 80 ± 10 %). An exposure time of 3 minutes was used and a total of 20 - 25 mosquitoes per net piece for each strain.

Trail 1 used PermaNet 3.0 and PermaNet 2.0 nets. For the PermaNet 3.0 samples, 1 roof piece and 1 side piece were tested, and 1 side piece was used for PermaNet 2.0 samples. Two different *Anopheles gambiae s.l* mosquito strains were used. The Kisumu strain was originally collected in Kisumu, Kenya in 1975 and is pyrethroid-susceptible. The Tiassalé strain was established in 2012 after collection in Tiassalé, Côte d'lvoire and shows multiple insecticide resistance underpinned by *kdr* mutations, *Ace-1* mutation and elevated P450 expression, and shows PBO synergism of pyrethroids in WHO tube bioassays. Both strains are maintained in Liverpool at the LITE facility.

In trial 2, either a side piece or a roof piece was randomly selected for cone assay. The Tiassalé strain detailed above was used initially, however due to problems at the time with reestablishment of the colony after maintenance, which caused a drop in resistance (notable via a marked drop in knockdown resistance mutation frequency) the colony was substituted with FuMoz. FuMoz is an *Anopheles funestus s.s* strain originally colonised at the National Institute for Communicable Diseases in South Africa and reared in LITE since 2013. FuMoz is pyrethroid resistant, primarily underpinned by elevated P450 expression, and shows PBO synergism of pyrethroids in tube bioassays.

For trial 3 an *Anopheles gambiae* strain originally collected in 2018 from Busia, Uganda, was used in study 3, this work was completed by Mechan *et al* (4), where full details of cone testing

methods can be found. The strain shows multiple insecticide resistance such *kdr* mutations and elevated levels of P450s.

2.4. Chemical content analysis

2.4.1. Sample preparation

For complete A.I content, following WHO guidelines, samples were taken from different panels of the nets (12). For PermaNet 2.0, a sample was taken from each side plus a piece from the roof and placed into the same tube. For PermaNet 3.0 the same method was followed, however a sample from all the sides were used for the side sample then 3 pieces taken from the roof were used for the roof sample. Similar to PermaNet 2.0 nets, Veeralin nets were sampled by taking a sample from each panel as per WHO methods (13). Olyset Plus nets had been analysed by Mechan *et al* (4) a sample of the roof panel was cut for this analysis. Samples were cut into circles using a die cutter (Sizzix) and weighed. For surface A.I content, the sample that had previously been cone tested was selected, 3 circles from the sample were placed into the same tube. PermaNet 2.0 samples were not surface tested as these are coated rather than incorporated, methods for coated bioavailability are not yet available.

2.4.2. A.I content extraction and quantification

Complete A.I was extracted using a solution of heptane with internal standard dicyclohexyl phthalate (DCP) dissolved in 1-propanol (1mg/ml) giving a final volume of 100µg/ml. 5ml was added to each sample, which was then placed on a heat block at 85°C for 45 minutes. Surface A.I was extracted using methanol with internal standard DCP (1mg/ml), 5ml was added and the samples were sonicated for 15 minutes at room temperature. This method is used as methanol does not cause the fibres to swell, which results in only surface active ingredients being extracted. The methods then followed the same procedure of 1ml being added to a new tube and evaporated to dryness at 60°C. The samples were then resuspended in 1ml acetonitrile,

filtered using a 0.2µM filter and syringe, then added to glass vials for HPLC analysis. A standard curve was produced for both pyrethroid insecticides and PBO. HPLC conditions for all net types were as follows: 70% acetonitrile 30% water mobile phase; flow rate 1ml/min; 226nm wavelength; and injection volume of 20µl.

2.5. Statistical analysis

Statistical analyses were performed in R 4.5.3. Plots were produced using *ggplot2* 4.0.2. The proportion of deltamethrin lost over time from roof samples compared to side samples was analysed using a linear mixed effects models (LMM) in *lme4* 1.1.38 with net number included as a random intercept. Fixed effects were evaluated using a Type III ANOVA using *lmerTest* 3.2.0. To analyse the relationship between different active ingredient amounts and knockdown or mortality generalised linear mixed-effects models (GLMMs) in *lme4* 1.1.38), with net age as a random effect. Dose response curves were produced using *drc* 3.0.1, with *ggplot2* used to plot the curves. The number of parameters used in the model was adjusted for the best fit for each dataset. Active ingredient amounts were rounded into 14 groups to allow for dose response analysis to be conducted. Pearson rank correlations were conducted to compare surface active ingredients with complete active ingredients.

3. Results

3.1. Regeneration of active ingredients over time

Complete deltamethrin and PBO on unused PermaNet 3.0s remained within target range specified for the full 36 months, as shown in Table 2. However, surface PBO increased by 610%

from 0.50g/kg at 7 months to 3.55g/kg at 36 months. Similarly, the surface deltamethrin increased by 88.1% from 0.42g/kg at 7 months to 0.79g/kg at 36 months.

Table 2 - Complete and surface content of deltamethrin and PBO on unused PermaNet 3.0 nets which had been stored and collected at timed intervals.

Net Type	Age (months)	Complete Delta Content (g/kg) 95% CI	Surface Delta Content (g/kg) 95% CI	Complete PBO Content (g/kg) 95% CI	Surface PBO Content (g/kg) 95% CI
PermaNet 3.0 - Roof	7	3.88 (3.58-4.19)	0.42 (0.345-0.50)	18.67 (16.14-21.20)	0.49 (0.31-0.68)
	14	4.42 (3.35-4349)	0.74 (0.63-0.86)	24.64 (23.34-25.92)	2.74 (2.52-2.96)
	24	3.89 (3.73-4.05)	0.89 (0.71-1.07)	21.38 (20.00-22.75)	2.68 (1.99-3.37)
	36	3.59(3.28-3.91)	0.79-0.73-0.86)	17.60 (15.65-19.55)	3.55 (2.81-4.28)

A comparison was made between complete A.I and surface A.I for PermaNet 3.0 samples collected from the field to assess regeneration over time during use (Figure 1A). Complete PBO declined rapidly, reducing below target range after 7 months of use, complete deltamethrin reduced less rapidly over time and remained within target range up to 24 months. This rapid decline was also shown in surface PBO and surface deltamethrin. Indeed, both showed a similar correlation between complete and surface declines (deltamethrin: $r=0.517$, $n=94$, $p=0<0.001$; PBO: $r=0.572$, $n=94$, $p<0.001$).

Total PBO on roof panels declined by 71.32% between 7 months and 36 months as displayed in (Figure 1). Similarly, surface PBO declined (Figure 1B) by 73.68% over the same time period. Surface deltamethrin shown in Figure 1B, declined by 86% from 7 months to 36 months. Whereas complete deltamethrin (Figure 1A) declined by 33.87% over the same time period. Complete content for used and unused side panels of PermaNet 3.0 are found in Figure S2.

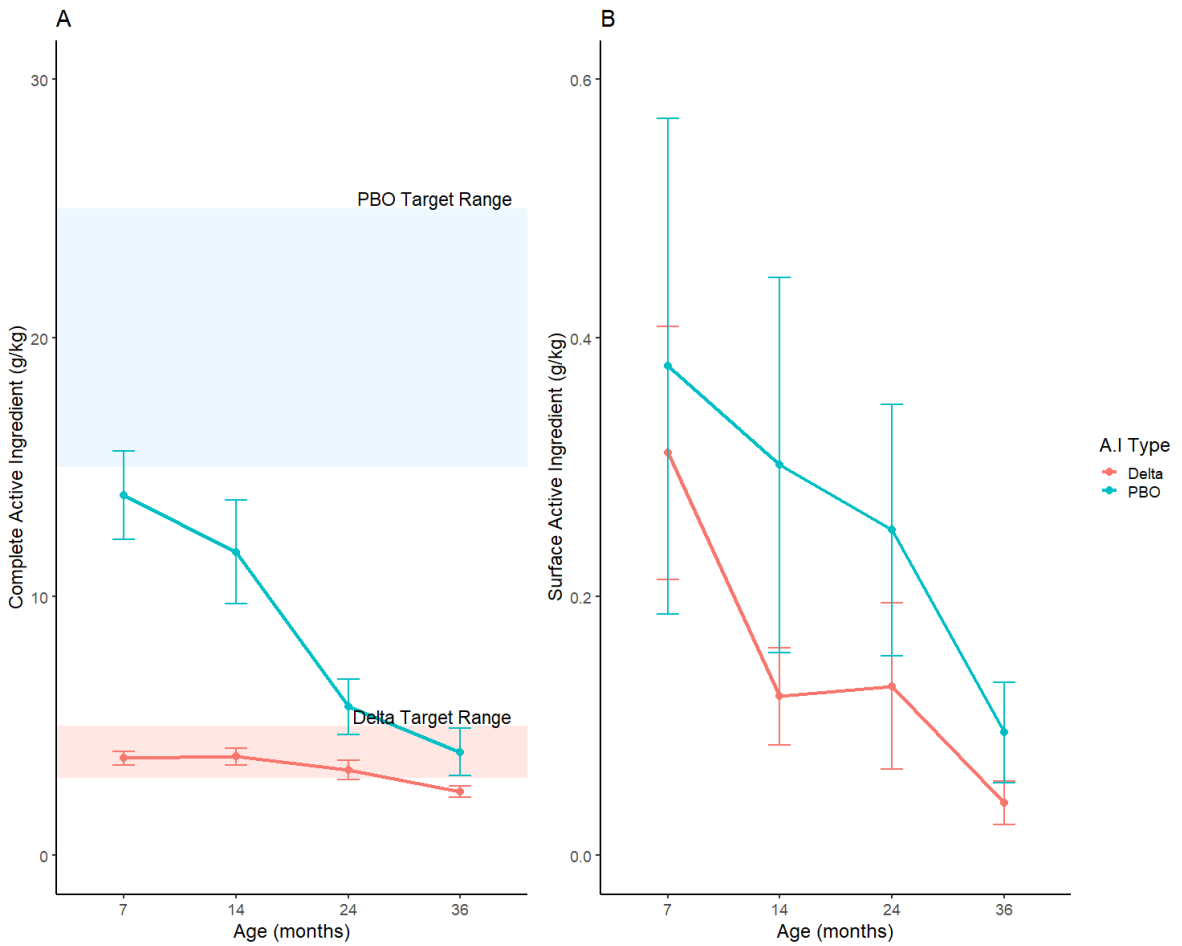


Figure 1 - Active ingredient (A.I) on used PermaNet 3.0 roof panels of different post distribution age. Left shows complete deltamethrin and complete PBO with acceptable target ranges (±20%), right shows surface deltamethrin and surface PBO. Error bars indicate 95% confidence intervals.

As the roof and sides of PermaNet 3.0 nets are treated in a different method with insecticide, this allows for a comparison of complete insecticide over time, comparing deltamethrin reduction from incorporated (roof) verse coated (sides).

After 7 months of use in the field, the incorporated roof panels retained substantially more deltamethrin than coated side panels, corresponding to proportional losses of 6.25% and 55.23%, respectively, as shown in Figure 2. The model (full results in Table S2 & Table S3) supported this: roof panels retained an estimated 49.1 percentage points more of the manufacturer target insecticide content than side panels at 7 months ($\beta = 49.1$, $p < 0.001$).

Retention declined significantly over time across both panel types ($p < 0.001$), falling by an estimated 36.9 percentage points at 36 months compared with 7 months ($p < 0.001$). There was no evidence of a panel × age interaction ($p = 0.92$), indicating that although roof panels consistently retained more insecticide than side panels, the rate of decline in retention over time did not differ between panel types.

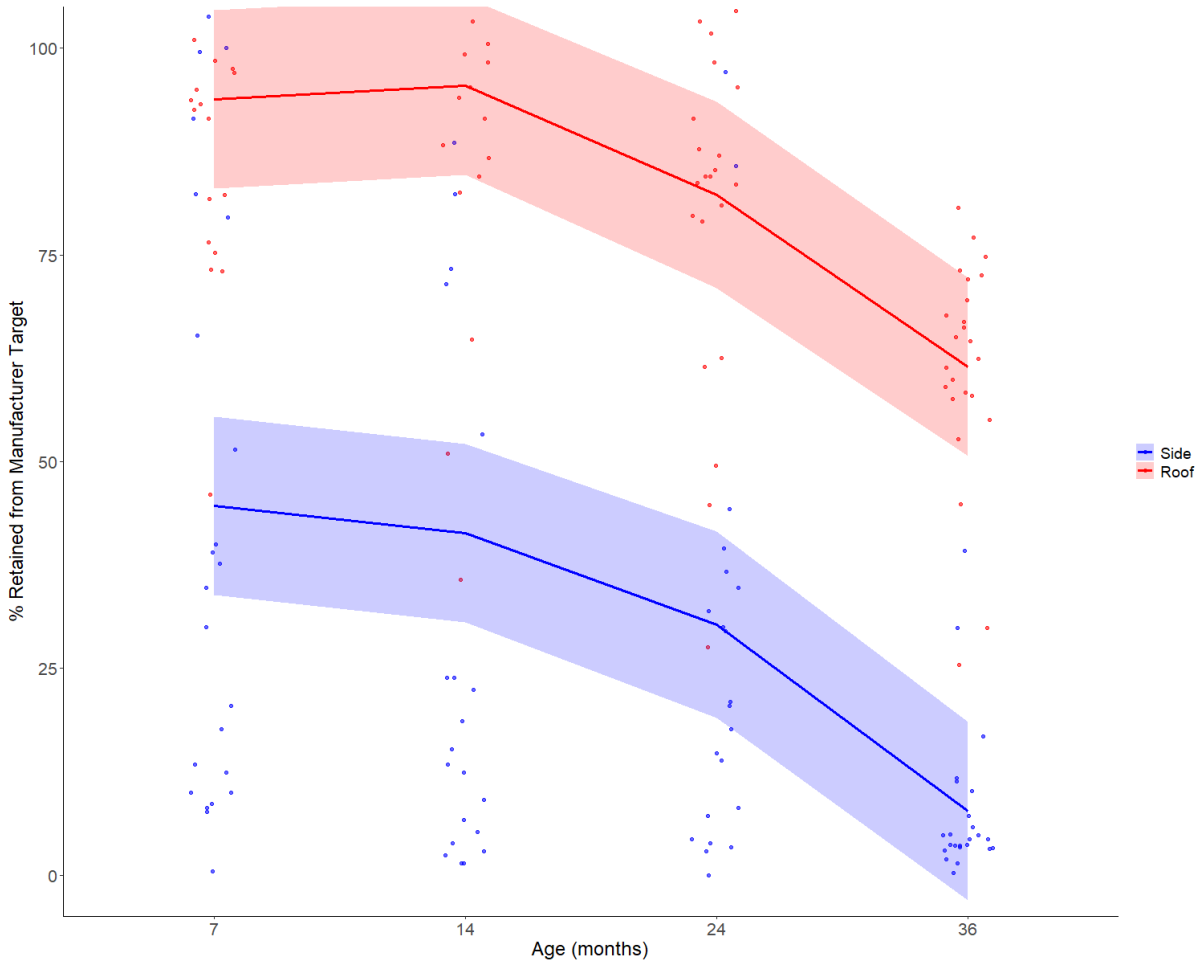


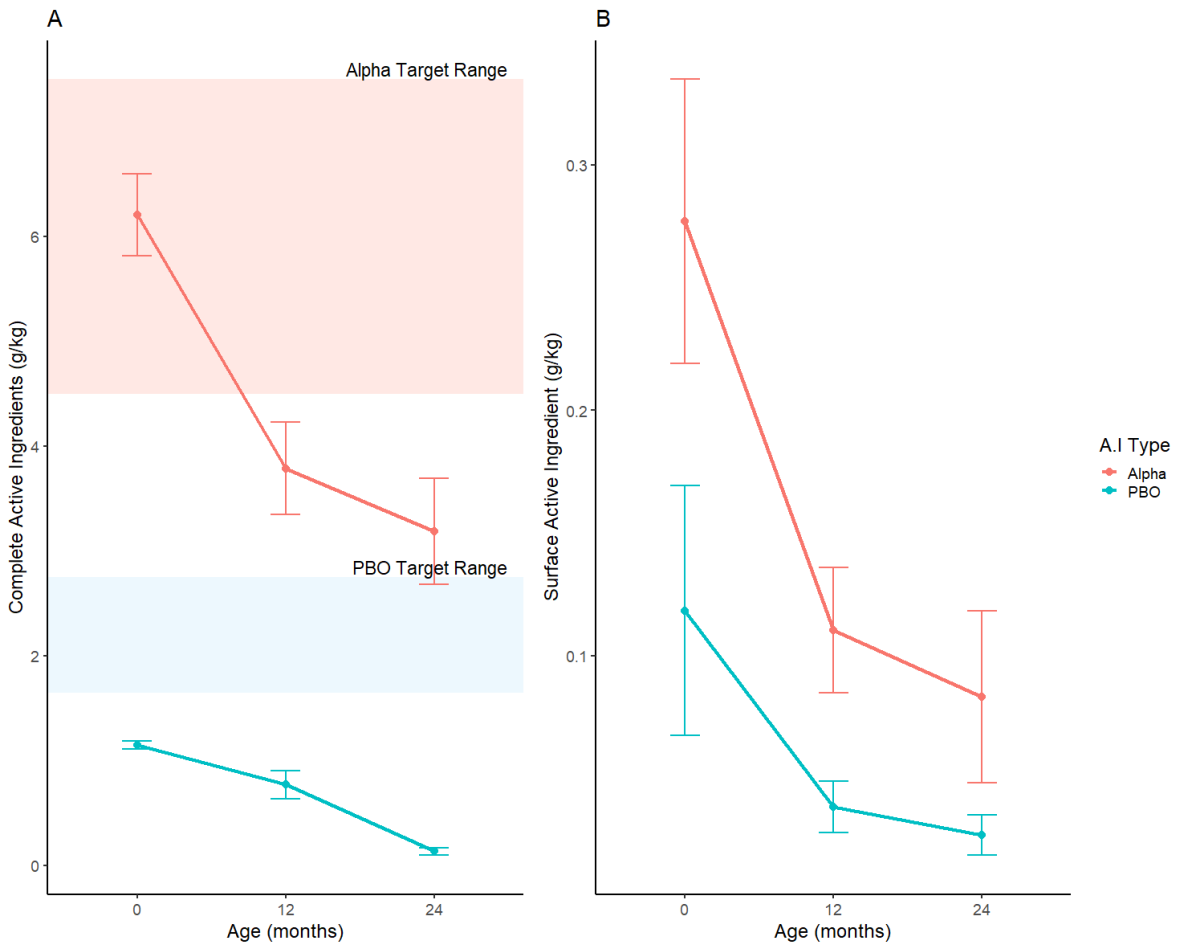
Figure 2 – Percentage of compete deltamethrin retained from manufacturer target dose over time of PermaNet 3.0 roof portions and side portions of the same net samples. 95% confidence intervals produced from mixed effects model full results displayed in Table 3. Points show observed data. Data assumes all nets started with 100% of target dose.

On Veeralin nets, the complete PBO content decreased rapidly as the nets increased in age, as did that on the surface albeit less rapidly as shown in Figure 3. In each case, the correlation between surface and complete A.I. was significant (ACM: r=0.56, p<0.001; PBO: r=0.629,

p<0.001). The compete alpha-cypermethrin (ACM) content on Veeralin nets declined rapidly after 12 months but remained consistent into 24 months. However, the surface ACM content remained consistent as the nets increased in age, showing only a slight decrease.

Despite having a target dose of 2.2g/kg of PBO, the unused nets in this study contained only 1.15g/kg (95% CI: 1.11-1.19) of PBO, which reduced by 33.04% after 12 months then again further by 83.12% after 24 months. Unused baseline nets contained 6.20g/kg of ACM which reduced by 38.87% after 12 months, reducing further by 16.09% after 24 months as displayed in Figure 3.

The surface PBO had a reduction of 66.67% after 12 months, which further reduced by 25% after 24 months. Surface ACM reduced by 60.71% after 12 months of use, then reducing by 27.27% after 24 months.



264

265 Figure 3 - A comparison of Alphacypermethrin and PBO on the surface and complete of Veeralin
266 nets collected at different operational ages. Error bars indicate 95% confidence intervals.

267

268 In Olyset plus nets, the complete permethrin content as shown in Figure 4A, remained close to
269 target for all assessed timepoints, but the surface content was low and declined with age
270 (Figure 4B), and the two measures were uncorrelated (r=0.21, p=0.489). However, for PBO, the
271 full content reduced rapidly as the nets increased in age, and the surface PBO also decreased
272 but at a much slower rate (r=0.87, p<0.001).

273

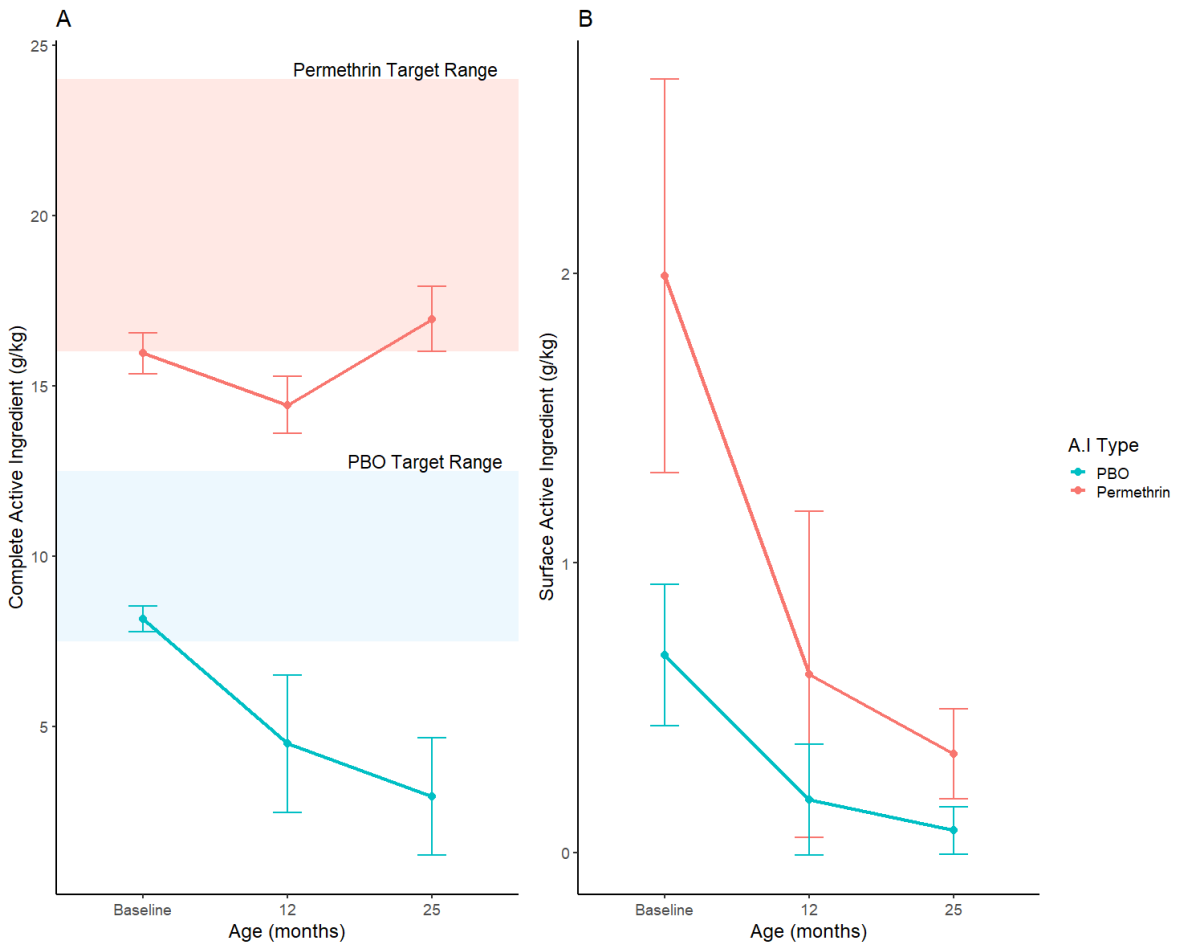


Figure 4 - A comparison of Permethrin and PBO on the surface and complete of Olyset Plus nets. Error bars indicate 95% confidence intervals.

3.2. Net efficacy in bioassays

Tiassalé knockdown on PermaNet 3.0 nets, declined as net age increased with both surface and complete active ingredient concentrations also reducing (Figure 5). Total deltamethrin content was a strong predictor of knockdown ($p < 0.001$), while surface PBO also significantly increased knockdown ($p = 0.003$). Surface deltamethrin showed a positive but small effect ($p = 0.082$). However, total PBO content did not independently predict knockdown ($p = 0.82$) with full model results displayed in Table S4. As with knockdown, the mortality reduced as the nets increased in age and thus reduced in A.I (Figure 5). Mortality after 24 hours was more strongly associated with surface measures than total content. Surface deltamethrin was a significant

predictor of mortality (p = 0.004), as was surface PBO (p = 0.009). Total PBO content also showed a strong positive association with mortality (p < 0.001), consistent with its role as a synergist. Total deltamethrin showed a weaker, borderline effect (p = 0.060), full model results shown in Table S5.

Overall, these results indicate that surface insecticide content more closely reflects bioavailable insecticide and provides a stronger predictor of both mosquito mortality and knockdown, than total insecticide content.

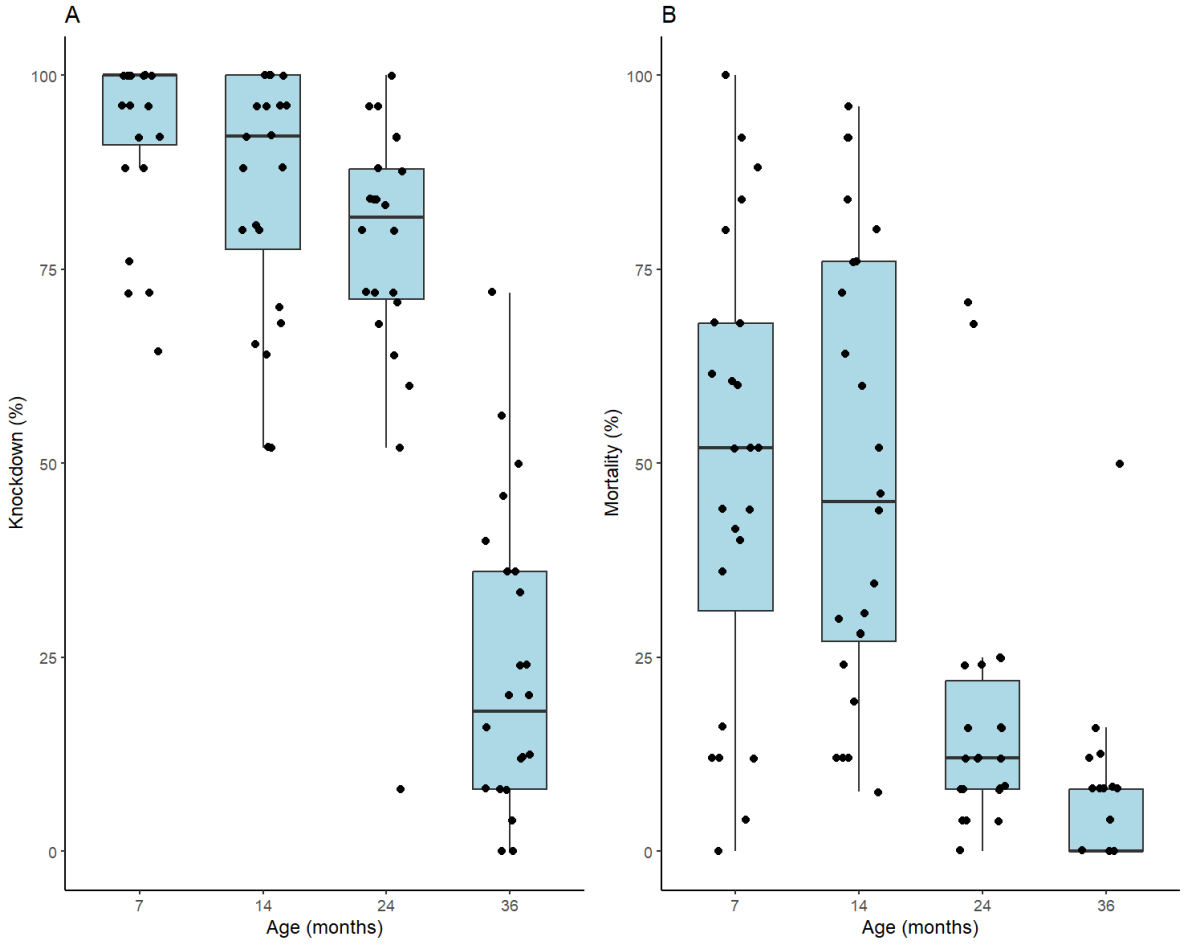


Figure 5 - Knockdown and mortality percentages for both surface and complete of the roof panels of PermaNet 3.0 that have been used in the field. Error bars show 95% confidence intervals.

When exposed to Veeralin nets knockdown with the FuMoz strain declined with increasing net age (Figure 6) however overall knockdown following exposure was low at baseline. Despite this low susceptibility, surface ACM remained the strongest predictor of knockdown ($p < 0.001$). Total ACM content did not independently predict knockdown once surface ACM was accounted for ($p = 0.83$). Surface PBO showed a positive but non-significant association with knockdown ($p = 0.078$), while total PBO had no independent effect, full model results shown in Table S7. FuMoz mortality was uniformly low across all net ages and showed limited variation with ageing (Figure 6). Perhaps due to this limited range in mortality detected, mortality was weakly predicted by total ACM content ($p= 0.008$), whereas neither surface ACM nor PBO (surface and complete) independently predicted mortality, full model results shown in Table S8.

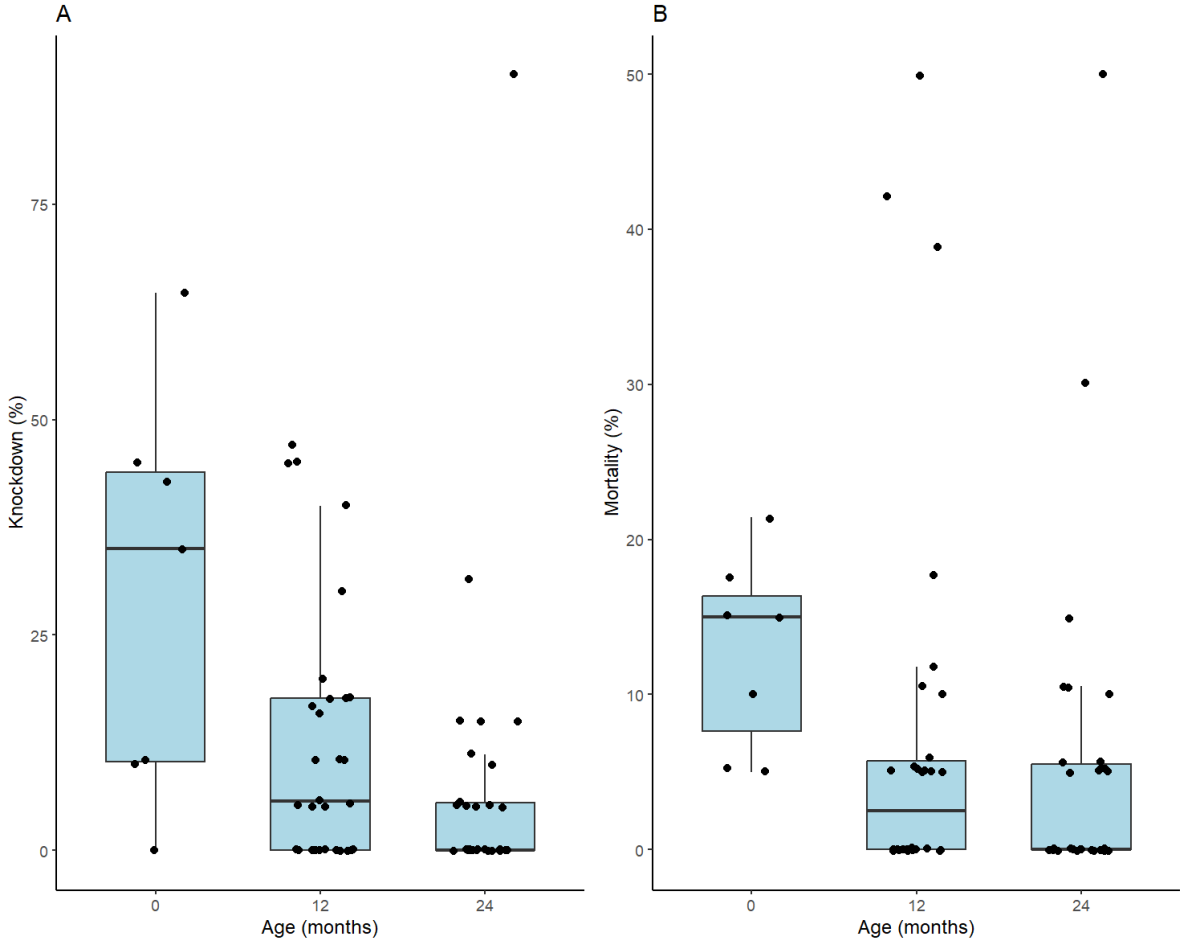


Figure 6 - Knockdown and mortality percentages of FuMoz mosquitoes for both surface and complete A.I from Veeralin Nets. Error bars indicate 95% confidence intervals.

Low sample sizes of Olyset Plus nets prevented models to be conducted, results for knockdown and mortality can be found in Figure S3.

3.3. Dose response analysis

Displayed in Figure 7 are the dose response curves for knockdown with the different active ingredient amounts from PermaNet 3.0 roof samples. Both surface deltamethrin (Figure 7C) and surface PBO (Figure 7D) have steeper and sharper curves indicating a consistent relationship with knockdown. Furthermore, LC_{50}s were lower with narrow ranges, 31.7 (95% CI:25.6-37.8) and 109.9 (95% CI:92.5-127.2) respectively. Observed data points are also more closely clustered around the curve in surface A.I plots indicating that surface A.I is a stronger predictor of knockdown on these net samples.

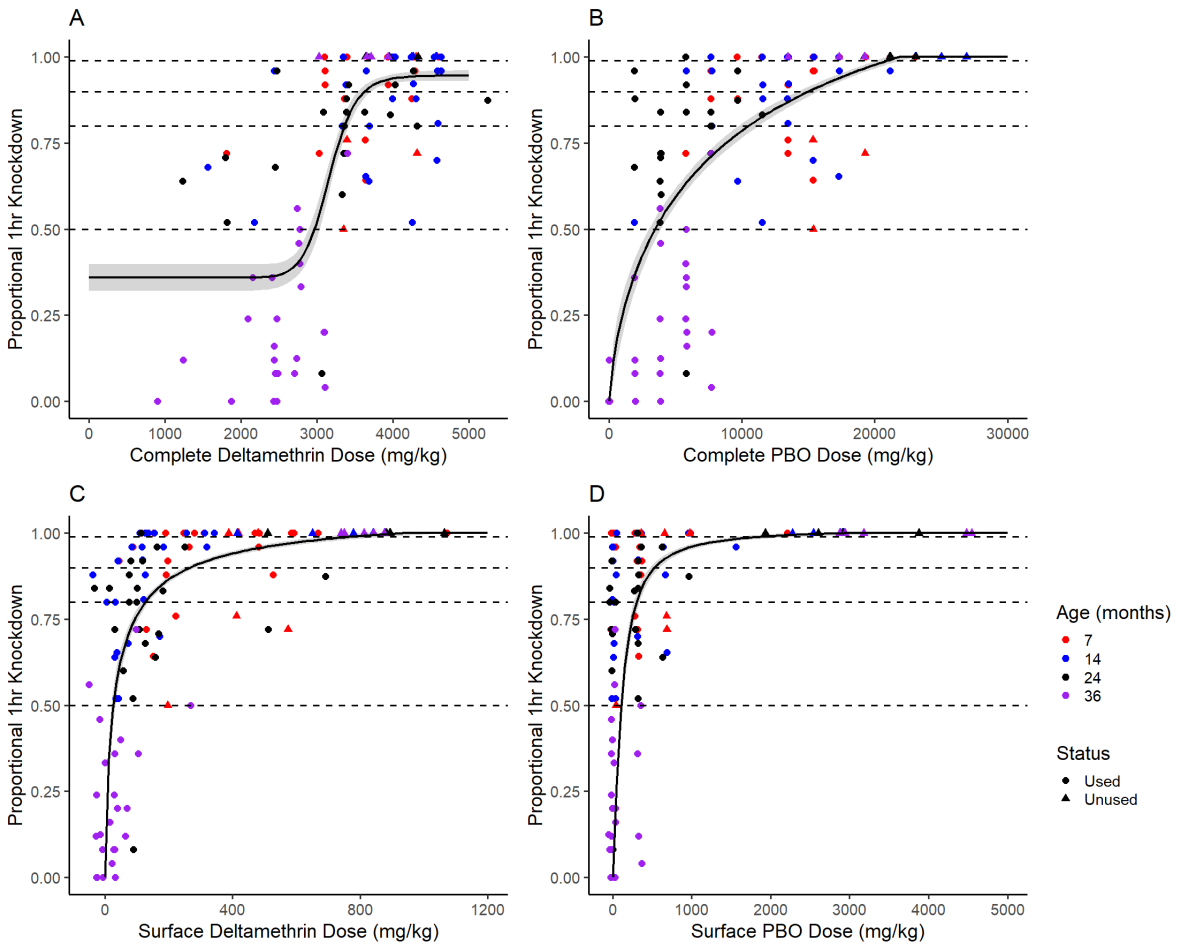


Figure 7 - Dose response curves for 1 hour knockdown for each active ingredient on PermaNet 3.0 roof samples with LC values highlighted. Grey shading indicates 95% CI. Active ingredient data grouped to enable analysis. Points indicate observed data for all 4 ages of used and unused nets. Results are for the Tiassalé strain.

Figure 8 displays dose response curves for mortality with the different degrees of active ingredient. As with knockdown, both the surface deltamethrin (plot C) and surface PBO (plot D) display more well-defined sigmoid curves. LC_{50} for surface deltamethrin remained low with small confidence intervals (195.3 95% CI:182.2-208.4) as did surface PBO (415.5 95% CI:389.3-441.8).

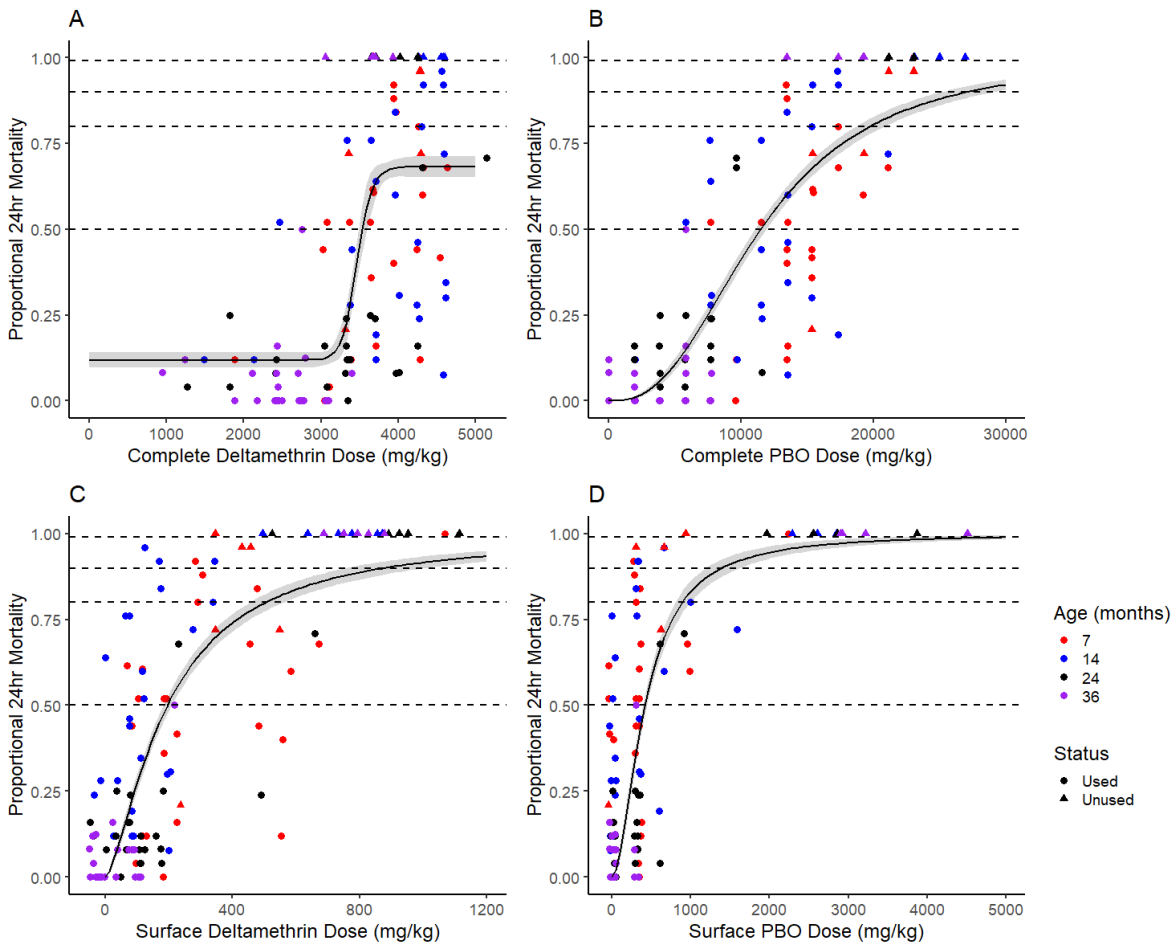


Figure 8 – Dose response curves for each active ingredient on PermaNet 3.0 roof samples with LC values highlighted. Grey shading indicates 95% CI. Active ingredient data grouped to enable analysis. Points indicate observed data for all 4 ages of used and unused nets. Results are for the Tiassalé strain.

4. Discussion

Here we observed the surface content of both PBO and pyrethroid content decreasing over time with operational use. This decline was observed for all assessed net brands. The concurrent decline in total A.I over this same time period demonstrates that regeneration is indeed occurring however the rate of regeneration is insufficient to compensate for the rate surface A.I is lost. Furthermore, we demonstrate this decline in surface A.I content is statistically correlated with a decline in bioefficacy against pyrethroid-resistant mosquitoes.

The surface A.I content of unused, undistributed samples of the same nets was observed to increase over the same time period. At the 36-month timepoint, the surface content of PBO on undistributed samples the PermaNet 3.0 roof was approximately ten times that measured at 7 months (and 30 times that of 36-month samples from the field). This large increase in surface content while in unrefrigerated storage demonstrates that A.I may continue to migrate to the surface despite nether being neither used or washed. This continued regeneration occurred for both PBO and deltamethrin; with PBO increasing at a more rapid pace. This phenomenon highlights that there is not necessarily an upper limit on regeneration, which may have implications for shelf life of net products prior to being deployed in the field.

The results of this study show that after use in the field PermaNet 3.0 incorporated roof panels lost complete PBO faster than complete deltamethrin. A greater loss of PBO compared to pyrethroid has been widely documented (4, 14), and may be attributable to PBO being more sensitive to environmental degradation factors such as sunlight and heat (15), furthermore, PBO is described in FAO/WHO pesticide specification literature as an oily liquid at room temperature, with its melting point listed as liquid at room temperature (16), meaning more PBO could be lost from nets during washing than that of the pyrethroids, other studies have also shown that due to the properties of PBO this enables more PBO to be lost during washing and may affect the regeneration time (17).

When compared to unused samples of the same age, it is shown that this loss is due to use of the ITNs as all unused samples remained within target, however more research is required to pinpoint the mechanisms by which the A.I is lost. As expected, the total amount of insecticide did not change, only the ratio between surface content and internal content which is consistent with previous studies.

Previous studies have shown PermaNet 2.0 nets after 36 months maintaining approximately one quarter of deltamethrin (18). A study from Burkina Faso comparing PermaNet 3.0 sides and

roof panels found similar complete insecticide losses to that of this study however no surface testing was conducted (19). Mechan *et al* (4) also compared net roof samples of PermaNet 2.0 and PermaNet 3.0 after distribution in a field trial. The results of their study follow the same trend as displayed here, with PermaNet 2.0 showing more degradation of pyrethroid and PermaNet 3.0 showing quicker rates of PBO degradation than the pyrethroid.

As PermaNet 3.0 nets have a deltamethrin incorporated roof, and deltamethrin coated sides, this allows for a comparison of coated and incorporated losses over time. The coated sides displayed a larger relative loss in insecticide after 7 months of use in the field when compared to incorporated samples from the same nets, however after this initial loss both net types lost insecticide at a proportional rate. The coated sides of PermaNet 3.0 have a starting concentration of 2.2g/kg, whereas incorporated roof panels start with 4g/kg. To ensure nets have enough insecticide to last for 3 years coated samples could be treated with a higher dose to factor for the initial loss.

The Olyset Plus dataset had a limited number of samples resulting in a small dataset, whilst analysis was completed to compare surface A.I to complete A.I and their effect on mortality, the conclusions are made with caution due to small sample size. Further improvements would be the use of baseline nets from the Sud-Ubangi study, whilst the unused controls could be viewed as a proxy for baseline, collecting nets and following the same test methods would give a baseline reading for both surface insecticide and mortality rate, further improving prediction models. This would also allow a better comparison to Veeralin study and Olyset Plus study which both had baseline nets collections. Improvements could be made to mortality models by combining the effect of insecticide and synergist, as with resistant populations both compounds are required to effect mosquitoes.

Extracting the surface active ingredient of incorporated nets via solvent washing has been used in a number of studies to date (17, 20), this helps to understand how much insecticide is

399 available to the mosquitoes, making the assumption that all on the surface is bioavailable. In
400 coated nets, all active ingredient is located on the surface, but it is currently unknown how
401 much is bioavailable to host seeking mosquitoes. An estimate can be made using bioassay
402 data however results are often heterogeneous due to variations between the colonies used,
403 test conditions, location or unattributed stochasticity among apparently replicated assays (10).
404 The use of solvent stripping would not be suitable for coated nets as it would remove all
405 insecticide, and this may exceed that which is bioavailable. Methods using a colorimetric test
406 (21, 22), and a recently developed before-and-after wash method (17) have the potential to
407 estimate bioavailability on both coated and incorporated nets. However, more work is needed
408 on this to validate which of the candidate methods are suitable for the different ITN chemistries
409 in use.

410 5. Conclusion

411 A major novel finding of our study is that operationally used nets did not regenerate surface
412 insecticide content sufficiently to replace losses from use, including exposure to the abiotic
413 environment. Surface active ingredient content is a good predictor of mortality efficiently
414 describing mortality decline over time, this method would be more relevant to use for chemical
415 durability studies. Unused nets continue to regenerate insecticide when stored in certain
416 conditions, which could have implications for self-life of stored nets. Furthermore, the methods
417 used in this study are found to be suitable for incorporated nets but highlight a need for the
418 development of such methods for coated nets.

419 References

420 1. Organization WH. World malaria report 2025: Addressing the threat of antimalarial drug
421 resistance. Geneva; 2025.

2. Bermejo A, Veeken H. Insecticide-impregnated bed nets for malaria control: a review of the field trials. Bull World Health Organ. 1992;70(3):293–6.
3. Organization WH. Updated WHO recommendations for achieving and maintaining universal coverage with long-lasting insecticidal nets for malaria control. World Health Organization; 2017 24/03/2026.
4. Mechan F, Katureebe A, Tuhaise V, Mugote M, Oruni A, Onyige I, et al. LLIN evaluation in Uganda project (LLINEUP): The fabric integrity, chemical content and bioefficacy of long-lasting insecticidal nets treated with and without piperonyl butoxide across two years of operational use in Uganda. Curr Res Parasitol Vector Borne Dis. 2022;2:100092.
5. Martin JL, Mosha FW, Lukole E, Rowland M, Todd J, Charlwood JD, et al. Personal protection with PBO-pyrethroid synergist-treated nets after 2 years of household use against pyrethroid-resistant Anopheles in Tanzania. Parasit Vectors. 2021;14(1):150.
6. Brake S, Gomez-Maldonado D, Hummel M, Zohdy S, Peresin MS. Understanding the current state-of-the-art of long-lasting insecticide nets and potential for sustainable alternatives. Current Research in Parasitology & Vector-Borne Diseases. 2022;2:100101.
7. Awolola ST, Adeogun AO, Olojede JB, Oduola AO, Oyewole IO, Amajoh CN. Impact of PermaNet 3.0 on entomological indices in an area of pyrethroid resistant Anopheles gambiae in south-western Nigeria. Parasit Vectors. 2014;7:236.
8. Staedke SG, Kamya MR, Dorsey G, Maiteki-Sebuguzi C, Gonahasa S, Yeka A, et al. LLIN Evaluation in Uganda Project (LLINEUP) - Impact of long-lasting insecticidal nets with, and without, piperonyl butoxide on malaria indicators in Uganda: Study protocol for a cluster-randomised trial. Trials. 2019;20(1).
9. Tan KR, Coleman J, Smith B, Hamainza B, Katebe-Sakala C, Kean C, et al. A longitudinal study of the durability of long-lasting insecticidal nets in Zambia. Malar J. 2016;15:106.
10. Harvey GF, Praulins G, Akoupo S, Amlalo GK, Anthony S, Azizi S, et al. Multi-site phenotypic characterisation of Kisumu colonies for comparison of insecticide susceptibility between testing centres. bioRxiv. 2026:2026.07.21.739746.
11. Organization WH. Implementation guidance (Modules 3 and 5): Bioassay methods for insecticide-treated nets: Cone test, version 1.3. Geneva, Switzerland; 2024.
12. Organization WH. Guidelines for laboratory and field testing of long-lasting insecticidal nets. Geneva; 2013.
13. Organization WH. Guidelines for monitoring the durability of long-lasting insecticidal mosquito nets under operational conditions. Geneva: World Health Organization; 2011.
14. Gichuki PM, Kamau L, Njagi K, Karoki S, Muigai N, Matoke-Muhia D, et al. Bioefficacy and durability of Olyset® Plus, a permethrin and piperonyl butoxide-treated insecticidal net in a 3-year long trial in Kenya. Infectious Diseases of Poverty. 2021;10(1):135.
15. Center NPI. Piperonyl Butoxide (PBO) general fact sheet. 2017.
16. Nations FaAOotU. Piperonyl butoxide (062). Rome; 2001.
17. Skovmand O, Dang DM, Tran TQ, Bosselmann R, Moore SJ. Timing is everything: a simple chemical method to determine the bioavailable surface concentration of insecticide for insecticide-treated net evaluation. Malaria Journal. 2025;24(1):362.
18. Hiruy HN, Irish SR, Abdelmenan S, Wuletaw Y, Zewde A, Woyessa A, et al. Durability of long-lasting insecticidal nets (LLINs) in Ethiopia. Malaria Journal. 2023;22(1):109.
19. Raharinjatovo J, Dabiré RK, Esch K, Soma DD, Hien A, Camara T, et al. Physical and insecticidal durability of Interceptor®, Interceptor® G2, and PermaNet® 3.0 insecticide-treated nets in Burkina Faso: results of durability monitoring in three sites from 2019 to 2022. Malaria Journal. 2024;23(1):173.
20. Smith SC, Zhou C, Stevie FA, Garcia R. Imaging and quantitative analysis of insecticide in mosquito net fibers using Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS). PLoS One. 2018;13(12):e0209119.

21. Green MD, Atieli F, Akogbeto M. Rapid colorimetric field test to determine levels of deltamethrin on PermaNet® surfaces: association with mosquito bioactivity. Tropical Medicine & International Health. 2009;14(4):381–8.
22. Dieval F, Bouyer J, Fafet J-F. An improved extraction method for surface dosage of insecticides on treated textile fabrics. Malaria Journal. 2017;16(1):14.

Declarations

Ethics approval and consent to participate

Not Applicable

Consent for publication

Not Applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding

This work was supported, in whole or in part, by The Gates Foundation (Investment ID INV-017131). Under the grant conditions of the Foundation, a Creative Commons Attribution 4.0 Generic License has already been assigned to the author's accepted manuscript version that might arise from this submission.

Author' contributions

ER, JH, DW conceptualised the study. ER conducted the bioassays and HPLC testing. ER, FM, GH, DW interpreted the data. VN, NM, FA, JZ provided the net samples. ER, FM, DW wrote the first draft. All authors read and approved the final manuscript.

Acknowledgments

We are grateful to the staff supporting PMI-VectorLink and the LLINEUP team for their assistance in collecting and providing the nets used in the study.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementary.docx