Efficacy of Aquatain™, a Monomolecular Surface Film, against the Malaria Vectors Anopheles stephensi and An. gambiae s.s. in the Laboratory

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Abstract. Monomolecular films are used for mosquito control because of their asphyxiating effect on larvae and pupae. Compared with other films, Aquatain mosquito formulation (AMF™) has an improved spreading ability and flexibility on a water surface. In the laboratory, AMF™ showed larvicidal, pupicidal, and oviposition deterrent effects against the malaria vectors Anopheles stephensi and An. gambiae. At a dose of 1 mL/m², the median lethal time to death for late larval instars was 3.02 (95% confidence interval [CI] = 2.76–3.25) and 0.98 (95% CI = 0.75–1.20) days for An. stephensi and An. gambiae, respectively. None of the treated larval pupated. Pupal mortality reached 100% within two hours for both species. AMF™ repelled gravid females from ovipositing in treated oviposition cups. Without the choice of an untreated cup, the lowered water surface tension caused most females to drown while attempting to oviposit. This physical control method has the potential to become a safe, cost-effective, and resistance-proof malaria vector control tool.

INTRODUCTION
Monomolecular layers differ from other mosquito control agents because of their ability to target multiple stages in the mosquito life cycle. All stages that come in contact with the water surface (e.g., eggs, larvae, pupae, emerging adults, and ovipositing females) are affected by the lowered surface tension caused by such layers. As a result, these layers can provide the combined benefits of larval and adult control, which leads to reduction in mosquito density and longevity.

Ethoxylated isostearl alcohols are plant-derived monomolecular layers that have been rigorously tested in laboratory and field settings. Commericially known as Arosurf® MSF (monomolecular surface film) and Agnique® MMF (monomolecular film), these layers were found to be efficient in killing pupae and late larval instars in addition to causing drowning of eggs and ovipositing females. Their mixing with the water surface (e.g., eggs, larvae, pupae, emerging adults, and ovipositing females) are affected by the lowered surface tension caused by such layers. As a result, these layers can provide the combined benefits of larval and adult control, which leads to reduction in mosquito density and longevity.

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Aquatain™ is a monomolecular film that has been designed for mosquito control because of its asphyxiating effect on larvae and pupae. Compared with other films, Aquatain mosquito formulation (AMF™) has an improved spreading ability and flexibility on a water surface. In the laboratory, AMF™ showed larvicidal, pupicidal, and oviposition deterrent effects against the malaria vectors Anopheles stephensi and An. gambiae. At a dose of 1 mL/m², the median lethal time to death for late larval instars was 3.02 (95% confidence interval [CI] = 2.76–3.25) and 0.98 (95% CI = 0.75–1.20) days for An. stephensi and An. gambiae, respectively. None of the treated larval pupated. Pupal mortality reached 100% within two hours for both species. AMF™ repelled gravid females from ovipositing in treated oviposition cups. Without the choice of an untreated cup, the lowered water surface tension caused most females to drown while attempting to oviposit. This physical control method has the potential to become a safe, cost-effective, and resistance-proof malaria vector control tool.

MATERIALS AND METHODS
Mosquitoes. Anopheles stephensi eggs (Strain STE 2, MRA no. 128, origin India) were obtained from the Malaria Research and Reference Reagents Resource Center, Centers for Disease Control and Prevention (Atlanta, GA), placed in water trays (30 × 15 × 7 cm), and kept in a climate-controlled chamber maintained at a temperature of 27 ± 1°C, 12L:12D photoperiod, and a relative humidity of 80 ± 5%. Larvae were fed on Liquifry No 1 (Interpet Ltd., Dorking, Surrey, United Kingdom) for the first three days and Tetramin® for the rest of their larval development. Pupae were collected and transferred in small cups to cages (30 × 30 × 30 cm) for emergence. Adults had ad libitum access to 6% glucose. Females, when 4–5 days old, were offered blood from the forearm of a volunteer, and an oviposition cup with wet filter paper was then placed inside the holding cage for egg collection. An. gambiae s.s., (Suakoko strain, courtesy of M. Coluzzi) were reared under similar conditions.

Experimental design. All experiments were conducted in a climate-controlled room at 27 ± 1°C, a 12D:12L photoperiod, and a relative humidity of 80 ± 5%. Tap water was kept in open plastic trays a day before every experiment to remove chlorine. Aquatain™ and AMF™ were used as recommended by the manufacturer. Eucalyptus oil provides an additional toxicity mode of action to AMF™ and is an oviposition repellent.

Larvicidal effect. The larvicidal effect of AMF™ was tested against L1–L2 (young, 1–3 days) and L3–L4 (old, 4–8 days)
instars separately. Four plastic trays (13 × 15 × 7 cm) were filled with 1 liter of water and 50 larvae were added to each tray along with Tetramin®-water solution (0.2–0.3 mg/larva/tray/day). The surface area of water was 0.035 m². Using the recommended dose of 1 mL/m², 35 µL AMF™ was applied to one of the trays. Half (17.5 µL) and double (70 µL) the recommended dose was pipetted in a second and third tray. One tray remained untreated and served as the control. Mortality was checked daily and recorded for ten days. Three replicates were performed. Tetramin®-water solution was adjusted accordingly to the daily mortality.

**Pupicidal effect.** Four plastic oviposition cups (5 cm diameter × 3.2 cm height) were filled with 40 mL of water. Fifteen pupae were added to each cup. Surface area was calculated to be 0.002 m². On the basis of the recommended dose, 2 µL of AMF™ was required. A total of 2 µL, 1.0 µL, and 0.5 µL was pipetted into three cups. A fourth cup was not treated and served as the control. The number of dead pupae was counted after every 15 minutes for 2 hours. Four replicates were performed. A pupa was considered dead if it did not show the characteristic stretching reaction on slight dipping.

**Effect on oviposition.** Female mosquitoes, 4–8 days old, were blood fed. For An. gambiae, females were blood fed twice. After 2 days, 12 gravid females were transferred to a cage that contained a 6% glucose water bottle and two oviposition cups (similar to those described above). One cup was treated with 2 µL of AMF™ (recommended dose) and the other served as the control. The cups were placed at two opposite corners of the cage (30 × 30 × 30 cm) and their positions were switched between replicates to avoid positional effects. After 48 hours, the eggs laid in each oviposition cup were counted. Four replicates were performed. A pupa was considered dead if it did not show the characteristic stretching reaction on slight dipping.

**Effect of eucalyptus oil.** To compare AMF™ and Aquatain™ for larvicidal and pupicidal effects, bioassays were performed with L₁–L₂ larvae and pupae. Treatments included AMF™ (with eucalyptus oil) and Aquatain™ (no eucalyptus oil). The minimal dose was used (17.5 µL for larvae and 0.5 µL for pupae), to pronounce any difference in effect in the same settings as described above. Three replicates were performed. Aquatain™ was also tested in the choice and no-choice oviposition experiments. In case of the choice experiments (four replicates), the gravid females had a choice between an untreated and Aquatain™-treated oviposition cup. In the no-choice (three replicates) experiment, gravid females had access to either an untreated, AMF™-treated, or Aquatain™-treated cup. AMF™ or Aquatain™ was applied at the lowest dose (0.5 µL).

**Statistical analysis.** In case of more than 10% mortality in the control, the percentage mortality data for the corresponding treatments was corrected for natural mortality by Abbott’s formula to calculate median lethal time to death (LT₅₀) values by probit analysis.²³,²⁵ The effect of species, larval stage, and treatments was analyzed using Cox regression and Kaplan-Meier pairwise comparisons.²⁶ A one-way analysis of variance was used to detect significant differences between the number of eggs laid in each cage. All analyses were performed using SPSS version 15 software (SPSS Inc., Chicago, IL).

**RESULTS**

**Larvicidal effect.** For all treatment doses, a minimum of 69% mortality was observed in the 10-day period after exposure (Figure 1). The effect on larval stages was significant. For both species, the LT₅₀ values for the L₁–L₂ larvae were higher than those for L₃–L₄ larvae at corresponding doses (Table 1). The hazard ratio (HR) (95% confidence interval [CI]) for the L₁–L₂ stage was 3.6 (3.14–4.20) and 1.9 (1.74–2.28) times that for L₃–L₄ stage of An. gambiae and An. stephensi, respectively. This difference was also observed by pairwise comparison between survival curves of both stages (P < 0.001 for each species). Cox regression showed no increase in the HR for the larvae of An. stephensi at the L₁–L₂ stage compared with An. gambiae except for the dose of 17.5 µL (HR = 2.2, 95% CI = 1.4–3.5) but a significant increased HR at the L₃–L₄ stage for all treatments. At the L₁–L₂ stage, the HR (95% CI) for An. gambiae was 9.8 (4.0–23.7), 3.1 (1.2–7.5), and 7.4 (3.0–17.8) for the 17.5-µL, 35-µL, and 70-µL treatments, respectively, compared with An. stephensi. All treatments were significantly different from the controls (P < 0.001) but an increase in concentration did not show a systematic increase in mortality because the survival curves for various treatments did not differ in any specific pattern (Figure 1). None of the larvae in any of the treatments, apart from the control, molted into a pupa (Table 1).

**Pupicidal effect.** For pupae, the species effect was more pronounced (Figure 2). The HR (95% CI) for An. gambiae pupae was 6.9 (4.5–10.5) times that for An. stephensi. The species effect is also apparent from the LT₅₀ values (Table 2). For An. gambiae, no significant difference was found between the different doses tested. For An. stephensi, however, the 1-µL treatment was significantly (P < 0.05) more effective than the other treatments. Although none of the pupae for both species at all concentrations did so, all of the pupae in the control treatment emerged into adults.

**Effect on oviposition.** In the choice experiment, gravid females had an option of laying eggs in an AMF™-treated cup and/or an untreated oviposition cup. Both species did not lay any eggs in the treated cups. A mean ± SE of 378 ± 91 eggs and 227 ± 39 eggs were laid in the control cups by An. stephensi and An. gambiae, respectively (Table 3).

In the no-choice experiments, gravid females were provided with a single cup that was either untreated or treated with one of the concentrations (0.5 µL, 1.0 µL, or 2.0 µL). Most females drowned in treated cups in an attempt to oviposit; only one An. gambiae drowned in a control cup. In contrast to complete absence of oviposition in the treated cups, a mean ± SE of 567 ± 126 and 217 ± 86 eggs were laid in the control cages by An. stephensi and An. gambiae, respectively. All females were dissected and eggs developed up to Christopher IV and IV–V transition stages were counted. There was no significant difference in the total number of eggs, laid and unlaid, in any treatment cage for An. stephensi (F = 3.482, degrees of
freedom (df) = 3, 8, P > 0.05) and An. gambiae (F = 0.295, df = 3, 8, P > 0.05).

**Effect of eucalyptus oil.** No additional effect of eucalyptus oil was found for larvae or pupae in terms of mortality when directly compared with Aquatain™. However, in case of oviposition, unlike AMF™, in the choice experiments a mean ± SE of 1.5 ± 0.6 females of *An. stephensi* and 8.3 ± 1.3 females of *An. gambiae* drowned while attempting to oviposit, although they had the choice of an untreated oviposition cup (Table 3). This finding shows that Aquatain™ by itself does not repel the gravid females. Another difference was that a mean ± SE of 11.8 ± 7.8 eggs were laid in the treated cups; egg laying was not observed with AMF™ treatment.

In the no-choice experiment (Table 4), no eggs were found in AMF™-treated cups and only 27 eggs were found in one of the cups treated with Aquatain™. In the control, a mean ± SE of 233.0 ± 34.9 and 178.7 ± 57.5 eggs were laid by *An. stephensi* and *An. gambiae*, respectively. No significant difference was found in the total number of eggs per cage when the dissected eggs were also taken into account for *An. stephensi* (F = 0.889, df = 2, 6, P > 0.05) and *An. gambiae* (F = 0.303, df = 2, 6, P > 0.05).

**DISCUSSION**

Our results show the efficacy of Aquatain™ against multiple life cycle stages of *An. stephensi* and *An. gambiae* in the laboratory. In the larval experiments, the L₃–L₄ stage was more susceptible to AMF™ than the L₁–L₂ stage. The same response was reported by Das and others for *An. stephensi* when treated with Arosurf® MSF. The late stages are affected more because of their reduced ability to use dissolved oxygen. The failure of larvae to pupate in any of the treated trays was a persistent result. A possible reason may be the reduced larval fitness because they showed stunted growth and had a loss of appetite, which was apparent from the accumulation of food in the treated trays. Corbet and others showed that larvae treated with PDMS spend more time on the surface; this behavioral change affects their fitness. The pupal stage showed a more drastic effect of AMF™ because they lack the ability to use dissolved oxygen completely. Arosurf® MSF also affected the pupae more than the larvae.

The unsystematic effect of different concentrations on the mortality of larvae and pupae may be caused by the mode of action. Polydimethylsiloxane is a bimodal agent. It changes...
the surface tension of the water and floods the respiratory organs, which results in the tail-nibbling behavior observed. The flooding feature is more dominant. All larvae on the surface at the time of treatment are likely to be instantly effected because of the flooding feature. As long as the amount of AMF™ is enough to flood the trachea of the larvae, further increase in the concentration of AMF™ probably has no additional effect.

Aquatain™ and AMF™ showed no difference in their effect on larvae and pupae, which suggests that Aquatain™ without the addition of a repellent has an equal potency to act as a control agent. Eucalyptus oil has a concentration-dependent oviposition repellent effect, which is why in the choice experiment with AMF™ the females were repelled from the treated cups. However if gravid females are not repelled, as in case of Aquatain™, it rules out the chance that the females will search for an alternative site to lay eggs. This non-repellent effect is useful because it provides an increased chance of drowning a gravid female. It will thus be preferable to apply Aquatain™ for mosquito control.

In addition to these results, Aquatain™ has certain properties that indicate the advantages that will be associated with using it as a control agent. Aquatain™, unlike contemporary control agents, has a physical mode of action. It is therefore less likely that resistance will develop against it. Other monomolecular

Table 1

<table>
<thead>
<tr>
<th>Species</th>
<th>Stage</th>
<th>Dose (µL)</th>
<th>Slope ± SE</th>
<th>LT₅₀ (95% CI)</th>
<th>χ²</th>
<th>% Mortality</th>
<th>% Pupation</th>
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<td>An. stephensi</td>
<td>L₁−L₂</td>
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<td>3.3</td>
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<td>1.60 (1.3−1.8)ᵈ</td>
<td>19.4</td>
<td>99.3</td>
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</table>

⁻ NA = not applicable.
⁺ Values without letters in common differ (P < 0.05) with respect to survival curves.
‡ A heterogeneity factor was used in the calculation of the CIs.

Figure 2. Cumulative percentage survival for pupae of Anopheles stephensi (As) and An. gambiae (Ag) in untreated (control) and Aquatain™-treated (0.5 µL, 1.0 µL, and 2.0 µL) oviposition cups.

Table 2

<table>
<thead>
<tr>
<th>Species</th>
<th>Dose (µL)</th>
<th>Slope ± SE</th>
<th>LT₅₀ (95% CI)</th>
<th>χ²</th>
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<tr>
<td>An. stephensi</td>
<td>0.5</td>
<td>3.41 ± 0.20</td>
<td>45.8 (44.0−47.5)ᵇ</td>
<td>23.3</td>
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<td>1</td>
<td>4.08 ± 0.26</td>
<td>44.0 (42.4−45.5)ᵇ</td>
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<td>3.29 + 0.19</td>
<td>47.5 (45.6−49.3)ᵇ</td>
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<td>An. gambiae</td>
<td>0.5</td>
<td>4.28 + 0.47</td>
<td>20.4 (19.2−21.5)ᵇ</td>
<td>13.4</td>
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<td>1</td>
<td>4.86 + 0.62</td>
<td>20.4 (19.3−21.3)ᵇ</td>
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<td>2</td>
<td>4.76 + 0.62</td>
<td>20.0 (18.9−20.9)ᵇ</td>
<td>0.13</td>
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* All treatments resulted in 100% mortality within 2 hours.
† Values without letters in common differ (P < 0.05) with respect to survival curves.

surface films such as Arosurf® MSF and Agnique® MMF have the same mode of action and resistance has not been reported thus far. Owing to its safety and non-toxicity, Aquatain™ will be suitable for all kinds of breeding sites and no personal protection will be required during application.19,20 The self-spreading feature will make it easier to apply Aquatain™ over large and otherwise inaccessible areas.21 This feature will be particularly useful in controlling larvae and pupae of the African vector An. funestus, which occupies larger, more permanent sites.13 An additional advantage in applying Aquatain™ will be the reduced water evaporation as the breeding sites are often economically and domestically important.20

Surface films are more effective at the late larval and pupal stage in contrast to Bti and Bs.22 Because application of Bti and Bs has been found to be cost-effective, we compared their cost with Aquatain™ when applied on a hectare of breeding site for a year.21 Considering the fact that Aquatain™ applied at a dose of 0.5 mL/m² caused 100% pupal mortality within two hours and ensured no pupation in the larval trays, we based our calculations on this concentration. Furthermore, we assumed that once a breeding site is treated, the film remains effective for 10 days (according to supplier specifications). On the tenth day, if a female successfully lays eggs, the resulting larvae need at least seven days before developing into pupae. Therefore, subsequent treatment can be delayed for an additional seven days.

The cost of Aquatain™ at a rate of 0.5 mL/m² per hectare after every 17 days for a year is $1,890 ($18 per liter), which is comparable to the water-dispersable granule ($1,300–1,825) and commercial corn granules ($1,466–1,955) formulations of Bs if applied after every 14 days per hectare per year.21 The cost of similar formulations of Bti are $208–313 and $563–782 for the water-dispersable granule and commercial corn granules formulations, respectively, but this estimate does not include the labor cost involved with repetitive application. The required application frequency of Aquatain™ is less than half that for Bti. However, because these estimates do not consider untreated periods that can be incorporated without compromising efficiency of a control program, actual costs would be lower than these estimates. We are working on various formulations of Aquatain™ that can further reduce the application frequency to approximately once a month in permanent breeding sites. Another logistic advantage of Aquatain™ will be the shelf life, which for various formulations of Bs and Bti is 2–3 years when stored at a temperature below 25°C. High temperature reduces their efficacy and must be considered during transportation.22,23 Aquatain™ has no such limitations. It has been reported to remain stable after being kept at 54°C for 2 weeks.24

The laboratory results, properties, and comparison with well-tested larvicides suggest that Aquatain™ is a promising control agent. However, for a more realistic overview, field trials need to be conducted. Field trials will also provide an opportunity to detect any non-target effects; although none have been reported so far when Aquatain™ was used in Australia.25 In the event of the expected field results, Aquatain™ may be incorporated, as a new control tool, into integrated mosquito control programs.

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