AN EVALUATION OF PYRIPROXYFEN FOR CONTROL OF *CULEX QUINQUEFASCIATUS* SAY UNDER SEMI-FIELD CONDITIONS IN AUSTRALIA.

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Summary

The insect growth regulator pyriproxyfen in two formulations (Sumilarv 90 CS and Sumilarv 5 GR) was evaluated in semi-field trials for the control of immature stages of *Culex quinquefasciatus* Say. In the first trial, the two formulations were compared with the industry standard Prolink 50 CS (50g/L s-methoprene) at the field application rate for Prolink (ie. 11gai/ha or 3.7 ppb) in 50 L tubs. All three products produced 100% emergence inhibition of a single generation. In the second trial, Sumilarv 5 GR was evaluated in two-litre buckets over various nominal concentrations from 500ppb down to 0.5 ppb for five successive generations over 60 days. When applied as a granule, Sumilarv 5 GR produced 100% emergence inhibition across 5 generations. However, where serial dilutions of Sumilarv 5 GR treated water were tested, efficacy was greatly reduced. To investigate this phenomenon, the dilution process was repeated and samples analysed for pyriproxyfen content. The dilution process generated just 6 ppm (against the nominal 500 ppb) indicating that the granules were very stable in water and the pyriproxyfen was not readily released. Further, the pyriproxyfen content of water over 10 weeks following application of granules (nominal 500 ppb) showed an erratic but gradual release of active ingredient and a peak concentration of just 8.5 ppb at 1 week declining to below 1 ppb at 10 weeks. Pyriproxyfen was very effective in inhibiting emergence of *Cx. quinquefasciatus* adults for multiple generations at less than 10 ppb, and probably much lower. Sumilarv 5 GR when applied directly to water at 0.1g/L (nominal 500ppb) provided emergence inhibition for at least five generations over 60 days. These results demonstrate that these products hold great potential to assist the management of mosquitoes of pest and public health concern.

Key Words: pyriproxyfen, s-methoprene, *Culex quinquefasciatus,* Sumilarv, Prolink

INTRODUCTION

Culex quinquefasciatus Say is widely distributed in Australia and is closely associated with freshwater habitats, particularly those containing polluted waters, including stormwater retention pits, sewerage treatment ponds and septic tanks (Lee *et al.* 1989). The adult mosquito will bite a range of different hosts including humans and other mammals, birds and lizards (Elizondo-Quiroga *et al*. 2006, Garcia-Rejon *et al.* 2010, Kay *et al*. 1985, Niebylski and Meek 1992, Samuel *et al.* 2004) although in Australia it appears to be mostly ornithophilic (Jansen *et al*. 2009). *Cx. quinquefasciatus* is a known vector of a number of pathogens of human health concern including the parasites that cause lymphatic filariasis as well as St Louis encephalitis and West Nile viruses (Bartholomay *et al*. 2010, Hayes *et al*. 2005, Kent *et al*. 2010). However, it appears to be a poor vector of viruses more typically present in Australia such as Murray Valley encephalitis, Kunjin, Barmah Forest and Ross River viruses (Boyd and Kay 2000, Kay *et al*. 1982). Notwithstanding the potential to transmit pathogens of human health concern, it is also considered a nuisance species to humans in Australia, especially when large populations of adult mosquitoes occur in close proximity to urban areas with adult mosquitoes often entering buildings. The mosquito is also known to transmit pathogens of veterinary importance such as species of *Plasmodium* associated with avian malaria as well as potentially vectoring dog heartworm, certain viruses afflicting lizards and Myxoma virus to rabbits (Lee *et al*. 1989).

In Australia there appears to be little or no focus on control of *Cx. quinquefasciatus,* presumably due to its perceived relatively low public health importance but also the operational challenges of delivering effective treatment given the diversity of potential breeding sites in urban environments. Nevertheless, *Cx. quinquefasciatus* is susceptible to a range of commonly used larvicides including *Bacillus thuringiensis israeliensis* (Bti), Temephos, methoprene and pyriproxyfen (Mian *et al*. 2017, Russell *et al*. 2003). It is important to note that while Bti is one of the most commonly used larvicides in Australia, its operational efficacy in urban habitats suitable for *Cx. quinquefasciatus,* containing water of a high organic content or where many weeks of residual control is required, may be reduced (Becker *et al*. 1992; Russell *et al*. 2003). In these circumstances, insect growth regulators may provide more reliable control of mosquito populations (Mian *et al*. 2020).

Semi-field trials were conducted in Sydney (Australia) in 2010 to evaluate two formulations of

the insect growth regulator Pyriproxyfen, Sumilarv 90 CS (90g/L capsule suspension) and Sumilarv 5 GR (5g/kg granule) for control of immature *Cx. quinquefasciatus* in small containers (2L buckets and 50L tubs) which emulate the preferred type of larval habitats that occur in residential and commercial environments.

MATERIAL AND METHODS

In trial 1, Sumilarv 90 CS and Sumilarv 5 GR (manufactured by Sumitomo Chemical Co, Japan or its subsidiaries) were compared with the standard IGR product used in Australia (Prolink Liquid Larvicide – 50 g/L capsule suspension of s-methoprene), manufactured by Wellmark, USA. Prolink is used at 220 mL/ha (11 gai/ha) assuming water depth up to 30 cm and clean water. Trial 2 involved only Sumilarv 5 GR. The first trial was to determine relative efficacy of all the products at the standard field application rate for Prolink and the second to evaluate the relative efficacy of different rates of Sumilarv 5 GR.

All mosquito larvae were sourced from a laboratory colony maintained by the Department of Medical Entomology, NSW Health Pathology, Westmead Hospital, Sydney and transported to the University of Technology Sydney trial site. The strain originated from Marsfield Park (Sydney) and has been cultured since 1998.

The two trials were commenced simultaneously in mid-October 2010 with trial 1 completed in 12 days while trial 2 extended over 60 days. Water temperatures in the tubs used in trial 1 ranged from 16 ºC to 22 ºC whilst the water temperature in the buckets used in trial 2 ranged from 15 ºC to 23 ºC. Mean daily minimum and maximum temperatures were 11.9 ºC and 22.7 ºC respectively during trial 1 and 11.9 ºC and 24.0 ºC respectively during trial 2.

Trial 1: Relative efficacy of Sumilarv 90 CS, Sumilarv 5 GR and Prolink (Semi-field trial)

Plastic tubs with a capacity of 50L were placed under an outdoor shelter to prevent dilution by rainfall but otherwise exposed to natural conditions including sunlight and wind during at least part of the day. Tubs were filled with Sydney tap water and water conditioner (Prime Freshwater and Saltwater Conditioner, Seachem Laboratories) added and allowed to stabilize for 2 days. Larvae were provided with a combination of food items – fish flakes, brewer's yeast and sugar, together with 3 floating goldfish pellets (Wardley Premium Goldfish Floating Pellets, The Hartz Mountain Corporation) in each tub at the beginning of the trial. Fifty 3rd instar larvae were placed in each tub with 5 replicates of each treatment. Prolink was applied at the standard rate equivalent to 11gai/ha based on 30cm water depth as were the two formulations of Sumilarv (90 CS and 5 GR) giving a nominal concentration of 3.7 ppb (AI) for all three treatments. Once pupation was observed, fine gauze netting was placed over each tub to prevent adults escaping on emergence. Total adult emergence was determined at the end of the 2 week period following treatment.

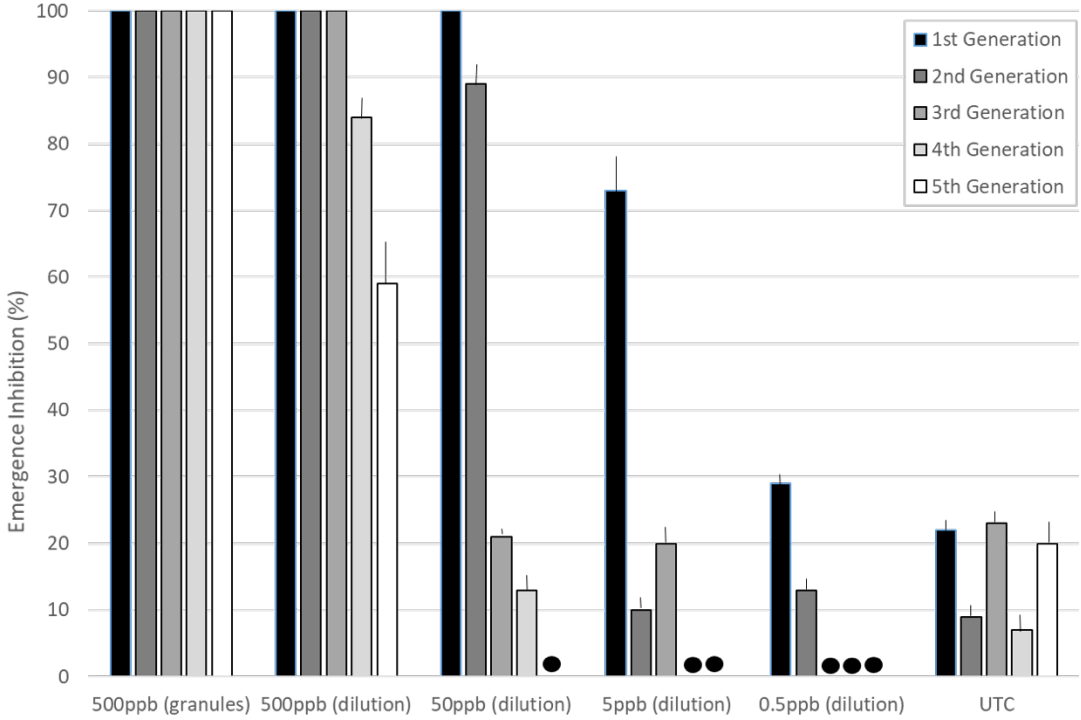
Trial 2: Efficacy of Sumilarv 5 GR (Semi-field trial)

A semi-field trial was conducted to evaluate the emergence inhibition of various dilutions of Sumilarv 5 GR in small 2L plastic buckets. Buckets were placed under an outdoor shelter to prevent dilution by rainfall but otherwise exposed to natural conditions including sunlight and wind during at least part of the day. Buckets were filled with Sydney tap water and water conditioner added and allowed to stabilize for 2 days. There were 5 replicates for each treatment. Larvae were provided with a combination of food items – fish flakes, brewer's yeast and sugar, together with one floating goldfish pellet in each bucket at the beginning of the trial.

Following treatment, five consecutive generations of late-instar larvae were exposed to each of the treatment rates over a total period of 60 days. For each generation, 20 3rd instar larvae were placed in each bucket. Once pupation was observed, fine gauze netting was placed over each bucket to prevent adults escaping on emergence. Buckets were monitored daily for adult emergence. After each round of adult emergence was completed in all buckets, the next generation of 3rd instar larvae were added. Once emergence inhibition fell below 80% for two consecutive generations that treatment rate was discontinued.

In one treatment, 0.2 g product was added to each bucket giving an effective concentration of 500 ppb. Given that Pyriproxyfen is known to be effective at very low levels on *Cx. quinquefasciatus* (Ali *et al*. 1999, Amalraj *et al*. 1988, Ansari *et al*. 1991, Mulla *et al.* 1986, Schaefer and Mulligan 1991, Schaefer *et al.* 1988) it was thought to be logistically difficult to measure such minute quantities of granules

Figure 1: Emergence inhibition for Sumilarv 5 GR and nominal dilutions 500ppm down to 0.5ppm for 5 generations over 60 days (n=5). Vertical bar is the SEM. Black dot indicates that no assay conducted.



accurately for the lower concentrations. Instead, granules were placed into water (0.1 g/L = 500 ppb) and vigorously shaken for 1 hour after which the diluent was extracted, and serial dilutions down to 0.5 ppb (nominal) pyriproxyfen (equivalent to 0.2 mg product/bucket) were prepared.

During the trial it became clear that larvae in those treatments containing diluents, particularly lower concentrations, remained healthy with low rates of mortality occurring as evident by the high number of mosquitoes emerging as adults. This was in direct contrast to the treatment involving direct granule application that achieved 100% emergence inhibition over 5 generations. This result suggested that the nominal treatment rates were not achieved by the process used to create the diluent stock. Therefore, the dilution procedure was duplicated so that the starting diluent concentration (nominal 500 ppb) could be analysed to determine Pyriproxyfen content. There were four test containers and two 5 mL aliquots were removed from each for analysis. Aliquots were taken from 2 cm below the surface in the center of the jars.

To better understand the concentration decline of pyriproxyfen in Sumilarv 5 GR treated water over time, 0.2 g product (nominal 500ppb) was added to each of four 2 L buckets under the same conditions as the original trial and water samples extracted over a 10-week period (specifically at 1 hr, 6 hr, 2 d and 1, 2, 4, 6, 8 and 10 wk after treatment). A single 5mL aliquot was removed from each bucket at each time period, taken from 5 cm below the surface in the centre of the bucket. Bucket weights were measured at the beginning of the trial and at the end to determine evaporation rates. There was no intent to maintain the buckets at 2 L, reflective of natural conditions under cover.

Laboratory Analysis: Water samples were supplied to ACS Laboratories (Australia) (Kensington, Victoria) for analysis of pyriproxyfen content. Each sample was diluted 1:1 Acetonitrile and then filtered

Figure 2: Pyriproxyfen concentrations in test solutions (4 replicates). Range is shown by the black vertical bar (n = 2 samples).

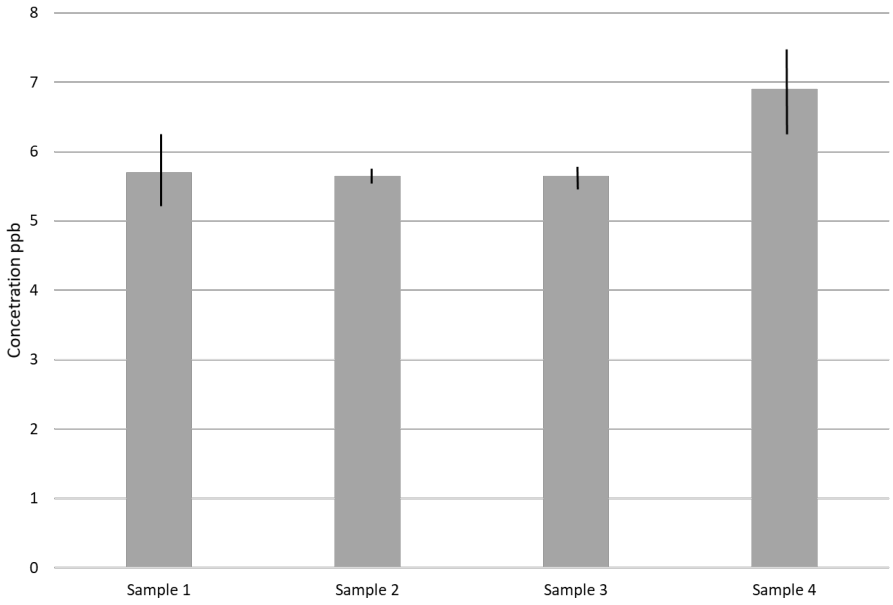
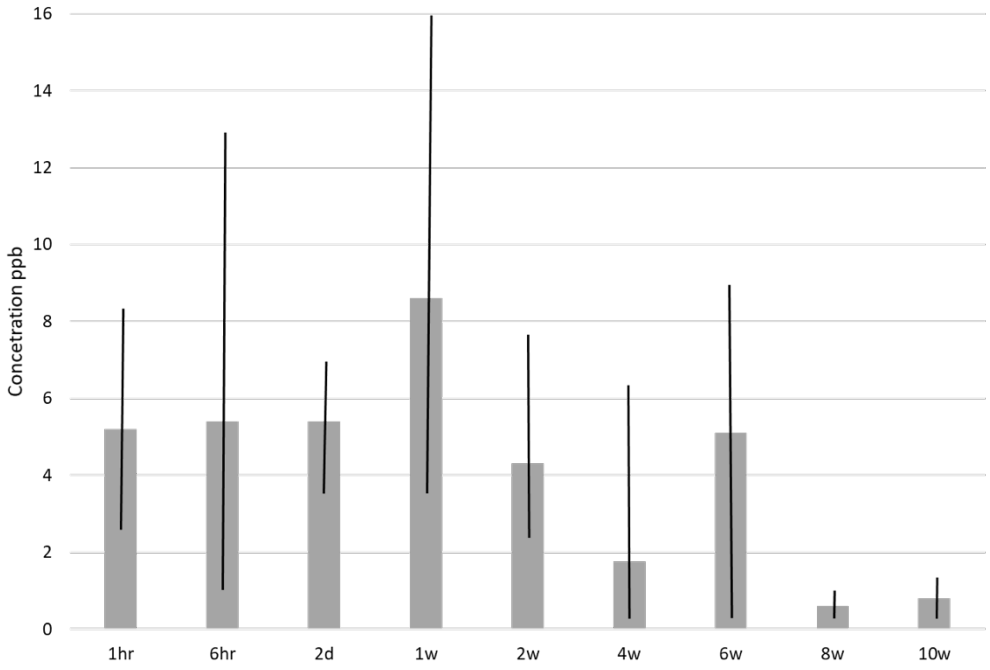


Figure 3: Mean (and range) of pyriproxyfen concentrations in stored 2 L containers (n = 4 replicates, single sample) over a 10 week period following application of Sumilarv 5 GR granules. Range is shown by the black bar.



through a 0.2 µm Teflon syringe filter into a 2 mL glass vial. Two microliters of the diluted sample was then injected onto a Waters BEH C18 column (1.7 µm, 50 x 2.1 mm) using a Waters Aquinity UPLC-MS-MS (ultra-high pressure liquid chromatography with Tandem mass spectrometry detection). Pyriproxyfen was eluted with a mixture of acetonitrile and 5 mM trifluoroacetic acid (75:25) at 0.5 mL/min and quantified by external calibration. Limit of detection (LOD) was 0.05 ppb.

RESULTS

Trial 1

In the semi-field study using 50 L tubs, Prolink, Sumilarv 90 CS and Sumilarv 5 GR all provided complete emergence inhibition of *Cx. quinquefasciatus* at the equivalent of 11 gai/ha (nominal 3.7 ppb). Mean emergence for the control treatment was 70.4% (± 6.8 SD).

Trial 2

Sumilarv 5 GR, applied as a granule, remained effective in inhibiting adult emergence over 5 generations (60 days) (Figure 1). However, an aqueous dilution of the product at the nominal rate of 500 ppb provided 100% emergence inhibition for 3 generations only and for 50 ppb only one generation. Laboratory assays of test solutions showed that the effective dose levels were much less than expected (Figure 2) with an overall mean and SE of 5.975 ± 0.260 ppb. Hence, the beginning concentrations in the test solutions was actually only ca. 6ppb (or only 1.2% of nominal) with subsequently serial dilution to ca. 0.6, 0.06 and 0.006 ppb.

Concentrations of pyriproxyfen derived from water samples taken for 2 L buckets treated with 0.2 g/2 L Sumilarv 5 GR granules measured over 10 weeks showed that concentration during the initial stages was highly volatile (Figure 3) between replicates and over time. Over this period, pyriproxyfen concentration in water, varied widely from 0.2 to 16 ppb (range) and 0.5 to 8.6 ppb (mean) and there was no clear decline curve (Figure 4). Nevertheless, the general direction was a decline in detected residues over the 10 week period. The initial results also closely match the concentrations demonstrated in the repeated test dilutions (Figure 2). Note also that total evaporation of moisture over 10 weeks was ca. 20% (19.95% ± 1.65% SE) which suggests the residue decline over 10 weeks was probably understated.

DISCUSSION

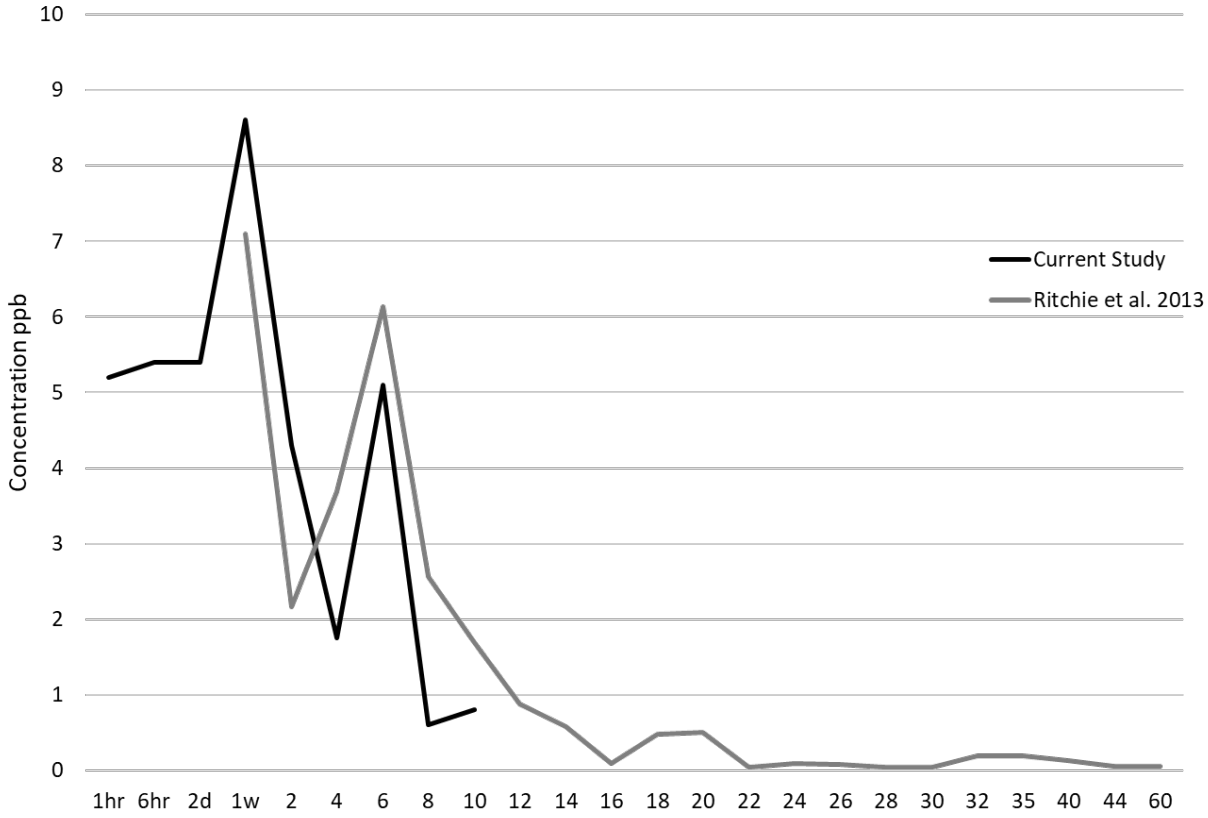
The results of these experiments demonstrate the effectiveness of these insect growth regulators to control populations of *Cx. quinquefasciatus* and, potentially, other mosquitoes of pest and public health importance associated with urban habitats.

The results of trial 1 demonstrated that the two formulations of Sumilarv provided comparable efficacy in controlling immature stages of *Cx. quinquefasciatus* to the standard application of Prolink. Sumilarv 90 CS and 5 GR formulations, when applied at the equivalent rate to Prolink (11gai/ha or 3.7 ppb), provided complete emergence inhibition of one generation of *Cx. quinquefasciatus* larvae, equivalent to Prolink. Note that chemical analysis of treated water from trial 2 would suggest that the actual pyriproxyfen concentration in the Sumilarv 5 GR treatment was most likely to be much less than 3.7 ppb, further confirming the efficacy of this product for mosquito control.

In trial 2, Sumilarv 5 GR, when applied as a granule directly into water, provided emergence inhibition of 5 successive generations of *Cx. quinquefasciatus* for 60 days. Over this period, pyriproxyfen concentration in water, varied widely from 0.2 to 16 ppb (range) and 0.5 to 8.6 ppb (mean) and there was no clear decline curve (Figure 4). The range of values coincided closely with the earlier study on *Aedes aegypti* L. (Ritchie *et al.* 2013) where similar methodology was used and in their study concentrations in water were also erratic during the first 8-10 weeks. It should be noted that this study was conducted prior to that reported by Ritchie *et al.* (2013). That study benefited from the knowledge that serial dilution was not an appropriate technique and utilized actual granules, weighed with a laboratory 4-place balance. It also mirrored the results of a similar and contemporaneous study on *Aedes vigilax* Skuse where the same rates of Prolink and Sumilarv 5 GR were applied (Webb *et al.* 2012). The result reported here has important implications for other researchers undertaking testing of comparable control agents and using the serial dilution method.

Our attempt to simulate lower concentrations by vigorous shaking of granules in water and then serial dilution was not successful because only limited amounts of pyriproxyfen were liberated in the process – only ca. 1% of total pyriproxyfen (ca. 5 ppb) was liberated which matches what we saw from the laboratory assessment of the diluent and of the natural release of pyriproxyfen from granules in both this study and that of Ritche *et al.* (2013). This suggests that the release of pyriproxyfen from granules is slow and that even vigorous shaking does not cause the granules to disintegrate or release

Figure 4: Decline in Pyriproxyfen concentrations (ppb) over time following application of Sumilarv 5 GR granules at 0.1g/L in the present study compared with Ritchie *et al.* (2013).



additional active ingredient. The level of pyriproxyfen in the water over the course of the study was sufficient to completely inhibit emergence for 5 consecutive generations over 60 days. For *A. aegypti*, Ritchie *et al*. (2013) achieved 8-10 weeks emergence inhibition at nominal 50 ppb and 40 weeks with 500 ppb. While the two species have different levels of susceptibility, it is likely that the length of emergence inhibition of *Cx. quinquefasciatus* may have been much longer than 60 days, had the trial continued.

Pyriproxyfen has been shown to be very effective against *Cx. quinquefasciatus* in a wide range of laboratory and field trials. In laboratory bioassays, LC_{50} values have ranged from 0.018 to 0.29 ppb on Cx. q*uinquefasciatus* of various strains and from various countries (Ali *et al*. 1999, Amalraj *et al*. 1988, Ansari *et al*. 1991, Mulla *et al.* 1986, Schaefer and Mulligan 1991, Schaefer *et al.* 1988) and the maximum LC_{90}/LC_{95} value was 1.1 ppb (Ali *et al.* 1999). Under field conditions, Sumilarv 5 GR has similarly shown high levels of emergence inhibition of *Cx. quinquefasciatus* under a range of environmental conditions (Amalraj *et al.* 1988, Ansari *et al*. 1991, Chavass *et al*. 1995, Jambulingham *et al*. 2008, Mulligan and Schaefer 1990, Nayar *et al.* 2002), decreasing with quality of water and water movement. Amalraj *et al*. (1988) found that rates of 10 ppb provided up to 6 weeks emergence inhibition in concrete water tanks but 50 ppb was required to give 1 week emergence inhibition in heavily polluted cesspits. Chavasse *et al.* (1995) treated open polluted breeding sites in Dar es Salaam (Tanzania) with Sumilarv 5 GR at the rate of 100 ppb and achieved 11 weeks of complete emergence inhibition. In Pondicherry (India) Sumilarv 5 GR was applied to disused wells, cesspits and drains at various rates from the equivalent of 10 to 167 ppb (Jambulingham *et al*. 2008) and the predicted EI_{80} values ranged from 4 weeks for slow moving drains treated at ca. 167 ppb to 28 weeks for disused wells treated at ca. 33 ppb. All of these studies focused on mosquito control relative to application rate and hence there is no indication of the actual concentration of pyriproxyfen in water or speed of release from the granules. Further, pyriproxyfen is known to affiliate with organic material either in suspension, in the substrate or in the walls of containers in which the tests are

conducted (Amalraj *et al*. 1988, Jambulingham *et al*. 2008, Mulligan and Schaefer 1990, Suman *et al*. 2013, Vythilingam *et al*. 2005). The results from this study and those of Ritchie *et al.* (2013) suggest that concentration in water following an application of Sumilarv 5 GR may only be a fraction of the applied rate. Yet, a large number of trials indicate that emergence inhibition over a long period may be achieved with very low doses. Granules may continue to release pyriproxyfen over a long period of time or pyriproxyfen affiliated with organic material may dissociate over time or more simply, foraging mosquito larvae may ingest organic material containing pyriproxyfen (Mulligan and Schaefer 1990).

With an emphasis on improved water storage and management in urban areas, there is the potential for the creation or enhancement of conditions suitable for mosquitoes of pest and public health concern such as *Cx. quinquefasciatus*. The results of this study have demonstrated that long periods of effective control of immature mosquito populations in these habitats can be achieved through the use of pyriproxyfen in the formulations tested here. Consideration by local mosquito control authorities should be given to the incorporation of these products into their integrated mosquito management programs.

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