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EFFICACY, ENVIRONMENTAL PERSISTENCE AND NON-TARGET IMPACTS OF PYRIPROXYFEN USE AGAINST *AEDES VIGILAX* IN AUSTRALIA

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Abstract Pyriproxyfen is an insect growth regulator, a juvenile hormone analogue, widely used for the control of container and freshwater mosquito species throughout the world and is potent at very low levels. To date its use in natural brackish environments has been limited. In Australia the dominant nuisance mosquito species is *Aedes vigilax* which has a distribution encompassing most of the country and the bulk of control activity is focused on this one species. *Aedes vigilax* is a vector of Ross River fever and Barmah Forest virus which are both increasing in prevalence in Australia. Studies have been conducted on the efficacy of Pyriproxyfen against *Aedes vigilax* and potency at very low concentrations has been demonstrated. This paper also briefly reviews studies on the persistence of Pyriproxyfen in water-sediment systems and the possible impact on non-target aquatic organisms, specifically in relation to crustaceans.
Key Words Pyriproxyfen, Ross River fever, Barmah Forest virus

INTRODUCTION

The saltmarsh mosquito, Aedes vigilax (Skuse) is the dominant pest mosquito in Australia and a significant vector of arboviruses such as Ross River virus and Barmah Forest virus (Brown et al., 1999; Chapman et al., 1999; Kay, 1982; Russell 2002). Control programs in Australia are based primarily on larvicide applications of Bti (*Bacillus thuringiensis israeliensis*) and S-methoprene (Russell and Kay, 2008).

Pyriproxyfen is a potent mosquito larvicide used widely around the world for freshwater and container breeding species (Invest and Lucas, 2008). In Australia, Pyriproxyfen is not yet approved for use against mosquitoes but is approved for use in agricultural and urban pest markets under various brand names.

A series of studies were conducted to evaluate efficacy of Pyriproxyfen against the saltmarsh mosquito *Aedes vigilax* relative to the existing IGR product S-Methoprene (trade name ProlinkTM Liquid Larvicide, 50 g/L S-Methoprene). A further study was conducted to evaluate the length of persistence of Pyriproxyfen and S-methoprene in a water-sediment system which might provide insights into relative residual activity or potential for impacts on non-target aquatic organisms.

MATERIALS AND METHODS

Efficacy

The methodology for assessing efficacy of Pyriproxyfen against *Aedes vigilax* has been reported elsewhere (Webb et al. in press). However, in brief, a dose-response study was conducted to determine the emergence inhibition (EI_{50} and EI_{95}) values for Pyriproxyfen. Two further trials, one in the laboratory and the other in a semi-field situation evaluated efficacy (emergence inhibition) at the recommended field rate for the existing IGR product on the market in Australia (Prolink^{TM} Liquid Larvicide -50 g/L S-Methoprene).

Persistence in Water

A semi-field trial was conducted to evaluate the fate of Pyriproxyfen 90 CS (90 g/L microencapsulated formulation) in brackish water and sediment and compare it with S-Methoprene. One litre glass jars were placed in a shaded and sheltered environment and filled with 1cm of field collected mangrove sediment and topped up

to 1L with field collected sea water. Jars were shaken vigorously and sediment allowed to settle for 24 hours during which time the water clarified. After 24 hours both formulations were added to the jars at the currently registered rate of S-Methoprene equivalent to 11gai/ha. Stock solution, derived by serial dilution, was injected with a syringe on the surface of the water to simulate field application. There were 5 replicates for each treatment.

All jars were placed under cover to prevent dilution by rainwater and each jar was covered by porous gauze clothe to prevent debris from entering and also to prevent breeding of mosquitoes and other insects. Jars were subjected to dappled UV light at least up to ca. noon each day.

Five milliliter samples were extracted from each jar at periodic intervals up to 42 days after introduction of the test material. Samples were collected from two locations in the jar, 1cm below the surface of the water and at the water-sediment boundary. Samples collected from the water-sediment boundary were collected using a 5ml syringe with an attached 10 cm long PVC extension to avoid excess disturbance of the sediment layer. The 5 ml samples from each replicate were combined into a 25mL samples of both surface water and water -sediment boundary material and evaluated in the laboratory for residual Pyriproxyfen or s-Methoprene concentration.

Water samples were analysed for Pyriproxyfen and S-Methoprene residues by use of solid-phase extraction (SPE) with liquid chromatography tandem mass spectrometry (LC-MS/MS) detection.

Each sample was shaken and an aliquot loaded onto a Phenomenex StrataX SPE cartridge (60 mg/3 ml). Samples were washed with water/methanol, and Pyriproxyfen and S-Methoprene residues were eluted with methanol/formic acid. The extracts were then diluted with mobile phase. The instrumental analyses involved chromatographic separation of the target analytes via reverse-phase Liquid Chromatography on a C18 column, and identification and quantitation of residues via triple-quad mass spectrometry (LC-MS/MS) in selected reaction monitoring mode, using external calibration. The limit of quantification was determined to be 1 ppb.

RESULTS AND DISCUSSION

Efficacy

Webb et al. (in press) reported EI_{50} and EI_{95} values for Pyriproxyfen (as TG and 90 CS product) and Prolink (Figure 1). Pyriproxyfen TG was the most potent with EI_{50} and EI_{95} values of 0.019 ppb and 0.076 ppb respectively.

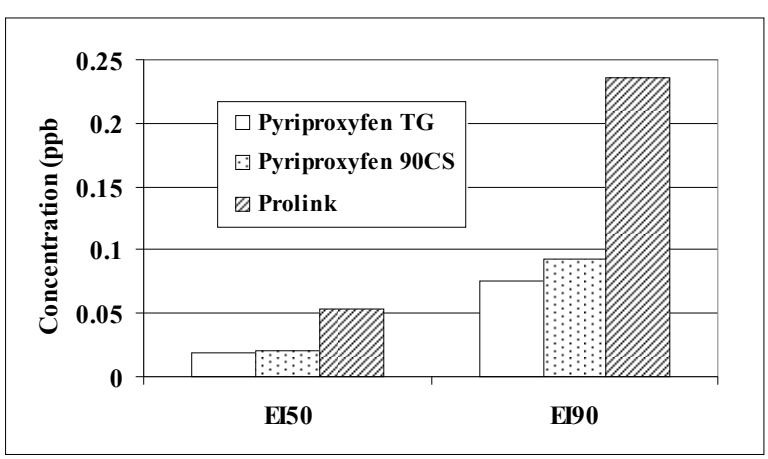

Figure 1. EI_{50} and EI_{95} values for Pyriproxyfen TG, Pyriproxyfen 90 CS and Prolink.

When compared in laboratory and semi-field conditions at the recommended field rate of S-Methoprene (ie. 11 gai/ha), Pyriproxyfen in two formulations (90 CS and 5 GR) provided 100% emergence inhibition of 3rd instar larvae, equivalent to S-methoprene. There have been few studies on the efficacy of Pyriproxyfen on saltmarsh or brackish water species. Jeffrey et al. (2007) reported EI_{50} and EI_{95} values of 0.036 ppb and 0.227 ppb respectively for *Verrulina funerea* in Australia which were very similar to *A. vigilax*. In Korea, Lee (2001) applied Pyriproxyfen 5 GR to natural brackish water ponds and achieved > 80% emergence inhibition for up to 70 days after application at nominal concentrations of 10-500 ppb with natural rainwater replenishment.

Persistence in Water

Having established that Pyriproxyfen is active in the same concentration range as S-methoprene for *A. vigilax*, a study was conducted to evaluate persistence in water to establish whether Pyriproxyfen is likely to offer enhance residual activity. The nominal application rate for both products was 7 ppb. The degradation profiles for Pyriproxyfen and S-methoprene were similar (Figure 2), starting in the range of 6-8 ppb 1 hour after application and generally declining to below the limit of quantification within ca. 7 days. This is consistent with the known half-life for aqueous photolysis of Pyriproxyfen (Sullivan and Goh 1998). Pyriproxyfen appeared to be slightly more persistent in both surface water and at the water-sedment boundary than S-methoprene, probably reflecting the greater UV stability of Pyriproxyfen (Henrick, 2007; Sullivan and Goh, 1998). Pyriproxyfen also appeared to partition to the sediment layer quicker than S-methoprene. However, they both declined below the limit of quantification (1 ppb) at similar times (5.5-7.5 days after application). Pyriproxyfen is known to bind very strongly with organic material and quickly degrades to less toxic metabolites (Hirano et al., 1998; Sullivan and Goh, 1998).

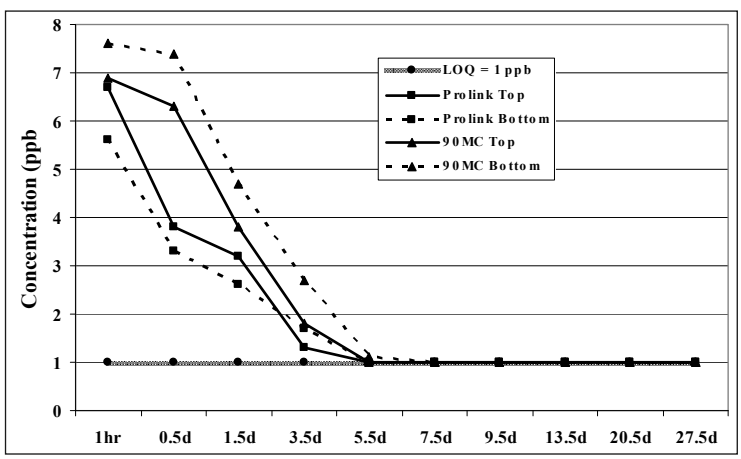

Figure 2. Degradation of Pyrpiproxyfen and S-methoprene in water.

Various laboratory and field studies have confirmed that Pyriproxyfen degrades relatively rapidly under UV light and partitions to the sediment layer (Miyamoto et al., 1993; Schaeffer and Miura, 1990; Schaeffer et al., 1988; Schaeffer et al., 1991) even when applied at rates much higher than the anticipated rate for *A. vigilax*. Schaeffer and Miura (1990) applied Pyriproxyfen at 110 gai/ha (as an emulsifiable concentrate formulation) to artificial ponds in a rice field (effective rate of 22 ppb) twice, 21 days apart, and monitored the residual concentration in pond water and sediment. No Pyriproxyfen was detected in sediment at any time during the study but the limit of detection was set at 10 ppb, close to the application rates. Residue levels in water were similar to nominal input up to 2 days after application but then declined below the detection limit. Similarly, Schaeffer et al. (1991) applied Pyriproxyfen (as an emulsifiable concentrate formulation) to dairy waste water ponds at 110 gai/ha (effective dilution of 17 ppb). The detected concentration in water at 1 hr after application was 1.3 ppb and residues in water were not detected at any subsequent time during the study. Pyriproxyfen rapidly partitioned to the organic material and then slowly declined. The half life of Pyriproxyfen in organic matter was determined to be 7.47 days. It is therefore likely that Pyriproxyfen was strongly bound to organic material in the highly polluted dairy waste water and partitioned quickly to the water-sediment interface.

EFFECTS ON NON-TARGET AQUATIC ORGANISMS

Both Pyriproxyfen and S-methoprene are juvenile hormone analogues (JHA). Hormone systems based on juvenile hormone (JH) or JH-like compounds are present in most arthropods but most highly developed in the higher (holometabolous) insects such as Diptera and Lepidoptera which have three distinct life phases (Miyamoto et al. 1993, Wright 1976). JHAs are also known to mimic the crustacean juvenile hormone methyl farnesoate (MF), a non-epoxidised precursor to JH III, the dominant juvenile hormone in insects (Miyamoto et al. 1993). So the key

non-target aquatic organisms of concern are likely to be insects that spend at least some part of their larval life in water, and crustaceans.

Pyriproxyfen is not acutely toxic to fish, at least not at the environmental concentrations generated by potential mosquito control operations. Brown et al. (1998 and 2002) and Pinder (1991a) examined the acute toxicity to local Australian species in relation to potential field rates of application. For Pacific blue-eye (*Pseudomugil signifer*), Crimson-spotted rainbowfish (*Melanotaenia duboulayi*) and Gobie (*Pseudogobius olorum*) the LC_{50} values were 106, 12.5 and 490 times the likely field concentration. For Crimson spotted rainbowfish there was no acute toxicity evident up to the highest dose (100 ppb) (Brown et al., 2002).

There has been very little published, but the toxicity of Pyriproxyfen to aquatic plants is known to be low based on regulatory studies submitted to various government regulatory agencies (Webb pers. obs.). Aside from those studies, the only available data appears to be that of Trayler (1991) who found no effect on growth and standing crop of the alga *Chlorella emersonii* at 10 ppb.

Pinder et al. (1991a) conducted acute toxicity bioassays on two common aquatic insects in freshwater lakes in Western Australia. They reported LC50 values for the Corixid bug (Micronecta robusta) and the mayfly (*Cleon fluviatile*) of 1250 ppb and 170 ppb respectively and no emergence inhibition of late instar mayfly was observed at 10 ppb. However, Hirano et al. (1998) later noted that the dragonfly Orthetrum albistrum speciosum exhibited much higher sensitivity to Pyriproxyfen in the last instar than earlier instars. Various field studies (Davis et al., 1990; Mulla et al., 1986; Pinder et al., 1991b; Schaeffer et al., 1988; Schaeffer and Miura, 1990) have concluded that Pyriproxyfen may have a short-term impact on certain non-target organisms but it does not compromise survival and dynamics of the non-target aquatic community. Schaeffer and Miura (1990) concluded that cladocerans and Podocopa were affected by the applications of Pyriproxyfen at 45 and 110 gai/ha (9 and 22 ppb) but not eliminated. They also concluded that abnormalities in adult Chironomids, and Odonata (Anisoptera and Zygoptera) between day 4 and 10 only, were probably due to sublethal dosing with Pyriproxyfen at the time of metamorphosis. Overall, they concluded that Pyriproxyfen was safe to the aquatic community, although some components were at least temporarily reduced. In contrast, Schaeffer et al. 1988 and Mulla et al. (1986) found no apparent effects on any elements of the aquatic communities studied. Davis et al. (1990) and Pinder et al. (1991b) provide the only known field data for aquatic insects from Australian environments. Davis et al. (1990) evaluated the emergence of the mayfly *Tasmanocoenis tillyardi* from ponds treated with Pyriproxyfen 5GR at 50 gai/ha (12.5 ppb) in two separate trials. In one, there was no significant difference in emergence between the control and treated plots and in the other emergence declined over 21 days post-treatment. In the same location Pinder et al. (1991b) evaluated the impact of Pyriproxyfen 5 GR at the same rate on various aquatic organisms including the caddisfly *Ecnomus pansus*, the mayfly *T. tillyardi* and the midge *Nilobezzia* sp. There appeared to be no obvious effects on abundance of these organisms nor on overall diversity. There was no impact on phytoplankton abundance. Emergence studies on the caddisfly (*Ecnomus pansus*) and the mayfly (*Tasmanocoenis tillyardi*) indicated no changes to overall emergence success nor pattern of emergence.

Acute toxicity studies on crustacean species have covered a range of taxa including a number of native Australian species - the cladoceran *Daphnia carinata*, the ostracod *Candonocypris novaezelandiae*, the amphipod *Austrochiltonia subtenius* and the estuarine shrimp *Leander tenuicornis* (Brown et al., 1999; Pinder et al., 1991a; Trayler, 1991; Trayler and Davis, 1996). The most extensively studied group is the cladocerans including *Daphnia magna* and *Daphnia carinata* which appear to be the most sensitive to methyl farnesoate and JHAs. Trayler and Davis (1996) determined an EC_{50} concentration for *D. carinata* of 80 ppb (10x the effective field rate). Margins of safety were even higher for *C. novaezelandiae* and *A. subtenius* with EC_{50} values of 6210 ppb and 120 ppb respectively (Pinder et al., 1991a). Brown et al. (1999) determined for *L. tenuicornis* an EC_{50} value of 98 ppb or 12 times the effective field rate.

Crustaceans are known to be more sensitive to JHAs using reproductive and growth endpoints than for mortality and behavioural endpoints. Miyamoto et al. (1993) determined a NOEC for survival of *Daphnia pulex* at the highest dose (1.8 ppb) but of reproductive success at 0.03ppb. However, the effect was reversible and reproductive success returned to normal after a 7 day depuration phase. For Copepods the NOEC was a high as >100 mg/L (Wang et al., 2005b).

Many earlier studies measured reproductive success in terms of neonates/adult and did not record sex ratios. Recently there has been a significant focus on the influence of MF on the initiation of male neonates and the ability of JH-like compounds to alter sex ratios, even though overall reproductive success may be maintained (Matsumoto et al., 2008; Oda et al., 2006; Olmstead and LeBlanc, 2001, 2002, 2003, 2007; Petersen et al., 2001; Tatarazako et al., 2003; Wang et al., 2005a).

Daphnia reproduce primarily by cyclic parthenogenesis (Tatarazako et al., 2003). Under favourable conditions most Daphnid offspring are female and the population expands by asexual reproduction, maximizing population growth. Environmental stress initiates the production of male neonates and the population undergoes a cycle of sexual reproduction that usually precedes dormancy during periods of low resource availability (moisture or food). MF is the key male sex determinant in crustaceans and production by the mandibular gland is triggered by environmental stress. Not surprisingly, JHAs such as Methoprene, Fenoxycarb and Pyriproxyfen have been found to induce male production by mimicking MF. A range of studies have shown the effects of JHAs on male neonate production and several have studied Pyriproxyfen specifically (Matsumoto et al., 2008; Mu and LeBlanc, 2004; Oda et al., 2006; Olmstead and LeBlanc, 2003; Rider et al., 2005; Tatarazako et al., 2003; Wang et al., 2005a).

Like other JHAs, Pyriproxyfen has been shown to shift neonate production to a male bias at relatively low exposure levels when exposure timing is precisely targeted. There appears to be a short critical 12 hour developmental window during which the oocyte is susceptible to influence (Wang et al., 2005a), corresponding to the latter stages of oocyte development (Le Blanc, 2007) and there is no effect once the eggs are transferred to the brood chamber. There is also evidence that MF and JH analogues may also act as anti-ecdysteroids interfering with embryo development (Mu and LeBlanc 2004) but they are significantly less potent as anti-ecdysteroids than as juvenoids effecting sex determination.

While exposure to JHA during early embryonic growth may trigger excess male production, it does not appear to be permanent. Matsumoto et al. (2008) found that male bias was transitory and that production of female neonates resumed in the second generation after cessation of exposure. Similarly Tatarazako et al. (2003) showed that within 8 days of cessation of exposure all offspring were again female. McKenny (2005) noted that estuarine mysid shrimp (*Mysidopsis bahia*) had slightly elevated male production in the first generation exposed to 43 ppb Fenoxycarb but in the subsequent generation of non-exposed individuals, male production actually declined below 50%. Therefore the interaction between JHAs and crustacean reproduction may be very sensitive to timing and is likely to be reversible once exposure is removed. Further, breeding is not necessarily synchronous and not all of the population may be susceptible at the time of exposure.

While controlled exposure under laboratory conditions to precisely defined reproductive stages can generate quantifiable changes in reproductive parameters, and certainly under multigenerational constant exposure studies (flow through and static renewal) such changes are evident and amplified, it does not represent a real life exposure scenario. A range of field studies at doses significantly higher than those illiciting effects in the laboratory fail to show long term impacts on crustacean populations and benthic communities overall (Pinder et al., 1991b; Schaeffer and Miura, 1990; Trayler, 1991). This is largely due to the fact that such studies are based on a single pulse exposure followed by declining field concentrations which emulates a typical exposure scenario following field application. Even using slow-release pellets, Butler et al. (2010) found that S-methoprene concentrations in stormwater retention pits were generally less than 1ppb and no impacts were evident on either natural retention pit benthic communities nor artificially created communities.

CONCLUSIONS

Aedes vigilax has been show to be highly susceptible to Pyriproxyfen. At an application rate similar to that of S-methoprene, Pyriproxyfen would provide high levels of emergence inhibition under field conditions. The degradation profile of Pyriproxyfen is also very similar with persistence in water above 1ppb for 5-7 days after application at a field rate of 11gai/ha (equivalent to a starting concentration of ca. 7 ppb). With correct timing of application this corresponds with latter instar development, the most sensitive period for larval mosquitoes with respect to juvenile hormone analogues. At the likely field rate, Pyriproxyfen does not present an undue acute toxicity hazard to aquatic organisms. Crustaceans, particularly Cladocerans, are at higher risk of reproductive effects under prolonged exposure to juvenile hormone mimics. However, a range of factors are likely to mitigate against long-term impacts by use of Pyriproxyfen for saltmarsh mosquito control. These include photodegradation, high affinity to organic matter and the short duration of exposure.

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