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The naturally derived insecticide spinosad is highly toxic to *Aedes* and *Anopheles* mosquito larvae

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Abstract. Spinosad is a naturally derived biorational insecticide with an environmentally favourable toxicity profile, so we investigated its potency against mosquito larvae (Diptera: Culicidae). By laboratory bioassays of a suspension concentrate formulation of spinosad (Tracer®), the 24 h lethal concentration (LC_{50}) against *Aedes aegypti* (L.) third and fourth instars was estimated at 0.025 p.p.m. following logit regression. The concentration–mortality response of third- and fourth-instar *Anopheles albimanus* Weidemann did not conform to a logit model. The LC_{50} value of spinosad in *Anopheles albimanus* was 0.024 p.p.m. by quadratic linear regression. A field trial in southern Mexico demonstrated that spinosad 1 p.p.m. compared with the standard temephos (Abate®) 1% granules 100 g/m^3 water prevented *Ae. aegypti* breeding in plastic containers of water for 8 weeks; at 10 p.p.m. spinosad prevented breeding for $>$ 22 weeks. In another field trial, spinosad at 5 p.p.m. and temephos both completely eliminated reproduction of *Ae. aegypti* for 13 weeks. In contrast, the bacterial insecticide *Bacillus thuringiensis* var. *israelensis* (*Bti*, Vectobac® AS) performed poorly with just 2 weeks of complete inhibition of *Ae. aegypti* breeding. Spinosad also effectively prevented breeding of *Culex* mosquitoes and chironomids in both trials to a degree similar to that of temephos. We conclude that spinosad merits evaluation as a replacement for organophosphate or *Bti* treatment of domestic water tanks in Mesoamerica. We also predict that spinosad is likely to be an effective larvicide for treatment of mosquito breeding sites.

Key words. *Aedes aegypti*, *Anopheles albimanus*, *Bacillus thuringiensis israelensis*, *Culex* spp., Chironomidae, inhibition of reproduction, larvicide, spinosad, temephos, Mexico.

Introduction

The success of insecticide-based control programmes in reducing the prevalence of insect vector-borne diseases (WHO, 1995; Curtis & Davis, 2001) has been accompanied by growing interest regarding the harmful effects of widescale and prolonged use of synthetic insecticides on human health and the environment (Attaran *et al*., 2000; Walker, 2000). Mosquito resistance to a number of conventional chemical insecticides is also a matter of current concern (Sina & Aultman, 2001).

Spinosad is a mixture of tetracyclic macrolide neurotoxins, spinosyn A and D, produced during the fermentation of the soil actinomycete *Saccharopolyspora spinosa*. As such, it may be considered as a bioinsecticide (Copping & Menn, 2000). Spinosad is highly toxic to Lepidoptera, Diptera and some Coleoptera and has a unique mode of action involving the postsynaptic nicotinic acetylcholine and GABA receptors (Salgado, 1998; Watson, 2001). The compound has been developed by Dow Agroscience (http://www.dowagro.com) as an agricultural insecticide for control of field crop, orchard and turf pests. Spinosad has a very low mammalian toxicity and a favourable environmental profile with low

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persistence and low toxicity to a number of predatory insects (Miles & Dutton, 2000; Williams *et al*., 2003). As a result, the United States Environmental Protection Agency has classified spinosad as a reduced risk material (Thompson *et al*., 2000).

The insecticidal properties of *S. spinosa* metabolites were first detected in a qualitative mosquito bioassay, during routine screening of soil samples for biologically active compounds in the early 1980s (Thompson *et al*., 2000). However, as far as we are aware, this product has not been subjected to testing for control of mosquito species of medical importance.

In this study, we aimed to determine the susceptibility of *Aedes aegypti* and *Anopheles albimanus* to spinosad. These species were selected because of their importance as vectors of dengue virus and *Plasmodium vivax*, respectively, in Mesoamerica. Until recently, control of *Anopheles* spp. in Chiapas State, Mexico, was based on the use of DDT, which has recently been phased out in favour of household applications of organophosphates and pyrethroids (Arredondo-Jiménez *et al*., 1993). Control of *Ae. aegypti* is achieved by pyrethroid fogging of residential areas and granular formulations of the organophosphate temephos (Abate®, Clarke Mosquito Control, www.cmosquito.com) placed in domestic water tanks.

The objectives of this study were two-fold. First, we aimed to determine the concentration–mortality relationship for *Ae. aegypti* and *An. albimanus* exposed to spinosad in the laboratory. Second, we tested the duration of protection offered by spinosad when applied to urban breeding sites to inhibit the reproduction of *Ae. aegypti* in southern Mexico. For this, we included treatments involving established mosquito control substances: temephos and a commercial formulation of the bacterial insecticide *Bacillus thuringiensis* var. *israelensis* (*Bti*), Vectobac® (www.valentbiosciences.com).

Materials and methods

Insects, spinosad and field site

Eggs of *Ae. aegypti* and *An. albimanus* were obtained from laboratory colonies held in the Centro de Investigación de Paludismo, Tapachula, Chiapas, Mexico. Mosquitoes used in the experiments described below were reared using filtered dechlorinated tap water. All laboratory procedures involving mosquitoes were performed at 26 ± 1°C, LD 12 : 12 h light cycle and 75–85% r.h.

Spinosad was obtained from an agrochemical supplier in Mexico in a commercial suspension concentrate formulation (Tracer® Naturalyte® Insect Control) comprising 480 g active ingredient (a.i)/L. This product is labelled for use as an agricultural insecticide for control of lepidopteran and thrips pests of vegetables in Mexico.

The field experiments were performed in the grounds of El Colegio de la Frontera Sur (ECOSUR). The ECOSUR laboratories are sited on the outskirts of the town of Tapachula (population ~200 000), Chiapas, Mexico in a lowland coastal tropical region, 22 km from the Pacific coast and 10 km from the border with Guatemala. The temperature is typically 33–36 °C by day and 22–25 °C at night with high humidity (75–90%) and almost daily precipitation during the rainy season (~350 mm/month from May to mid-November).

Laboratory bioassay

The susceptibility of each species of mosquito to spinosad was tested in the laboratory using a methodology adapted from the Elliot larval test (WHO, 1975). Groups of 25 larvae of the third and fourth instar were placed in 150 mL plastic cups containing a solution of spinosad at one of the following concentrations: 0.001, 0.003, 0.01, 0.03, 0.1 p.p.m. active ingredient (a.i.). Four groups of larvae were assigned to each treatment. An additional cup contained water as a control. After 1 h exposure, larvae were transferred to cups containing 100 mL clean dechlorinated water. A small quantity of powdered soya bean and yeast was added to each cup as food. Mortality responses were recorded 24 h later. A larva was classified as dead if it did not move when gently touched with the point of a toothpick. The experiment was performed three times on different dates.

Field trial 1

A field trial was performed to determine the duration for which spinosad offered protection against the reproduction of *Ae. aegypti* in an urban environment. The trial commenced on 23 June and was terminated on 14 November 2003.

Circular brown plastic containers of 1.5 L capacity were filled with 1.0 L dechlorinated water, treated with a grass infusion, and assigned to one of the following treatments: (i) 1 p.p.m. spinosad, (ii) 10 p.p.m. spinosad, (iii) 0.1 g temephos 1% a.i. sand granules (Abate), equivalent to the recommended rate of 100 g granules/m^3 water (PAHO, 1981), (iv) water control. Each treatment was replicated eight times. The containers were placed in a Latin square design on metallic stands located in the shade of the overhanging eves of a laboratory building in the grounds of ECOSUR. Each container was carefully inspected at weekly intervals and living insects were counted and all living and dead insects were removed. Immature mosquitoes were classified visually to genus and other aquatic insects were classified to family (chironomids, predatory Coleoptera). Water lost through evaporation was replaced with clean dechlorinated water. The experiment was terminated at 22 weeks after the start.

Field trial 2

A second field trial was performed in an identical manner to the preceding experiment except with the following treatments: (i) 5 p.p.m. spinosad, (ii) 0.1 g temephos granules,

(iii) 1.3 μL Vectobac AS (*Bacillus thuringiensis israelensis*, Abbott Laboratories, U.S.A., equivalent to the recommended rate of 1 L/ha), (iv) water control. The experiment commenced on 7 July and was terminated on 2 December 2003, 21 weeks after the start.

Statistical analyses

Concentration–mortality results for *Ae. aegypti* were subjected to logit analysis and the Fieller macro present in the Generalized Linear Interactive Modelling program (GLIM, Numerical Algorithms Group, 1993). Scaling was performed to accommodate minor overdispersion. The behaviour of models was checked by examination of the distribution of residuals and fitted values. The results from *An. albimannus* did not conform to a logit model and were therefore subjected to quadratic linear regression of percentage mortality against $\log_e$ [concentration a.i.].

The numbers of *Ae. aegypti* larvae and pupae recorded in each field trial was pooled at intervals of 14 days and subjected to $\sqrt{x}$ transformation and univariate repeated measures analysis of variance. The datasets fulfilled the assumptions of homogeneity of variance, normality and sphericity of the covariance matrix. Numbers of other insects (chironomids, *Culex* spp.) were not included in the analyses.

The overall percentage inhibition of reproduction (I_r) was calculated as: $I_r = (1 - T/C)*100$, where T and C are the total numbers of immature stages observed in the treatment and control containers, respectively, during the course of the experiment.

Results

Laboratory bioassay

Logit regression was performed following correction for minor overdispersion in the mortality results for *Ae. aegypti* (scale parameter 1.20). Logit regression of mortality at 24 h post-treatment against $\log_e$ [concentration] gave an estimated LC_{50} value of 0.025 p.p.m. spinosad (range of 95% confidence limits: 0.023–0.027) for *Ae. aegypti*. Slope and intercept values (± SE), given in terms of the $\log_e$ odds ratio (p/q), were slope 1.920 ± 0.331, intercept 7.061 ± 0.086.

The results from *An. albimanus* did not conform to a logit model due to a high degree of overdispersion. This situation was not be improved through a series of standard transformations. Therefore, these data were subjected to quadratic linear regression of percentage mortality against $\log_e$ [concentration], which fitted observed values very closely ($R^2 = 0.9835$) (Fig. 1). From this regression, the 50% lethal concentration for *An. albimanus* was estimated at 0.024 p.p.m. a.i.

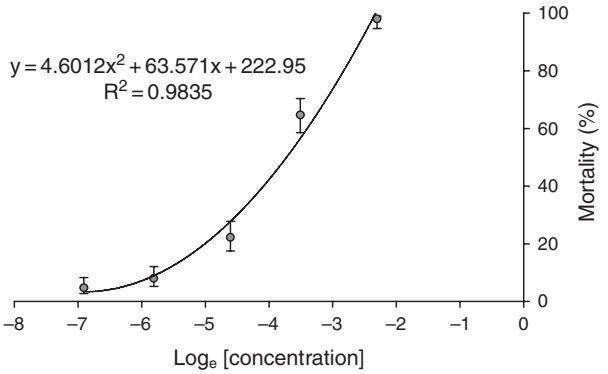

Fig. 1. Quadratic regression of $\log_e$ [concentration of spinosad (p.p.m.)] against percentage mortality response in third- and fourth-instar *Anopheles albimanus*. Bars represent S.E. of the mean and are asymmetrical.

Field trial 1

Aedes aegypti was by far the most prevalent species observed in experimental containers (Table 1). *Culex* spp. and chironomids were also common. Very low numbers of predatory Coleoptera (Hydrophilidae, Dytiscidae) were occasionally observed and a single example of *Toxorhynchitis teobaldi* but these were not considered further. The mean number of immature *Ae. aegypti* in control containers varied from 14 to 74 during the 22 weeks of the experiment (Fig. 2a). Numbers of *Ae. aegypti* were significantly reduced in all other treatments (Table 2). Water containing spinosad at 1 p.p.m. resulted in complete inhibition of reproduction of *Ae. aegypti* for 8 weeks, after which this treatment was similar to that of the control. Overall reproduction in containers treated with 1 p.p.m. spinosad was approximately half that observed in the control (Table 1). In contrast, no immature *Ae. aegypti* were observed in the 10 p.p.m. spinosad treatment at any stage during the experiment. Temephos completely inhibited *Ae. aegypti* reproduction for 8 weeks. Mean numbers of immature stages in temephos-treated containers stayed lower than observed in the control, resulting in a 91% overall inhibition in *Ae. aegypti* reproduction compared to the control treatment.

Other species were also inhibited from reproducing in water containing spinosad (Table 1). Mean numbers of *Culex* spp. in control containers varied from 0 to 24, whereas *Culex* spp. were not observed in the spinosad 1 p.p.m. for the first 15 weeks of the trial and were never observed in the spinosad 10 p.p.m. treatment. *Culex* spp. appeared sporadically in temephos-treated containers from week 14 onwards. The total numbers of immature *Culex* observed in all containers over the course of the experiment were reduced by over 90% in the 1 p.p.m. spinosad and temephos treatments and were completely inhibited in the 10 p.p.m. spinosad treatment.

Similarly, the mean density of chironomid larvae varied from 1.4 to 12 larvae/container in the control during the course of the experiment. Spinosad at 1 p.p.m. completely inhibited reproduction for 8 weeks but the overall effectiveness was low (22% inhibition). In contrast, no chironomids were observed in the 10 p.p.m. spinosad treatment during the course of the trial. Temephos was not particularly effective against chironomids, which appeared at week 3

Table 1. Duration of complete inhibition of reproduction and overall percentage reduction in reproduction of *Aedes aegypti*, *Culex* spp. and chironomids in containers treated with spinosad and temephos in the first field trial or spinosad, temephos and Vectobac (*Bacillus thuringiensis* var. *israelensis*) in the second trial

	Ae. aegypti	*Culex* spp.	Chironomidae
Field trial 1			
Total number observed in controls	5286	646	1014
Duration absolute inhibition (weeks)			
Spinosad 1 p.p.m.	8	15	8
Spinosad 10 p.p.m.	> 22	> 22	> 22
Temephos	8	13	2
Percentage overall inhibition			
Spinosad 1 p.p.m.	55	94	22
Spinosad 10 p.p.m.	100	100	100
Temephos	91	93	47
Field trial 2			
Total number observed in controls	2326	203	934
Duration absolute inhibition (weeks)			
Spinosad 5 p.p.m.	13	17	7
Vectobac	2	0	3
Temephos	11	16	2
Percentage overall inhibition			
Spinosad 5 p.p.m.	84	90	72
Vectobac	22	0	19
Temephos	87	85	72

Control containers were filled with dechlorinated water alone. Percentage overall inhibition of reproduction based on total numbers of each group of insects observed in treatment containers compared to the numbers observed in the control.

and the overall inhibition of reproduction was intermediate (47% inhibition) (Table 1).

Field trial 2

Total numbers of *Ae. aegypti* observed in the second field trial were approximately half that of the first trial (Table 1), probably because the second trial began at the end of the rainy season and lasted into the dry season when *Ae. aegypti* populations decline. This effect was also evident in mean numbers of *Ae. aegypti* in control containers, which declined from 20 to 50 larvae/container for the first 7 weeks of the trial to fewer than 15 larvae/container thereafter (Fig. 2b). Spinosad at 5 p.p.m. and temephos both completely eliminated reproduction of *Ae. aegypti* for 13 weeks and the overall degree of inhibition was 84% and 87%, respectively, compared to the control (Tables 1 and 2). The bacterial insecticide, Vectobac, performed poorly with just 2 weeks of complete inhibition of *Ae. aegypti* and overall numbers reduced by 22% during the course of the trial. However, small numbers of dead larvae were frequently observed in the Vectobac-treated containers during the trial, suggesting that the pathogen persisted and continued to cause a low prevalence of mortality of mosquito larvae.

Temephos and 5 p.p.m. spinosad also performed very similarly in inhibiting the reproduction of *Culex* spp. with 16 and 17 weeks of complete inhibition, respectively, and 85% overall reduction in *Culex* numbers compared to the control (Table 1). Vectobac was not effective against *Culex* spp.; overall numbers of *Culex* spp. in Vectobac-treated containers slightly exceeded those of the control.

Numbers of chironomids were not affected by the change from rainy to dry season during the second trial (Table 1). Spinosad 5 p.p.m. gave the greatest period of complete inhibition of reproduction of chironomids (7 weeks), which was 2–3 times more than the other treatments. Overall numbers of chironomids were reduced by 72% in both spinosad and temephos treatments, whereas Vectobac offered little inhibition of chironomid reproduction compared to the control (Table 1).

Discussion

Spinosad proved to be highly toxic to larvae of both species of mosquitoes in laboratory assays. The response of *Ae. aegypti* larvae closely followed the logit model, with an estimated LC_{50} of 0.025 p.p.m. spinosad. The LC_{50} value could not be estimated by logit regression for *An. albimanus* because the data distribution did not conform to a logit model (Fig. 1). The 50% lethal concentration estimated by the quadratic regression model for *An. albimanus* was 0.024 p.p.m. The results of preliminary studies on another *Anopheles* species, *An. pseudopunctipennis* also indicated significant deviation from a logit model, but an excellent fit to a quadratic model ($R^2 = 0.9877$), with an estimated 50% lethal concentration of 0.010 p.p.m. (preliminary results not shown, based on 200 insects/concentration). These observations indicate that the anopheline

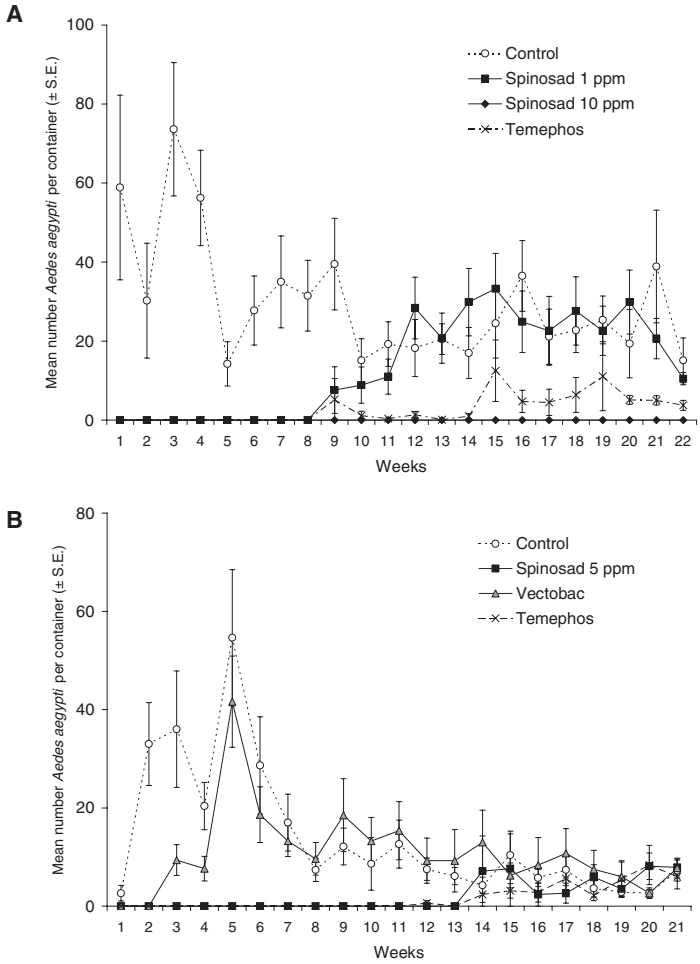

Fig. 2. Densities of immature *Aedes aegypti* observed in containers treated with (A) spinosad (1 and 10 p.p.m.), temephos granules (Abate) and water control in first field trial or (B) spinosad (5 p.p.m.), Vectobac (*Bacillus thuringiensis* var. *israelensis*), temephos granules and water control in second field trial. Vertical bars represent S.E. of the mean.

species were as susceptible to spinosad as was *Ae. aegypti*, although the nature of the anopheline concentration–response curve differed from that of a standard logit or probit model.

Spinosad is known to be highly active against Diptera and is registered for control of leafmining dipteran pests of crops in many countries. Due to the favourable United States EPA classification, spinosad is also used in a bait formulation over very large areas for control of the Mediterranean fruit fly, *Ceratitis capitata*, in Central America (Vargas *et al.*, 2001). Laboratory assays on *C. capitata* indicated LC_{50} values of 0.013 p.p.m. for neonate larvae continuously exposed in diet (Adán *et al.*, 1996), very similar to the values we obtained for the mosquito species that we tested.

Spinosad is slower acting than many conventional chemical insecticides, such that observations of mortality shortly after exposure may not accurately reflect the proportion of the population that have acquired a lethal dose of the compound (Williams *et al.*, 2003). Consequently, we performed evaluations at 24 h after initial exposure and ignored the 1 h observations suggested in the WHO protocol specifically to account for the speed of action of spinosad.

Field trials clearly demonstrated that spinosad at a concentration of 10 p.p.m. inhibited the reproduction of *Ae. aegypti* for the entire 22-week period of the first trial. In contrast, the chemical treatment, temephos, provided complete inhibition of this species for a considerably shorter period (8 weeks). The performance of spinosad at a concentration of 5 p.p.m. in the second trial was similar to that of temephos and far better than the bacterial-based insecticide, Vectobac (*Bti*). Spinosad was also effective at inhibiting the reproduction of *Culex* spp. and chironomids in both trials to a degree equal or better than observed in the temephos treatments. Spinosad is not a cheap product, but the very low concentration required to eliminate mosquito reproduction and the high cost of alternative biological insecticides

Table 2. Repeat measures analysis of variance of numbers of immature *Aedes aegypti* observed in water containers treated with 1 and 10 p.p.m. spinosad, temephos and water control in field trial 1, and 5 p.p.m. spinosad, Vectobac, temephos and water control in field trial 2

Source	Sum of squares	d.f.	Mean squares	*F*	*P*
Field trial 1					
Between Subjects					
Treatment	250.6	3	83.5	13.2	<0.001
Blocks	49.0	1	49.0	7.7	0.011
Rows	23.9	3	8.0	1.3	0.313
Columns	16.1	3	5.5	0.9	0.481
Error	132.6	21	6.3		
Within Subjects					
Time	252.9	10	25.3	14.3	<0.001
Time*Treatment	198.2	30	6.6	3.7	<0.001
Time*Blocks	33.7	10	3.4	1.9	0.047
Time*Rows	40.6	30	1.3	0.8	0.810
Time*Columns	47.7	30	1.6	0.9	0.625
Error	372.5	210	1.8		
Field trial 2					
Between Subjects					
Treatment	874.0	3	291.3	30.6	<0.001
Blocks	0.3	1	0.3	0.03	0.853
Rows	23.3	3	7.8	0.8	0.498
Columns	23.8	3	7.9	0.8	0.489
Error	199.5	21	9.5		
Within Subjects					
Time	121.3	10	12.1	3.8	<0.001
Time*Treatment	660.1	30	22.0	6.9	<0.001
Time*Blocks	30.0	10	3.0	0.9	0.493
Time*Rows	51.8	30	1.7	0.5	0.975
Time*Columns	53.7	30	1.8	0.6	0.968
Error	665.9	210	3.2		

For analysis, weekly records of numbers of immature *Ae. aegypti* were summed for 14 day intervals, square-root transformed and subjected to repeat measures ANOVA. Water containers were arranged in a Latin square design (rows × columns) consisting of four containers per treatment on each of two adjacent metallic stands (blocks).

with minimal mammalian toxicity, such as *Bti*, means that spinosad-based vector control may be economically viable in all but the poorest developing countries.

Clearly, the observed inhibition of reproduction of *Ae. aegypti* may have been due to two effects; a reduction in the attractiveness of spinosad-treated containers for *Ae. aegypti* and/or mortality of the immature stages prior to, or shortly after hatching. We observed large numbers of eggs on the sides of spinosad containers, suggesting that spinosad is not overtly repellent to *Ae. aegypti* at the concentrations tested and that the principal cause of reduced reproduction lies in the insecticidal properties of this compound. Ovicidal properties of spinosad have been reported for lepidopteran species (Bret *et al.*, 1997; Peterson *et al.*, 1998), although the magnitude of the ovicidal activity is lower in water compared to that observed when using organic solvents (Pineda *et al.*, 2000).

Mammalian toxicity of spinosad is extremely low ($LD_{50} > 5000$ mg/kg for rodents). Spinosad is also practically non-toxic to birds, whereas toxicity to fish is classed as slight or moderate with 96 h acute LC_{50} values between 5 and 30 p.p.m., depending on species (Thompson *et al.*, 2000). The advantages of spinosad use for larval mosquito control are clear in terms of the minimal risks to human health. However, the impact of this compound on aquatic non-target organisms is poorly understood. Spinosad is toxic to a number of aquatic invertebrates including *Daphnia* spp., chironomids, shrimp and molluscs (Pest Management Regulatory Agency, 2001). In demographic studies on *Daphnia pulex*, continuous exposure to spinosad resulted in population extinction at > 0.01 p.p.m. However, spinosad was at least five times less toxic than the organophosphate diazinon during continuous exposure studies (Stark & Vargas, 2003).

We conclude that spinosad merits detailed evaluation as a replacement for organophosphate treatment of domestic water supplies in Mesoamerica. For this, a slow-release formulation would be required for extended control in household water tanks, similar to the pellet and briquette formulations employed for slow release of *Bti* (WHO, 1999). Compared to *Bti*, spinosad also appears to provide longer lasting protection against reproduction of urban vectors such as *Ae. aegypti*. We also predict that spinosad is likely to be a highly effective larvicide for treatment of mosquito breeding sites, although this has yet to be demonstrated. The possible adverse effects of a spinosad-based larvicide on non-target aquatic invertebrates would have to be taken into consideration, given the toxicity spectrum of this biologically derived insecticide.

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