The Compatibility of Spindor Dust with \textit{Poecilia reticulata} for Integrated Mosquito Larviciding

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Abstract

\textbf{Background:} The compatibility of spindor dust (spinosad), a bio-rational larvicide derived from the fermentation of a soil bacterium \textit{Saccharopolyspora spinosa}, was investigated on the mosquito fish, \textit{Poecilia reticulata}, and larvae of both \textit{Anopheles gambiae} s.s. and \textit{Culex quinquefasciatus} mosquitoes.

\textbf{Methods:} Three replicates of each to different concentrations of spinosad dust under static bioassay were performed to determine the acute toxicity of the larvicide on each organism. To investigate the genotoxic and ultrastructural changes in \textit{P. reticulata}, the fish were exposed for 28 days to low concentrations of the test larvicide capable of killing 30\% and 70\% of \textit{Cx. quinquefasciatus} larvae. Thereafter, gill and intestinal cells were removed at days 3 and 28 respectively, and then processed for epifluorescent and transmission electron microscopic studies.

\textbf{Results:} Spinosad showed no lethal toxic effect on \textit{P. reticulata} but caused an appreciable mortality to \textit{Anopheles} and \textit{Culex} larvae with 24\% \textit{LC50} values of 59.34 $\mu$gL$^{-1}$ and 73.06 $\mu$gL$^{-1}$ respectively. The larvicide failed to significantly induce micronuclei in the fish as determined with acridine orange assay ($P>0.05$). Marked damage characterised by pyknotic nuclei, loss of cristae in mitochondria, dense and degraded cytoplasm was mostly found in the exposed intestinal cells of the fish and the damage severity increased with increasing concentration of spinosad.

\textbf{Conclusion:} Spinosad at 49 $\mu$gL$^{-1}$ seems to be the threshold above which severe damage occurred in the fish. Therefore, spinosad is only compatible with \textit{P. reticulata} for integrated mosquito larval control at concentration not greater than 49 $\mu$gL$^{-1}$.

Keywords: Spinosad; Larvicide; \textit{Poecilia reticulata}; \textit{Anopheles gambiae} s.s.; \textit{Culex quinquefasciatus}

Introduction

Current efforts at mosquito control in Nigeria and indeed the whole of Africa have gradually drifted away from the more traditional larviciding and environmental management to almost solely on disease management and domestic adulticiding since the discovery of the dangers of Dichlorodiphenyltrichloroethane (DDT). Despite ease of application, disease management and adulticiding alone have failed to interrupt mosquito vector burden on a nationwide basis evidenced by the continual establishment of new cases of mosquito related diseases and subsequently death, particularly amongst children under the age of five and pregnant women [1]. While it is true that mosquitoes cannot be totally eliminated in any ecosystem, good mosquito management practices that consider environmental issues must be adopted within the purview of Integrated Vector Management (IVM) to keep mosquito population below the level of public health concern. Integrated Vector Management is the targeted use of different vector control methods alone or in combination to achieve the greatest disease control benefits in the most cost effective manner while minimising negative impacts on the ecosystem e.g. depletion of biodiversity and adverse side effects on public health [2].

The larval stages of mosquito vector are the most vulnerable stages because they are confined by their nature to various aquatic media making control practices easier, more effective and highly sustainable. When mosquito larvae are effectively targeted, the transmission chain in the mosquito life cycle is broken, thereby resulting in a more sustainable mosquito control programme. Therefore, to achieve an effective and long lasting mosquito control programme there is a need to reprioritize larval control practice and adopt the "double barreled" approach inherent in a good IVM System. Existing and efficacious chemical larvical control methods can be combined with non-chemical larvical control methods such as the use of guppy fish that occupy several open drainages in Lagos, Nigeria. Guppies have been credited for their high larvivorus potential against mosquito vectors in many parts of the world [3-10]. As efficient biological control agent, guppies need to be protected from the deleterious effects of larvicides. Spinosad is an insect control product derived from the fermentation of a soil bacterium \textit{Saccharopolyspora spinosa} [11]. This compound was shown to cause cytogenotoxic damage to guppy species at higher concentration [12].

It was therefore pertinent to conduct an initial investigation on the genotoxic and ultra structural effects of the biolarvicide at low concentrations in bid to establish a dosage solution that is compatible to apply in an aquatic ecosystem particularly for integrated mosquito larval control practice where the integrity of the fish as a support control agent is to be ensured. Micronucleus (MN) is considered as the most suitable and effective method to use in fish when evaluating the genotoxic effects of xenobiotics because of its simplicity and ease of scoring [13-15]. The success of integrated mosquito larval control...
will rely greatly on the data generated from this study hence the aim of this research was to determine a concentration of spinosad that will effectively reduce mosquito vector population with minimal deleterious impact on the non-target biological control component of an IVM programme.

Materials and Methods

Collection of test organisms

*Poecilia reticulata* were collected in the morning hours from drainage at Christian Missionary Grammar School (CMS), Yaba, Nigeria (N6.533048, 3.388424E), and using fish net of mesh size 1.5 mm in diameter. Similarly, a five-litre capacity container was used to take five scoops of *Culex* larvae from an opposite drainage to the guppy’s collection site (N6.53427, 3.390012E). Fish and *Culex* larvae were transported to the Zoology Department, University of Lagos, Nigeria in different buckets. *Anopheles* larvae were collected from the University Biological Garden (N6.51841, 3.400679E) by setting up ten oviposition traps made out of plastic containers. The containers were placed randomly within the garden at 1.5 m height. They were half filled with dechlorinated tap water with few dried leaves placed at the bottom of each container to give a dull background, attractive to gravid *Anopheles* females. An oviposition substrate (Whatman No 1 filter paper) was then lined vertically inside the containers where gravid females preferred to lay eggs just above the water level. Traps were monitored daily for the presence of mosquito larvae that were then collected and taken to the laboratory for transparent plastics containing dechlorinated tap water. Approximately 1,100 *Anopheles* and 2,300 *Culex* larvae were collected.

Breeding/Rearing of test organisms

The fishes were released into a 200 L holding tank containing dechlorinated tap water at pH 7. They were reared under laboratory condition of 28°C ± 0.8, 72% ± 2% relative humidity and a 12:12 h light: dark regime. The tank was drained then washed and refilled with fresh dechlorinated tap water twice weekly to prevent the accumulation of fish metabolic wastes. After 8 days of acclimatization period, selected brood stocks were transferred into 5 litres plastic containers to obtain offspring. After 3-4 weeks, a cycle of reproduction was completed, and 2 day old juveniles were separated from adults and introduced into 2 litres of well aerated dechlorinated tap water where they were allowed to mature into adults, mean length 3.5 ± 0.2 cm. Mosquito species were separated into their respective types using the keys of Oyerinde [16]. Larvae from *Anopheline* and Culicine mosquitoes were then separately collected with nylon mesh and each poured into five 500 ml transparent plastic containers, half filled with dechlorinated tap water in which a mixture of 1.50 g mice pellets and 0.7 g yeast were added on the bottom of each container to give a dull background, attractive to gravid *Anopheles* females. Adults were initially fed on a 10% glucose solution soaked into cotton wool. Five adult mosquitoes from each type were collected and taken to the laboratory in transparent plastics containing dechlorinated tap water. Approximately 1,100 *Anopheles* and 2,300 *Culex* larvae were collected.

Selection of test concentrations for sub-lethal toxicity studies

Low concentrations of spinosad, 49 μgL⁻¹ and 110 μgL⁻¹ that were within the range that killed 30% and 70% of *Cx. quinquefasciatus* larvae respectively but did not cause mortality in guppy or impair the fish feeding potential during acute toxicity study were selected for Acridine Orange (AO) and Transmission Electron Microscopy (TEM) analyses. The *Culex* sp. was selected for study because it was more tolerant to spinosad than *Anopheles* sp., based on the result from acute toxicity test. For the Acridine assay, benzene (CAS: 71-43-2) which is a known mutagen was selected as the positive control at 0.8 μgL⁻¹ [17,18].

Acridine orange assay

Fish were not fed 24 h before testing and a 28-day static-renewal bioassay was utilized where the test media were renewed at the same concentration once every 48 h [19]. Fish of mean length 3.5 ± 0.2 cm were randomly selected and divided into 3 groups (21fish/group). At day 3 and 14 respectively, three fish were randomly selected from each replicate of treatment, negative and positive control groups respectively and immediately dissected to remove gill arches. Gill cells were processed for structural analysis using the induction of micronuclei as adapted by Cavas [15]. Gill cells were smeared on clean slides and fixed in three successive changes of methanol-acetic acid solution of ratio 1:3 v/v. After 24 h, phosphate buffer solution (pH 7.4) containing AO at a concentration of 0.003% was dropped on each slide, then covered with cover slips, sealed with transparent nail polish. Three slides were prepared from each randomly selected fish in each replicate with controls. Prepared slides were viewed for the presence of micronuclei with 63x/1.4 oil immersion under Olympus BX51 microscope. One thousand five hundred (1500) cells were scored from each slide. Micronuclei were detected as exhibiting yellow

Acute toxicity test

Spinosad with active ingredient 1.25 g/kg consisting of Spinosyns A (CAS: 131929-60-7) and D (CAS: 131929-63-0) was obtained as spinor dust from Nigeria Stored Product Research Institute, Yaba (NSPRI). A stock solution of the larvicide was prepared to a final concentration of 1.25 mgL⁻¹ using dechlorinated tap water as diluent. Therewith, serial dilutions were prepared for acute toxicity test against mosquito larvae as follows: 0 μgL⁻¹, 25 μgL⁻¹, 50 μgL⁻¹, 75 μgL⁻¹, 100 μgL⁻¹, 125 μgL⁻¹, 150 μgL⁻¹, and guppies: 0 μgL⁻¹, 20 μgL⁻¹, 40 μgL⁻¹, 100 μgL⁻¹, 250 μgL⁻¹, 500 μgL⁻¹, 1,500 μgL⁻¹. Prior to the start of experiment, the physicochemical characteristics (pH, dissolved oxygen, conductivity and temperature) of the test media and dechlorinated tap water (control) were analyzed with a pH meter (*©Mettler Toledo AG*), DO meter (*©Mettler Toledo AG*), Conductivity meter (*©Mettler Toledo AG*) and Stem Glass Thermometer (Uniscope) respectively. *Poecilia reticulata* were not fed 24 h prior to bioassay. Three replicates of 25 fish of mean size 3.5 ± 0.2cm were randomly selected and placed in different bioassay containers of capacity 1litre, each half filled with control and treated media at earlier stated concentrations. Similarly, active 4th instar mosquito larvae of *Anopheles* and *Culex* species in three replicates of 50 each were randomly selected and respectively placed in bioassay containers of capacity 1litre, each half filled with treated and untreated media at earlier stated concentrations. A fish or larva was classified as dead if it failed to move when gently probed with the edge of a glass rod. Mortality was less than 5% in each container holding control fish and larva.
green fluorescence under blue excitation using an FITC barrier filter. Micronuclei were described according to Al-Sabti and Metcalfe [14] and Cavas [15].

**Transmission electron microscopy**

At day 28, three fish were randomly selected from each concentration in the treatment group along with control fish for dissection to remove intestinal tissue. Tissue was immediately fixed with 1.25% glutaraldehyde (EMS, USA) in 0.10M phosphate buffer solution, pH 7.4 at 4°C for 1 h in the dark, and then rinsed three times in phosphate buffer solution pH 7.4. Each rinse lasted for 15 minutes in the cold and dark. Tissue was post-fixed in un buffered 2% osmium tetroxide for 2 h at room temperature in the hood, and then rinsed two times in distilled water for 5 minutes, each. This was followed by an ascending series of graded alcohol dehydrations (25%, 50%, 75%, 95% and 100%). After 24 h, tissue was in filtered with Spurr’s resin, 1:5 then 1:2 (ethanol: plastic) on a rotator, followed by two 100% plastic changes on a rotator. Each series of infiltration lasted for over 8 h. Tissue samples were then embedded using a siliconized rubber mould with Spurr and placed in 60°C oven overnight. Samples were removed after 24 h and allowed to cool. Hardness was checked followed by trimming of the blocks for sectioning. Thick sections (1μm) were cut with glass knife and stained with toluidine blue dye. The sections were then examined by light microscopy to select areas for fine structural study and photomicrography. Ultrathin sections (80 nm) were cut with an MT-2B ultramicrotome (Sorvall) using a glass knife. The ultrathin sections were taken on 300 mesh copper grid and stained with 2% Uranyl Acetate and Reynold’s lead citrate solution for 30 and 3 minutes, respectively. The samples were imaged at 80kV with Philips CM-10 Transmission Electron Microscope.

**Statistical analysis**

The dose mortality response of the 24 h toxicity test was analyzed with Probits while the Student paired sample T-test was used to analyze the significant differences in the frequency of micronucleus in treated and control media. Both statistical tools were obtained from SPSS Version 15.0 for Windows (SPSS Inc. Chicago, IL, USA).

**Results**

**Physicochemistry of the medium**

All treatments were maintained under the same conditions, resulting in nearly identical physicochemical values for the control and spinosad treated media (Table 1). Thus, indicating that spinosad was the only difference among the treatments.

**Acute toxicity and susceptibility of test organisms**

*An. gambiae s.s.* was the more susceptible target organism with a susceptibility factor (SF) of 1.00 and LC_{50} value estimated at 59.34 μgL^{-1} (13.43-104.39), compared to *Cx. quinquefasciatus* SF=1.2 and LC_{50} value at 73.08 μgL^{-1} (55.29 - 93.01) (Figure 1). Spinosad compound exerted significant (P>0.05) induced in spinosad treated gill cells except with the benzene group (Table 2). The patterns of induction of micronuclei are shown in Figures 2 and 3.

**Frequency of micronuclei with AO assay**

At both days 3 and 14, observations of micronuclei were not significantly (P>0.05) induced in spinosad treated gill cells except with the benzene group (Table 2). The patterns of induction of micronuclei are shown in Figures 2 and 3.

**TEM of Intestinal cells of guppy**

Figures 4-9 show the results of the TEM examination of intestinal cells of control and exposed fish. In the control group, the cytoplasm and nuclear membrane were with well-defined nucleus and one nucleolus just as in the mitochondria with distinct cristae and well defined matrices (Figures 4 and 7). In the treated cells however, there were cell distortions that became more severe with increasing concentration of the larvicide. Minimal damage occurred only in the nucleus at lower concentration of 49 μgL^{-1} including nucleus elongation with rearranged chromatin, presence of large secretory vesicles and electron dense cytoplasm (Figures 5 and 8). At higher concentration of 110 μgL^{-1} severe damage characterized by electron dense and degraded cytoplasm with...
inclusions, presence of pycnotic nuclei, ruptured lysosome and fewer cristae in mitochondria was found (Figures 6 and 9).

Discussion

Spinosad compound exerted no lethal toxicity on *P. reticulata* within the tested concentrations therefore a mean lethal concentration could not be determined implying that the compound can be used to kill mosquito larvae without causing mortality in the fish species making it a good larvicide for integrated mosquito larval control. Pest Management Regulatory Agency [20], reported the toxicity of spinosad to aquatic invertebrates including *Daphnia* sp, Chironomids, shrimp and molluscs, albeit, in comparison to an organophosphate, spinosad was 5 times less toxic to non-target species during continuous exposure studies [21]. Toxicity to fish by spinosad is classified as low to moderate with 96h LC₅₀ values between 5 and 30 p.p.m depending on the species.
Spinosad failed to inhibit growth in the fish at reduced concentration evidenced by their inability to significantly induce MN in the fish gill cells with AO assay however, with TEM analysis, the compound behaved differently. Marked difference from the control was observed at higher concentration of 110 µgL⁻¹ compared to 49 µgL⁻¹. The presence of large secretory vesicles and mucus cells that characterized the cells exposed at 49 µgL⁻¹ was likely to be an initial protective response by the fish to the impacts from the bio-larvicides. Al-Ghanbousi et al. [29], showed the hyper production of mucus in Aphanisius dispar following the exposure of the fish species to low concentration of deltamethrin. Another but similar report, demonstrated the increase of secretory vesicles in the gill of A. dispar upon exposure to temephos [30]. Researchers have suggested that mucus secretion by gills and intestines play a major role in the protection of these tissues from the environmental impacts of xenobiotics [31-33] however, it is likely that under high concentration or continuous exposures to larvicides, this protective ability in the fish may become compromised hence the need to establish a baseline concentration for effective and sustainable integrated larval control.

Additionally, the elongation of the nucleus under the lowest concentration of spinosad probably suggested that spinosad did not inhibit cell division in the fish which corroborated the result obtained with AO assay. It is true that at both tested concentrations, spinosad failed to inhibit growth in the fish gill with AO assay but TEM analyses of guppy's intestinal cells revealed otherwise especially the nucleus at the lowest concentration under the TEM analysis which severe harm occurred in the fish organelles hence, a concentration not greater than 49 µgL⁻¹ is suggestive for field integrated mosquito larviciding involving the use of fish as a support control agent.

Conclusion

In this study, 49 µgL⁻¹ of spinosad seems to be the threshold above which severe harm occurred in the fish organelles hence, a concentration not greater than 49 µgL⁻¹ is suggestive for field integrated mosquito larviciding involving the use of fish as a support control agent.

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[11]. Other reports have also shown that spinosad was safe to some non-target organisms [11,22-25] but the impact of spinosad on non-target aquatic organisms is still poorly understood [26] hence, future research on the compound will necessitate an in-depth sub-chronic and chronic toxicity test on different tissues of various fish species before a final recommendation is made on spinosad use for replacement of organophosphates in field mosquito larvicidal control.

The values of the 50% mean lethal concentration of 73.06 µgL⁻¹ and 59.34 µgL⁻¹ obtained for Cx. quinquefasciatus and An. gambiae s.s. respectively in this study compared favorably with those from other workers. Anthonio et al. [27], estimated a 24h LC₅₀ value for spinosad against Aedes aegypti as 0.060mg/AUL (range of 95% confidence limits 0.045 – 0.079). The 24 h toxicity of two formulations of spinosad under different water resources against 3rd larval instar of Cx. pipiens was obtained as 0.002 ppm and 0.007 ppm for liquid and dust formulations respectively and they concluded that the dust formulation had better initial kill on the Culex mosquitoes than the liquid form [28]. Statistically, Cx. quinquefasciatus and An. gambiae s.s. showed the same degree of tolerance to the compound contrary to our expectations considering that immunity may have been conferred on the Culex sp. based on the nature of their natural habitat which is often prone to contamination. However, the higher susceptibility value obtained for Cx. quinquefasciatus was suggestive of the concentration of larvicide to use for mosquito control practices. The concentration capable of killing the more tolerant larvae species, in this case, culex mosquitoes should be applied to ensure adequate control of other less tolerant species that may co-habit with them.

Spinosad failed to inhibit growth in the fish at reduced concentration evidenced by their inability to significantly induce MN in the fish gill cells with AO assay however, with TEM analysis, the compound behaved differently. Marked difference from the control was observed at higher concentration of 110 µgL⁻¹ compared to 49 µgL⁻¹. The presence of large secretory vesicles and mucus cells that characterized the cells exposed at 49 µgL⁻¹ was likely to be an initial protective response by the fish to the impacts from the bio-larvicides. Al-Ghanbousi et al. [29], showed the hyper production of mucus in Aphanisius dispar following the exposure of the fish species to low concentration of deltamethrin. Another but similar report, demonstrated the increase of secretory vesicles in the gill of A. dispar upon exposure to temephos [30]. Researchers have suggested that mucus secretion by gills and intestines play a major role in the protection of these tissues from the environmental impacts of xenobiotics [31-33] however, it is likely that under high concentration or continuous exposures to larvicides, this protective ability in the fish may become compromised hence the need to establish a baseline concentration for effective and sustainable integrated larval control.

Additionally, the elongation of the nucleus under the lowest concentration of spinosad probably suggested that spinosad did not inhibit cell division in the fish which corroborated the result obtained with AO assay. It is true that at both tested concentrations, spinosad failed to inhibit growth in the fish gill with AO assay but TEM analyses of guppy’s intestinal cells revealed otherwise especially the nucleus at higher concentration of 110 µgL⁻¹. It is also important to note that these concentrations did not result in physical death in the fish, and were within the range already recommended for field mosquito larviciding [34]. The reliance on mortality alone as diagnostic tool for assessing chemical toxicity/safety could be misleading therefore, the need to apply suite of biomarkers on various tissues of an organism for better informed decision. It is also essential to subject spinosad compound to further detailed evaluation.
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