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Zhong et al.: Agnique® MMF Evaluation

IMPACT OF AGNIQUE® MMF MOSQUITO LARVICIDE/PUPICIDE ON BRACKISH WATER NON-TARGET INSECTS

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Abstract

Agnique® monomolecular film (MMF) registered as mosquito larvicide and pupicide is a surfactant synthesized from an ethoxylated isosteary alcohol. The MMF lowers water surface tension and subsequently kills mosquito larvae and pupae by physical drowning. We assessed the acute and residual toxicities of MMF on two aquatic non-target species, water boatmen *Trichocorixa* spp. (Hemiptera: Corixidae) and water scavenger beetles *Tropisternus* spp. (Coleoptera: Hydrophilidae) in a brackish water marsh in Vero Beach, Florida. The data showed that 95% caged water boatmen and 64% caged scavenger beetles were killed after 48-h exposure. When freshly collected water boatmen in cages introduced into the MMF-treated pools at 2 days after treatment (2DAT) and 4 DAT, 25 – 50% and 82-95% of the water boatmen survived, respectively after 48-h exposure. When newly collected water scavenger beetles were added to the treated plots at 7 DAT, no mortality was observed.

Keywords: Monomolecular film, Agnique, Water Boatmen, *Trichocorixa*, Water Scavenger beetles, *Tropisternus*, Toxicity.

Introduction

Monomolecular films, registered as mosquito larvicide and pupicide, were developed in late 1970s (Garrett and Barger 1977). They produce a thin (monomolecular) film on the water surface that lowers surface tension and subsequently kills mosquito larvae and/or pupae by inhibiting proper orientation at the water surface and/or by wetting tracheal structures, thereby causing anoxia (Levy et al. 1981). Currently, two commercial products, Arosurf® monomolecular surface film (MSF) and Agnique® monomolecular film (MMF), are available with the same mode of action. Both products are ethoxylated alcohol surfactant, synthesized from isostearyl alcohol. Structurally MMF is similar to MSF but has been purified by removing polyethylene glycols (PEG) during the manufacturing process (Nayar and Ali 2003). PEGs, when mixed with water, can cause jellification that may clog the spray equipment (Mulla et al. 1983). MMF formulation, registered in 1997, is a clear, colorless, odorless liquid containing 100% active ingredient, insoluble in water and ready to use without dilution. The MMF molecule has a hydrophilic and a hydrophobic region that reduces the water surface tension at the air-water interface.

MSF has been reported as an effective larvicide and pupicide against *Aedes*, *Anopheles*, *Culex*, and *Ochlerotatus* mosquitoes (White and Garrett 1977, Levy et al. 1982a, b, c, 1985, 1986, Mulla et al. 1983, Das et al. 1986, Perich et al. 1990, Nayar and Ali 2003). As part of the integrated mosquito management (IMM), mosquito control districts are in favor source reduction to kill mosquitoes in larvae and/or pupae stages. However, several major mosquito larvicides, such as *Bacillus thuringiensis israelensis*, *Bacillus sphericus*, temephos, and S-Methoprene, fail to control mosquito pupae. The source reduction efforts are challenged by lack of effective pupicide and mosquito pupae will successfully emerge to adult stage for rapid dispersal. To enforce the IMM strategy, an effective pupicide will be needed to reduce adulticide applications. In the State of Florida, the MMF was normally used when pupae were present and only about 3.6% of the total ground larviciding acreages were treated with MMF (FDACS FY 2004-2005).

Previous laboratory studies showed that the MSF formulation has no effects on fish, snails, tree frogs, fiddler crabs, crayfish, shrimp and isopods. A common feature of these non-targets are not dependent on the air-water interface during any stage of their life cycle (Levy et al. 1982c, Webber and Cochran 1984, Hester et al. 1991). In field experiments, Mulla et al. (1983) reported that MSF had no apparent adverse effects on mayfly larvae *Callibaetis pacificus*, diving beetle adults *Berosus metalliceps*, ostracods and copepods. Karanja et al. (1994) found that MSF had no adverse effects on most commonly encountered aquatic non-target families: Dytiscidae, Hydrophilidae, Planorbidae, Ampullaridae, Corixidae, Notonectidae, Nepidae, Belostomatidae and Glossiphonnidae. However, Takahashi et al. (1984) showed that MSF produced acute toxicity on *Corisella* spp. (Corixidae), *Notonecta unifasciata* (Notonectidae), *Tropisternus lateralis* (Hydrophilidae) beetle adults and clam shrimp *Eulimnadia* sp., but the mayfly larvae *Callibaetis* spp., chironomid larvae and copepods were not affected.

In this research, we investigated the impact of the MMF formulation on two aquatic non-targets. The objective was to assess the acute and residual toxicity of MMF on water boatman (Corixidae) and water scavenger beetles (Hydrophilidae) in a brackish water marsh in east central Florida.

Material and Methods

Study Area

Field trials were conducted in the Impoundment 23 marsh, a brackish ecosystem located along the Indian River Lagoon in Vero Beach, Florida, U.S.A. The marshes are low land areas with many potholes and receive water from both tidal flooding and rainfall. The average monthly precipitation over 102 - 178 mm (4 - 7 inches) from May to October and 50 - 76 mm (2 - 3 inches) for the rest of the months. Winter and spring are the most frequent drying period when the tide do not inundate the marsh. During the mild dry period, some potholes still hold on the water. The area is historically a prolific mosquito producing area and is frequently treated with mosquito larvicide. Most of time, the salinity ranged from 5 to 25 $\%_0$ during the wet time and reached over 70 $\%_0$ during the dry time. Some non-target species including fish adapted to this tempo and harshness environment and struggled to survive during the dry period. Vegetations were predominantly Saltgrass (*Distichlis spicata*), smooth cordgrass (*Spartina alterniflora*), Saltwort (*Batis maritima*), and Glasswort (*Salicornia virginica).*

Two types of experimental mesocosms were constructed in the marsh: "artificial pools" and "natural pools". Both types of mesocosms were constructed from ~ 1.4-m diameter × 0.3-m high stiff plastic pools. Eight artificial pools were made by filling these pools to ~ 2/3 capacity with brackish marsh water and then placing a 0.09 m^2 (1 ft^2) vegetation island in the middle of each (Fig. 1). Eight natural pools were constructed by cutting off the bottoms of the plastic pools and anchoring the plastic pool ring into the brackish water sediments with four wooden stakes in an area with visible aquatic insect activity (Fig. 2). Four treated and four untreated control pools were randomly selected for both artificial and natural pool trials. The maximum labeled dose of 9.35 L/ha of MMF was applied to each treated pool using a micropipette (Fisher Scientific, Pittsburgh PA). Global positioning system (GPS) readings were 27º39'39.27"N and 80º23'02.48"W for artificial pool site, and 27º39'44.90"N and 80º23'06.03"W for natural pool site. Salinity, dissolved oxygen, temperature, and pH were monitored inside untreated pools and outside of the pools using an YSI multi-probe field meter (YSI Model number 556 MPS, Yellow Springs, OH).

Non-target Insects

Previous researches suggested that monomolecular films only affect those aquatic insects that use the water-air interface (Levy et al. 1982c, Webber and Cochran 1984, Hester et al. 1991), insects in the family Corixidae and Gerridae (Hemiptera), Dytiscidae and Hydrophilidae (Coleoptera) were identified as suitable bioassay candidates. Unfortunately, the individual numbers of Dytiscidae and Gerridae collected from the field were inadequate for the bioassay. Only two families, water boatmen, (Corixidae, Hemiptera) and water scavenger beetles (Hydrophilidae, Coleoptera) were selected. Water boatmen are common in freshwater ponds and lakes, and a few species occur in salt marsh brackish water. The body of water boatman is elongated-oval in dark gray color with cross-lined in dorsal surface. Water boatmen swim rapidly in erratic fashion, frequently get air at the surface of the water, carry a air bubble under water and cling to vegetation for rest. Most of them feed on algae and minute organisms, and the eggs are usually attached to aquatic plants (Borror et al. 1981). Hydrophilids are black and oval, and somewhat convex beetles. The adults are scavengers and walking underwater with opposite legs alternately moving. They fetch air from water surface and store in a silvery film over the ventral side of abdomen under the water. The larvae are predaceous and eggs are laid in silken cases attached to aquatic plants (Borror et al. 1981).

Water boatmen for the bioassay were collected from the shallow brackish waters immediately adjacent to the trial site using a 5.5 × 7.9-mesh per centimeter swimming pool net. Over 1,000 water boatmen were collected for each field trial. Only adult water boatmen were used for the testing. About 20 - 40 water boatmen were preserved as voucher specimens for each bioassay. Water scavenger beetles) were collected using a 0.35-L mosquito larvae dipper or a 30.5-cm 7.9-mesh per centimeter aquarium net. Because of the less abundant of the beetles adjacent to the trial site, the collection process was time consuming due to the short time (1-2 second) of the beetles emerged from water to get air bubbles. Over 200 beetles were collected. Voucher specimens were stored in 75% alcohol in the laboratory at Indian Rive Mosquito Control District for future reference. The specimen were further identified and confirmed by insect taxonomist from University of Florida and Florida A&M University.

Bioassays in Artificial Pools

Field-collected water boatmen and water scavenger beetles were placed in artificial pools to evaluate acute and residual toxicity of MMF. The insects were held in floating screened cages constructed with 7.9-mesh per centimeter screen and topped with plastic lids. Each screened cage was cylindrical (10.5-cm diameter and 11-cm height), with a 2.5-cm wide ring cut from a 0.95-L styrofoam cup glued on top of the cage. This styrofoam ring was used to stabilize the cage opening, keep the cage afloat and provide airspace at the top of the cage. The airspace allowed the caged insects to fetch the air bubble from the water surface. Water boatmen and scavenger beetles were exposed to 9.35 L/ha (1 lb/acre) maximum labeled dose of MMF in the artificial pool. After each application, the cages were submerged for 10 seconds to allow MMF to spread over the water's surface inside the cages. Ten adult water boatmen were carefully introduced into each of the two cages in each artificial pool. Eight artificial pools were used with four randomly assigned as treated and four as reference controls. Once the cages were repositioned on the surface, they were covered with plastic lids to prevent the escape. In these suspended floating cages, the insects were able to hold on the side or bottom of the screen, but unable to access vegetation and sediments. The experiment trials were configured as a worst-case testing scenario because by excluding the absorption or reduction by vegetative wicking action (Cognis 2004). Acute toxicity was assessed by monitoring the insect mortality at 24-h and 48-h respectively. Five field trials were conducted with a minimum one month apart to insure that all pools are free from MMF residue contamination. Bioassays using scavenger beetles followed the same field procedures except only one cage was placed in each artificial pool and one trial was conducted due to the limitation of beetle collection.

After the initial application, the impact of remaining MMF residue at 2 days after the treatment (2 DAT), 4 DAT and 6 DAT was continually assessed by adding freshly collected water boatmen into new floating screen cages with a same assay protocol as the acute assay. The residue impact was also evaluated after 24-h and 48-h exposure to the MMF residue in the pools. For water scavenger beetles, the similar residual toxicity evaluation was conducted. Because of the beetles survived for several days after the MMF exposure, the newly collected beetles were added at 7 DAT and evaluated after 24-h, 48-h, 72-h and 144-h exposure period.

Bioassays in Natural Pools

Effect of MMF on scavenger beetles in their habitats was assesses in the natural pools which allow the brackish water exchange between inside and outside from the bottom of the pool so that the water level will equilibrate. The natural pools are able to confine the MMF on the water surface layer inside the pools following the application. In addition, this type of mesocosms can be easily transferred to new assay locations to adapt water level fluctuation in temped salt marsh environment. Eight natural pools were placed in an area with visible scavenger beetle activity. The numbers of aquatic insects inside each pool were unknown and varied. No insects were artificially introduced the into the natural pools. Four pools were treated with maximum label dose (1gallon/acre) of MMF and four untreated controls were randomly assigned. The observation included the following: one-minute visual count for beetle number (actively fetch air bubble) prior to MMF application, 24h and 48 hr post-application. Those beetles that fetched air bubbles from the water surface were counted and were recorded. Subsequently, a 5-minute dead insect collection was conducted at 24 and 48 hrs post-application. All insect collections were returned to Indian River County Mosquito Control laboratory for identification, counting and recording. After the first trial, the site was totally dried due to decreasing water level in the marsh and the natural pools were left unchanged in the same area. Ten days later, the areas were flooded again to the proper water level though rain fall and high tide and a second field trial was conducted. To reduce the bias from previously treated and untreated pools, we randomly selected

4 newer treated pools contained 2 previously treated and 2 previously untreated pools. The 4 newest untreated pools were also contained the equal number of previously treated and untreated pools. Due to the limitation of the proper water levels and availability of the scavenger beetles in the same area, only two trials were conducted.

Statistical Analysis

Microsoft® Office Excel (Microsoft Corporation 2003) was used for data input and graphic presentation. Mean percents of survival numbers of both non-targets from artificial and natural pools were transformed by log10(x+1) and subject to analysis of variance (ANOVA) (STATISTICA, StatSoft, Inc. 2001). Mean percent separation for survival data were by Student-Newman-Keuls (Sokal and Rohlf 1981) ($P < 0.05$).

Results

Water Quality and Non-target Insect Identification

Water qualities inside the "natural" and "artificial" pools were similar compared to marsh water outside of the pools (Table 1). Two species of water boatman (Corixidae) were identified from collections as *Trichocorixa verticalis* and *T. sexcincta*. Both are listed as water boatmen of saline waters (Scudder 1976) and are common in Florida. Four water scavenger beetle (Hydrophilidae) species were also identified in collections - *Tropisternus quadristriatus, T. collaris, T. lateralis nimbatus* and *Berosus infuscatus*. All three *Trapisternus* spp. used for bioassay are blackish and relatively larger aquatic beetles with body size ranged ~ 8 - 10mm. Epler (1996) reported that they are widespread and common in Florida. *Berosus sp*. is a smaller aquatic beetles with body size ranged ~ 3.5 - 4.5mm in our collection and also common in Florida (Epler 1996). Due to the difficulties to identify the individual species from the genus *Tropisternus spp.* in field, beetles used for bioassay were simply grouped into the two genus *Tropisternus* and *Berosus*.

Bioassays in Artificial Pools

Field captured water boatmen and water scavenger beetles without MMF exposure reached excellent survival rate inside the screen cage enclosure floating on top of the water surface inside the pools. The highest mortality in untreated control cages was less than 6% after 48-hr assay. After the MMF application, the impacts on these aquatic insects who were exposed to MMF were immediately observed such as: abnormal grooming behavior, increasing the frequency of fetching air bubble from water surface, disorientation and subsequently died. Acute bioassay in artificial pools showed that MMF significantly ($F_{9, 30} = 67.5$, $p \leq 0.0001$) affected the survival of water boatmen after the exposure in the treated pools. The average survival rate of water boatmen was 21% and 4% in treated pools compared to 99% and 98% in untreated control pools after 24-hrs and 48-hrs exposure respectively. The average of survival rate for each trials was not significant from each other both treatment and control groups, except last trial, where 66% water boatmen survived 24-hr (Table 2). The effect of MMF on survival of water scavenger beetles was progressively increased following the exposing time increasing. The survival rates were 85%, 35% and 13% after 24-hr, 48-hr and 72-hr assay respectively (Table 3).

Residue toxicity bioassay indicated that the adverse impact of MMF on the water boatmen was gradually reduced following time and no observed effects were found after 4 days. Data showed that the survival rate of water boatmen exposed to MMF residue at 2 DAT increased to 25% - 53%, but the adverse impact was still statistically significant compared with the control (Table 4). When the insects exposed to MMF residue at 4 DAT, the water boatman survival rate reached 85 - 99% (24 hr) and 85 - 96% (48 hr) (Table 5). When water boatman exposed to the MMF residue at 6 DAT, the survival rate was 96% and 89% at 24 and 48 hr respectively (Table 6). When water scavenger beetles exposed to MMF residue at 7 DAT, 100% beetles survived up to 144 hr in a bioassay (Table 7). The residue bioassay indicated that the fast degradation of MMF at the natural salt marsh environment. However, we also observed the MMF residue on the water surface at 7 DAT by an indicator oil test (Cognis Corporation) in a separate reference pool. It was unknown why many water boatmen and water scavenger beetles survived after 4 DAT in the presence of MMF residue on the water surface that could reduce the water surface tension to drown the non-targets.

Bioassays in Natural Pools

Water quality parameters were similar between inside and outside of the natural pools because the water exchange was enabled from the bottom of the pools (Table 1). Data of visual observation showed that no significant differences ($p = 0.44$, $df = 8$) between the treatments and control groups (Table 8) for water boatmen and water scavenger beetles after the MMF application, despite the presence of scores of dead beetles on the water surface of the treated pools. We are unable to observe the dead water boatmen because they sank to the sediment bottom. The observation data in Table 8 were obviously contradictory to the acute toxicity result in artificial pools. We do not agree with this "no effect" conclusion in the Table 8 because we think the sampling technique was prone to error. We have observed that the water boatmen and water beetles fetched air bubble more frequently under the influence of MMF. The number of count for obtaining air bubble activity through visual observation may be biased by duplicated counting the same boatmen or beetle in treated pools.

Dead water boatmen sank into the bottom of natural pools were not collected from the mud sediment due to the unable to see the dead body from the water surface or distinguish it from other marsh sediment debris. Dead aquatic beetles floated on top of the water were collected by a pair of tweezers without disturbance the mud sediment in natural pools. The dead beetles were identified as water scavenger beetles in the genus of *Tropisternus* and *Berosus*. The MMF application affected survives of the beetles, the number of dead beetles in the treated pools was significantly greater ($p < 0.001, df = 8$) than that of the dead one in the untreated control pools (Table 9). 24-h after the application, an average of 29.0 ± 7.2 (Mean ± SE) dead *Tropisternus* spp. and of 10.3 ± 2.5 dead *Berosus* spp. were collected in each treated "natural pool". After 48-hr, the cumulative numbers of dead beetle collection reached 43.6 ± 8.0 (Mean ± SE) for *Tropisternus* spp. and 31.1 ± 10.5 for *Berosus* spp. (Table 9). In addition, observation of beetle activities showed that no beetle activity or very little activities were seen in the treated pools after 48 hr. We do, however, observed the beetle density in the treated pools was recovered and reach the similar levels of the pretreatment or controls atter 10 days of the initial MMF application. The number of *Tropisternus spp.* and *Berosus spp.* collected from previous MMF treated pools were 45.0 ± 10.0 and 8.5 ± 3.5 (Mean ± SE) which were not significantly different ($p = 0.924$, $df = 2$ and $p = 0.311$, $df = 2$) from that of the untreated control pools (45.5 ±4.5) and 16.5 ± 7.5 respectively at 48-h after the second treatment (Table 10). Even though we could not find a appropriate water levels to replicate this test event in next two years, this preliminary data showed the sign that beetle populations were able to rapidly rebound in treated pools.

Discussion

The mode of action is identical for Agnique® monomolecular film (MMF) and its predecessor, Arosurf® monomolecular surface film (MSF) that lower the water surface tension to affect the aquatic non-targets on water surface by drowning them. It has a low toxicity and safe to a variety aquatic non-targets under the water surface such as: fish, tadpole, chironomid, ostracods, copepods (Mulla et al. 1983, Takahashi et al. 1984). This study shows that MMF application at the maximum-labeled rate has a short-term and significant impact on adult water boatmen and water scavenger beetles on the water surface for air bubbles in brackish marsh habitats. The adverse impact could last 4 days in the worst case scenario with no vegetation or sediment mud in the artificial pools.

We did not conduct bioassay for the beetle larvae and immature water boatman. This is because that our initial field observation showed that they were not affected by the MMF application. For water scavengers beetles, it is easy to understand because it was difference life stage and the larvae do not fetch air bubble from the water surface. We are excited for the observation of immature water boatman were not affected by the MMF application. We observed that the juvenile water boatmen did not actively fetch air bubble from the water surface. The change of water surface tension by MMF application will not affect water boatman juveniles submerged in water. Tones (2006) reported the late summer diapause behavior of the water boatman *Trichocorixa verticalis* interiores in Saskatchewan saline lakes. If this summer diapause behavior apply to other water boatman species in Florida, the impact of the MMF for mosquito control can be greatly reduced in summer. However, this assumption remains to be investigated.

Residue toxicity bioassay indicated that the impact of MMF residue on water boatmen and water scavenger beetles were reduced following time. At 4 DAT of MMF, majority adult water boatmen and water scavenger beetles will able to survive and this will benefit the population recovery from the limited adverse effects. We have observed the rapid population rebound of the water scavenger beetles at 10 days after initial the MMF application during our natural pool trials.

Another note is that the non-target mortality created by the natural process of the salt marsh ecosystem is much higher than the mortality by the MMF application for mosquito control. During our field work in 2005-2006, large numbers of non-targets (fish, water boatmen, etc.)

were killed when blackish waters in the salt marsh dried. However, this did not have any detrimental effect on the non-target population. We observed that as soon as the marsh waters level rise, the non-target populations began to rapidly recover. The remarkable fluctuations of the non-target populations in the tempo zone reflect natural cycles of the salt marsh ecosystem. Therefore, we think that the ground MMF application for mosquito larval/pupa control in small pocked water hole will not likely cause a significant reduction of the water boatmen and water scavenger beetle population in a salt marsh ecosystem. As a safeguard, however, we recommend that the interval for two MMF applications should be at least 10 days to facilitate the population rebound for the affected species. The adverse impact from larger scale MMF application, especially by aerial, remains to be investigated.

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Table 1. Average water quality measurements during non-target field bioassays evaluating MMF toxicity in Impoundment 23 marsh, Vero Beach, Florida.

*Parameter **	*Marsh Water*	*"Natural Pools"*	*"Artificial Pools"*
Temperature (ºC)	26 (23–31 ºC)	26 (23–31 ºC)	26
PH	7.8	7.8	7.6
Salinity (ppt)	14	14	5
Dissolved Oxygen (mg/L)	0.38	0.38	1.2

All measurements made using an YSI multi-probe field meter (YSI Model number 556 MPS).

Table 2. Mean percent (± SE) survival at 24-h and 48-h of caged water boatman after application of Agnique (9.35 L/ha) in artificial pools filled with brackish water in a salt marsh at Indian River County, FL.

			% Survival (Mean ± SE)	
Trial Number	Treatment	N	24 h	48 h
1	T	4	15.0 ± 6.1b[1]	*7.5 ± 4.3b*
	C	4	95.0 ± 3.5a	*93.8 ± 3.1a*
2	T	4	6.3 ± 2.4b	*1.3 ± 1.3b*
	C	4	100 ± 0a	*100 ± 0a*
3	T	4	11.3 ± 3.8b	*2.5 ± 2.5b*
	C	4	100 ± 0a	*100 ± 0a*
4	T	4	3.8 ± 2.4b	*0 ± 0b*
	C	4	100 ± 0a	*100 ± 0a*
5	T	4	66.3 ± 19.1b	*7.5 ± 2.5b*
	C	4	100 ± 0a	*95 ± 0a*

[1]Treatment means in each column significantly different ($P \leq 0.05$). Student-Newman-Keuls test.

Table 3. Mean percent (± SE) survival at 24-h, 48-h and 72-h of caged water scavenger beetles (*Tropisternus spp.*, Hydrophilidae) after initial application of Agnique (9.35 L/ha) in artificial pools filled with brackish water in a salt marsh at Indian River County, FL.

		% Survival (Mean ± SE)		
Treatment	N	24 h	48 h	72 h
T	4	85.0 ± 11.9a	35.0 ± 15.0b[1]	12.5 ± 9.5b
C	4	97.5 ± 2.5a	97.5 ± 2.5a	95.0 ± 2.9a

[1]Treatment means in each column significantly different ($P \leq 0.05$). Student-Newman-Keuls test.

Table 4. Mean percent (± SE) survival at 24-h and 48-h of caged water boatman introduced into the new assay cages which were placed in the artificial pools at 2 days after initial application of Agnique (9.35 L/ha) in the artificial pools in a salt marsh at Indian River County, FL.

			% Survival (Mean ± SE)	
Trials	Treatment	N	24 h	48 h
1	T	4	31.3 ± 7.5b	25.0 ± 7.4b
	C	4	100 ± 0a	100 ± 0a
2	T	4	53.8 ± 9.0b	52.5 ± 9.5b
	C	4	100 ± 0a	97.5 ± 1.4a
3	T	4	33.8 ± 9.7b	31.3 ± 7.5b
	C	4	100 ± 0a	98.8 ± 1.3a

[1]Treatment means in each column significantly different ($P \leq 0.05$). Student-Newman-Keuls test.

Table 5. Mean percent (± SE) survival at 24 h and 48 h of caged water boatman introduced into the new assay cages which were placed in the artificial pools at 4 days after initial application of Agnique (9.35 L/ha) in the artificial pools in a salt marsh at Indian River County, FL.

			% Survival (Mean ± SE)	
Trials	Treatment	N	24 h	48 h
1	T	4	85.0 ± 4.6b[1]	85.0 ± 3.2b
	C	4	100 ± 0a	100 ± 0a
2	T	4	98.8 ± 1.3a	96.3 ± 2.4a
	C	4	100 ± 0a	95.0 ± 2.0a
3	T	4	97.5 ± 1.4a	95.0 ± 2.9a
	C	4	100 ± 0a	98.8 ± 1.3a

[1]Treatment means in each column with the different letter significantly different ($P \leq 0.05$). Student-Newman-Keuls test.

Table 6. Mean percent (± SE) survival at 24 h and 48 h of caged water boatman introduced into the new assay cages which were placed in the artificial pools at 6 days after initial application of Agnique (9.35 L/ha) in the artificial pools in a salt marsh at Indian River County, FL.

		% Survival (Mean ± SE)	
Treatment	N	24 h	48 h
T	4	96.3 ± 2.4a	88.8 ± 3.8b[1]
C	4	100 ± 0a	100 ± 0a

[1]Treatment means in each column with the different letter significantly different ($P \leq 0.05$). Student-Newman-Keuls test.

Table 7. Mean percent (± SE) survives of caged water scavenger beetles (*Tropisternus spp.*, Hydrophilidae) that were introduced into the artificial pools at 7 days after the initial application of Agnique (9.35 L/ha) in the artificial pools with brackish water in a salt marsh at Indian River County, FL.

		% Survival (Mean ± SE)			
Treatment	N	24 h	48 h	72 h	144 h
T	4	100.0 ± 0a	100.0 ± 0a	100.0 ± 0a	100.0 ± 0a
C	4	100.0 ± 0a	100.0 ± 0a	100.0 ± 0a	100.0 ± 0a

[1]Treatment means in each column with the same letter was not significantly different (P≤0.05) by Student-Newman-Keuls test.

Table 8. Numbers of aquatic insects (Mean ± SE) found on water surface where they were fetching air bubbles. The insects counts were lasted for 1min before and after the Agnique® MMF (9.35 L/ha) treatment in both treated and control "natural pools" located in brackish water of a salt marsh impoundment in Indian River County, FL.

		% Survival (Mean ± SE)		
Treatment	N	*Trichocorixa* spp	*Tropisternus* spp	*Berosus* spp.
Pretreatment	4	6.5 ± 1.4a	2.3 ± 0.5a	2.3 ± 1.0a
Pre-control	4	4.0 ± 1.7a	2.8 ± 1.1a	1.8 ± 1.0a
Post-treatment	4	2.5 ± 1.0a	2.3 ± 1.1a	2.3 ± 0.5a
Post-control	4	2.3 ± 1.9a	3.7 ± 1.9a	4.3 ± 2.3a

[1]Treatment means in each column with the same letter was not significantly different ($P \leq 0.05$) by Student-Newman-Keuls test.

Table 9 Cumulative numbers of dead beetles (Mean ± SE) collected in 5 minutes from the treated and untreated (control) natural pools after 24 and 48 h post treatment with the maximum label dose of Agnique® MMF (9.35 L/ha). The collections of dead beetles were limited to 5 minutes for each natural plot in a salt marsh impoundment at Indian River County, FL.

			Dead Beetles (Mean ± SE)	
Species	Treatment	N	24 h	48 h
Tropisternus Spp.	T	8	29.0 ± 7.2a[1]	43.6 ± 8.0a
	C	8	0.1 ± 0.1b	0.5 ± 0.2b
Berosus Spp.	T	8	10.3 ± 2.5a	31.1 ± 10.5a
	C	8	0.5 ± 0.4b	1.0 ± 0.6b

[1]Treatment means in each column with the different letter significantly different ($P \leq 0.001$) by Student-Newman-Keuls test for each beetle genus.

Table 10 Population recovery of two aquatic beetles, *Tropisternus* sp. and *Berosus* sp. in previous treated pools compared with the beetle densities in previous untreated pools ten days after initial Agnique®MMF treatment at 9.35 L/ha in a salt marsh impoundment at Indian River County, Florida. Cumulative numbers of beetles (Mean ± SE) were collected at 24 and 48-h from the natural pools after the second treatment of Agnique MMF

			Beetle Density (Mean ± SE)	
Species	Treatment[1]	N	24 h	48 h
Tropisternus Spp.	PT	2	37.0 ± 11.0a[2]	45.0 ± 10.0a
	PC	2	41.5 ± 0.5a	45.5 ± 4.5a
Berosus Spp.	PT	2	8.0 ± 3.0a	8.5 ± 3.5a
	PC	2	9.5 ± 5.5a	16.5 ± 7.5a

[1] PT = Previous Treated with maximum label dose of Agnique MMF. PC = Previous Control.
[2]Treatment means in each column with the same letter was not significantly different ($P \geq 0.05$) by ANOVA for each beetle genus.

Figure 1. "Artificial pools" were established with plastic pools filled with marsh water in a brackish marsh impoundment, Vero Beach, Florida. The center of each pool was placed an island consist of 30.5 cm by 30.5 cm (one square foot) marsh sediment with grass. Two floating bioassay cages with lid were placed in each pool.

Figure 2. "Natural pools" were established with each plastic ring anchored by 4 wooden stakes driven in at an angle to prevent movement due to tidal influx. Before Agnique® MMF application, the pools were checked to make sure the plastic rings stay in contact with the sediment soil in a brackish marsh impoundment, Vero Beach, FL.