Attract-and-kill strategy. Laboratory studies on hatched larvae of *Culex pipiens*

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Abstract: The attract-and-kill strategy is a new pest management technique that presupposes the intelligent combination of an attracting agent (e.g. pheromone) and a killing agent (e.g. insecticide). In the present study, the potential combination of the microencapsulated synthetic oviposition pheromone 6-acetoxy-5-hexadecanolide with an insecticide has been tested. Initially, polyurea microcapsules containing 6-acetoxy-5-hexadecanolide, the synthetic mixture of diastereomers of the oviposition pheromone of the mosquito species *Culex quinquefasciatus* Say (Diptera: Culicidae), were studied. Laboratory bioassays were performed to confirm the bioactivity of the microencapsulated pheromone on the oviposition activity of *Culex pipiens* L. biotype *molestus* Førskal (Diptera: Culicidae) with the aim of determining the optimum dose for oviposition response. Its effect was dose dependent, revealing an optimum dose of 300 mg of dried microcapsules. Attractancy over time was also studied. The microencapsulated pheromone was found to be sufficiently attractive to gravid female mosquitoes for a period of 40 days. Finally, the combination of the synthetic pheromone with the control agent temephos showed both an acceptable oviposition activity and sufficient larvicidal effect.

Keywords: polyurea microcapsules; oviposition mosquito pheromone; attract-and-kill strategy; *Culex pipiens* biotype *molestus*; slow release

1 INTRODUCTION

Extensive use of synthetic insecticides has resulted in serious environmental implications such as groundwater pollution and cases of human and animal poisoning. Depending on the method of application and climatic factors, only 10% of conventionally applied agrochemicals may reach their target in time and in sufficient quantities.[1] Thus, there is a persistent and unambiguous need to replace current synthetic insecticides with less harmful ones, together with the use of more environmentally friendly application techniques. Exploitation and use of insect pheromones present a challenging field of research to address both of these aspects.[2]

The study and the development of semiochemical products has created an intelligent approach, from the standpoint of drawing the insect to the poison rather than bringing the poison to the insect, the so-called 'attract-and-kill strategy'.[2] The word 'semiochemicals' is synonymous with 'signal substances' and is derived from 'semio' (Greek = sign). It refers to biomolecules that spread information between individuals.[3] This new strategy has many advantages: it employs an intelligent combination of pheromone and insecticide, it is species specific, it has targeted application, it protects beneficial organisms and it minimises the risk of resistance development.[2,4] As a result, the unnecessary spreading of toxic substances and severe chemical pollution can be avoided. To improve the potential of this technique further, controlled-release systems (CRSs) should be used to secure a slow pheromone release rate over an extended period of time. However, difficulties in developing CRSs have so far limited their use. This is mainly because CRS manufacturing should involve materials that are suitable for application over large areas under different conditions, biodegradable and relatively cheap.[5]

The use of such a combined approach in mosquito control would be of great value since these insects are known to be vectors in the transmission of human tropical diseases such as the West Nile virus disease. West Nile virus is mainly transmitted in natural cycles between birds and *Culex* mosquitoes, but it can also affect humans.[6,7] In the last decade, the spread of West Nile virus in the Western Hemisphere has resulted in hundreds of people dying and thousands being infected. The disease is believed to have had its first outburst in ancient times, and, according to a recent theory, Alexander the Great was possibly a victim of this disease.[8]

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Culex pipiens L. represents a mosquito species complex whose taxonomy is still a matter of argument.[9,10] The principal members of the complex are: *Culex pipiens* with two biotypes (*molestus* and *pipiens*) and *Culex quinquefasciatus* Say. Female *Culex* mosquitoes deposit their eggs in the form of egg rafts on the water surface. Some of these species form a droplet at the apex of each egg in the egg raft, which affects the oviposition behaviour of intraspecific gravid females.[11–13] The main volatile compound present in the apical droplets of the *Cx. quinquefasciatus* egg rafts is (−)-(5*R*,6*S*)-6-acetoxy-5-hexadecanolide, which acts as an attractant to other gravid females of the same species complex to oviposit near the pheromone source.[14,15] A mixture of the diastereomers of 6-acetoxy-5-hexadecanolide has been synthesised in the authors' laboratory from δ-valerolactone in an efficient, high-yielding and relatively low-cost way. Its bioactivity against *Cx. pipiens* biotype *molestus* has also been confirmed.[16] Recently, a slow-release system has been developed that incorporates (*Z*)-11-hexadecenyl acetate, the main component of the sex pheromone of several Noctuidae, by means of polyurea coating.[17]

The present study reports on the preparation and preliminary biological use of polyurea-microencapsulated synthetic 6-acetoxy-5-hexadecanolide [microencapsulated synthetic oviposition pheromone (MSP)]. Slow-release studies, bioactivity experiments and optimum dose evaluation were all investigated to assess the potential use of polyurea microcapsules as 'release carriers' for the synthetic pheromone. The main purpose of this research was to investigate for the first time the potential use of an attract-and-kill strategy against mosquitoes under laboratory conditions by combining temephos, a commercial larvicidal product commonly used in Greece, with the MSP.

2 MATERIALS AND METHODS

2.1 General

Synthetic pheromone was prepared according to the method described by Michaelakis *et al*.[16] The polyurea microcapsules were prepared by an interfacial polycondensation technique between a polyisocyanate [toluene-2,4-diisocyanate (TDI), tech., 80%] and a polyfunctional amine [diethylenetriamine (DETA)] in the presence of a non-ionic emulsifier (nonylphenol polyethylene glycol ether). All reagents were obtained from Sigma-Aldrich and used as received.

Gas chromatography analysis was carried out on a Hewlett Packard 5890 Series II gas chromatograph. The oven temperature programme was 50 °C for 2 min, 15 °C min^{-1} to 280 °C and then hold for 25 min. Splitless injections (1 μL) were made at an injector temperature of 250 °C.

2.2 Preparation of microcapsules

Microcapsules were prepared using TDI as the oil-soluble monomer, DETA as the water-soluble monomer and 0.25% (v/v) nonylphenol polyethylene glycol ether as the emulsifier. Octane was used as the oily liquid. Synthetic pheromone (100 mg) was dispersed in the organic media (∼5.3 g total mass) before emulsification. After addition of the water-soluble monomer (DETA), the emulsion was heated at 65 °C for 2 h. Then, polymeric particles were isolated and washed sequentially with water (until pH = 7), methanol and hexane. Extraction of the washings with organic media (ethyl acetate) revealed that the amount of pheromone trapped in the polymeric material was approximately 70%. The resulting microcapsules (∼2.5 g) were suspended in water for further analysis. The final weight of the aqueous suspension was 62 g.

2.3 Release rate determination

Dried microcapsules (50 mg after drying for 3–4 h on a filter paper in open-air conditions) were put in clean tea bags and kept at 20 ± 2 °C and 80 ± 2% relative humidity over a period of 25 days. At 2, 4, 7, 11, 15, 21 and 25 days, samples of microcapsules were removed from their bags and compressed with a broad glass bar, and the non-polar components were extracted with hexane. The extracts were evaporated to small volume (∼1 mL), and 10 μL of an internal standard (100 μg mL^{-1} hexadecane in hexane; C_{16}) was added. The resulting solution was injected for GC analysis. Compounds were identified by comparison of the retention times with those of authentic standards. Three samples were used in each experiment. For each recording, the ratio of the peak area of the pheromone to the peak area of the internal standard (SP/C_{16}) was estimated and the mass of the residual pheromone was calculated (Table 1). The pheromone release over a given time interval was calculated according to

Cumulative mass released

= initial residual mass of SP (ng)

− residual mass of SP (ng) on each recording day

where the initial mass was that on day 2. The results are summarised in Fig. 1.

Slow evaporation, with temperatures no higher than 30 °C during the solvent concentration step, and very careful and thorough solvent rinsing of all

Table 1. Amount of residual SP in microcapsules aged under laboratory conditions. Three samples were used in each experiment and mean values (± SE) were calculated

Time (days)	SP/C_{16}	Residual SP (ng)	SE (±)
2	1.335	1335	39.3
4	0.869	869	25.8
7	0.655	655	24.8
11	0.420	420	35.9
15	0.580	580	32.1
21	0.410	410	29.9
25	0.360	360	26.6

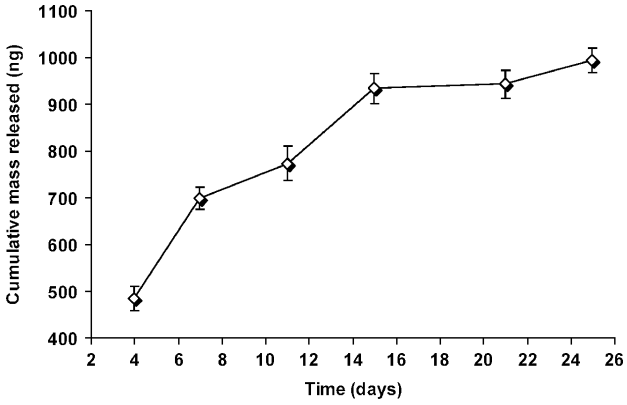

Figure 1. Plot of cumulative mass released, relative to the initial level at day 2, versus time of ageing (days) of microcapsules in cloth bags as determined by solvent extraction. Three replicate extractions were used for each ageing interval. Mean values and SE were calculated.

glass surfaces were required to ensure accurate and reproducible recoveries.

2.4 Mosquito rearing

A *Culex pipiens* L. biotype *molestus* Førskal colony, maintained in the laboratory of Benaki Phytopathological Institute (Kifissia, Greece) for more than 20 years, was used. Adults were kept in wooden-framed cages (33 cm ×33 cm ×33 cm) with 32 × 32 mesh at 20 ± 2 °C, 80 ± 2% relative humidity and 14:10 h light:dark photoperiod. Cotton wicks saturated with 10% sucrose solution were provided to the mosquitoes as food source. Females laid their eggs in round, plastic containers (10 cm diameter × 5 cm depth) filled with tap water (150 mL). Egg rafts were removed daily and placed in cylindrical enamel pans (35 cm diameter ×10 cm depth) in order to hatch. Larvae were reared under the same temperature and light conditions and were fed daily with baby fish food (0.25 g L^{-1} water) until pupation. Pupae were then collected and introduced into the adult rearing cages.

2.5 Laboratory oviposition bioassays

2.5.1 Determination of the optimum dose of MSP for oviposition response

Two-choice oviposition experiments were set in sieve-covered, iron-framed cages (90 cm ×50 cm ×33 cm). Two- to three-day-old mosquitoes of both sexes were removed from the maintenance cages (not containing oviposition pots) and introduced into the bioassay cages. Bioassay cages were kept under normal rearing conditions. Adult mosquitoes were left for 24 h in order to acclimatise in the new environment and also to achieve as much mating as possible. Afterwards, two glass pots (7.5 cm diameter ×6 cm depth), one containing distilled water (200 mL) and the other distilled water (200 mL) plus MSP in a tea bag, were placed into the bioassay cages, as centrally as possible, with approximately 60 cm distance between them in order to provide oviposition sites.

Tea bags containing microcapsules with SP were hung on the pot lips in such a way that part of the tea bag was submerged in the water. This was intended to achieve the best possible imitation of the natural situation where the pheromone is released near the surface of the water. Different doses of dried microcapsules (100, 150, 200, 250, 300, 350 and 400 mg containing approximately 2.67, 4.00, 5.34, 6.67, 8.01, 9.34 and 10.68 μg of SP respectively) were tested in the bioassay cages. All samples were kept moist under the experimental conditions for 1 day prior to introduction into the bioassay cage.

2.5.2 MSP ageing and oviposition response

Having found the dose that elicited the highest oviposition response under the test conditions, new laboratory bioassays were set up to study the persistence of the effect of MSP on the oviposition activity of *Cx. pipiens* biotype *molestus*. All the conditions and factors were the same as in the previous experiment, and all the MSP samples tested were kept moist for a period of 50 days. During this period, the ageing samples were applied in oviposition bioassays (three replicates) as in Section 2.5.1 on days 2, 5, 11, 15, 20, 25, 30, 35, 38, 41 and 50 after the beginning of the experiment. Newly emerged mosquitoes were added daily to the bioassay cages, and a 10% sucrose solution was provided *ad libitum*.

2.6 Laboratory implementation of the attract-and-kill strategy

2.6.1 Control agent

According to the literature, temephos appears to be highly selective for mosquitoes and causes minimum undesirable effects on the environment and public health.[18] Additionally, owing to its relatively low mammalian toxicity, it is used as a larvicide in many mosquito control programmes in various formulations, and its efficacy has been well documented.[19,20]

A commercial 500 g L^{-1} temephos EC (Abate 50EC; BASF) commonly used in Greece was used at 0.15 mL L^{-1} (75 mg AI L^{-1}). This was equivalent to the lowest recommended label rate for the active substance.

2.6.2 Oviposition bioassays

Two-choice oviposition experiments were set in sieve-covered, iron-framed cages (90 cm ×50 cm ×33 cm). Two- to three-day-old male and female adult mosquitoes were removed from the maintenance cages (not containing oviposition pots) and introduced into the bioassay cages, which were kept under the normal rearing conditions. Adult mosquitoes were left for one more day in order to acclimatise in the new environment and also to achieve as much mating as possible. Tea bags containing microcapsules with the pheromone were hung on the pot lips in such a way that part of the tea bag was submerged in the water. Temephos EC (150 μL L^{-1}) and MSP (300 mg of dried microcapsules containing approximately 8.0 μg of SP) were tested in each cage. Both aqueous temephos stock emulsions and MSP samples (wet

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form) were kept under the normal mosquito rearing conditions for a period of 50 days (three samples per test were used). Ageing samples were applied for oviposition bioassays on days 2, 7, 14, 21, 29, 37 and 43 after the beginning of the experiment: two glass pots (7.5 cm diameter ×6 cm depth), one containing temephos stock emulsion (200 mL) (control pot) and the other temephos stock emulsion (200 mL) plus MSP (test pot), were placed into the bioassay cages, as centrally as possible, at approximately 60 cm distance from one another in order to provide oviposition sites.

The pots with the oviposited egg rafts were removed from the bioassay cages 24 h after the introduction of the MSP bags into the bioassay cages. These pots, after counting egg rafts, were kept for observation under normal mosquito rearing conditions. Newly emerged mosquitoes were added daily into the bioassay cages, and a 10% sucrose solution was provided *ad libitum*.

2.7 Data recording and analysis

The number of egg rafts in the oviposition pots was recorded 24 h after the introduction of the MSP bags into the bioassay cages. The counted egg rafts were converted to percentages of the total number of rafts in both pots for each cage. The results refer to three experiments for each case.

For the determination of the optimum dose, three bioassays were carried out for each dose, and data (percentages of egg rafts laid in test pot) were subjected to probit analysis (SPSS 11.0). EC_{50} (concentration causing 50% increase in oviposition) values were also estimated.

3 RESULTS AND DISCUSSION

The first concern and absolute prerequisite for the successful application of the polyurea microcapsules was whether they were capable of releasing the pheromone through diffusion but could deter its rapid loss to give a steady release rate over a sufficient time period. Gas chromatograms were set for this purpose, and, as shown in Fig. 1, constant detection of the enclosed pheromone was observed over a period of 25 days. The initial time was set as the first recording (day 2) since a high rate of release during the first days had been observed. This was attributed to the fact that release carriers usually have pheromone not only at the inner face but also at their surface, thus aiding rapid release during the first critical days.[21] After this initial loss, pheromone release more closely approximated pseudo-first-order release rates. Within a period of 1 week, the residual pheromone was diminished to half of its initial amount, but pheromone was still detected for more than 2 weeks.

The results of the dose study (oviposition bioassays) are summarised in Table 2. It is clearly demonstrated that MSP is bioactive against *Cx. pipiens* biotype *molestus*. According to the literature, the choice of a proper oviposition site is a very important factor in the reproduction of species with aquatic larvae, such as mosquitoes.[22] Although the presence of pheromone provides a reliable cue that the water body offers suitable conditions for larval development, there is an upper limit to pheromone concentration. If this critical concentration is exceeded, the pheromone appears to act as a repellent, probably because mosquitoes avoid sites with high larval competition.[16,23,24] The same behaviour pattern was also observed in MSP bioassays. MSP was tested as oviposition promoter in seven different doses, from 100 to 400 mg of microcapsules, per test cage. Although oviposition was elicited at doses between 150 and 350 mg per cage (see Table 2), the highest response was recorded at the dose of 300 mg where the oviposition attractant level was almost 71%. This result was in agreement with the statistical analysis (EC_{50} 292 mg). In contrast, doses of 100 and 400 mg per cage did not elicit any significant oviposition response. More specifically, at 100 mg the amount of pheromone seemed to be inadequate to induce oviposition in a specific place, whereas 400 mg per cage resulted in either confusion or even deterrence of the gravid female mosquitoes. Therefore, the dose of 300 mg MSP per cage was considered to be the optimum dose for oviposition response, and it was employed in the second set of oviposition bioassays in order to estimate the bioactivity of MSP in a period of time.

Table 2. Mean percentage of egg rafts (±SE) in oviposition pots with different doses of MSP

MSP dose (mg)	mean % egg rafts laid in oviposition pots	SE (±)	no. of trials*
control	50.66	2.64	9
100	52.58	1.05	3
150	64.11	1.37	3
200	65.68	2.40	3
250	69.45	2.15	3
300	71.75	1.90	3
350	71.19	2.43	3
400	51.27	2.28	3

* Nine bioassays for control were carried out in contrast with MSP bioassays in which the replicates were three.

Oviposition responses of *Cx. pipiens* biotype *molestus* to aged MSP (using the dose of 300 mg per cage) compared with distilled water controls are shown in Fig. 2. The two-day-old MSP application elicited almost a 72% oviposition response. Although all the other applications elicited lower oviposition levels, the aged MSP showed attraction behaviour for almost 30 days. The oviposition results shown in Fig. 2 can be subdivided into three areas of interest. The first area, which contains the two-day-old MSP, showed the highest recorded attractancy (ca 72%). In the second area, day 5 to day 30, the oviposition response level decreased to a value of 68.5 ± 0.8% (mean value ± SE) and settled at that level for the next 25 days. After day 30, and for the next 8 days (38-day-old MSP), a linear decline in the rate at which gravid females laid their egg rafts in the oviposition pot was

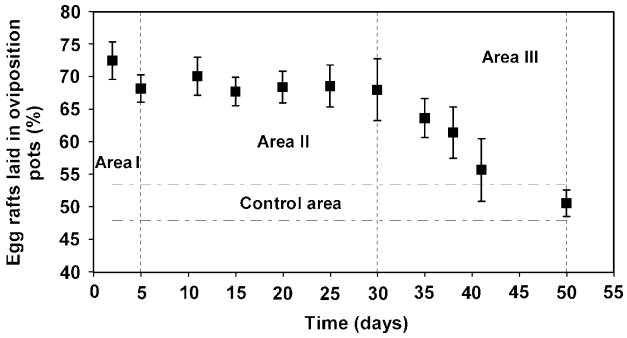

Figure 2. Oviposition responses of gravid females to aged MSP. The broken lines represent the upper and the lower values of the control mean ± SE (50.7 ± 2.8%, $n = 9$).

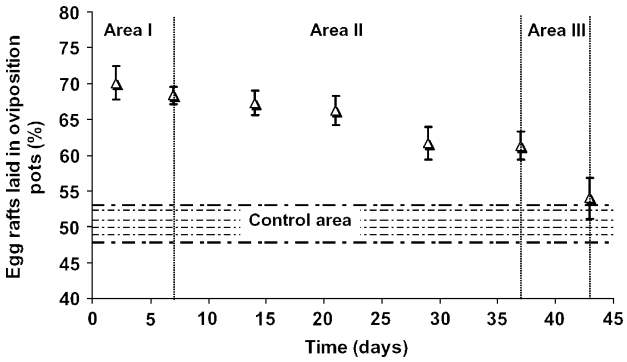

Figure 3. Oviposition responses to aged MSP in combination with temephos. The broken lines represent the upper and the lower values of the control mean ± SE (50.4 ± 2.6%, $n = 10$).

observed. In the last area, day 38 to day 50, almost all of the initial amount of pheromone had been delivered and the oviposition response level seriously decreased.

The results of the attract-and-kill-bioassays using a combination of MSP and temephos over a period of 43 days are shown in Fig. 3. The results indicated that the use of this organophosphate did not affect the oviposition bioactivity of the MSP. With the same bioassay pattern as the MSP (see Fig. 2), the mixture of the oviposition pheromone and the killing agent attracted the gravid female mosquitoes to lay their eggs in the test pot. The first application, on day 2, showed an oviposition attractancy level of approximately 70%. Even though all other days elicited lower oviposition levels, the attraction was significantly different from the control. In particular, the oviposition attractancy level was around 65% (65.0 ± 3.3%) for the seventh up to the 37th day. The last application (day 43) revealed no attraction, and the oviposition results were in the range of the control, probably because all of the initial amount of pheromone had been delivered into the environment. All of the egg rafts obtained from the oviposition bioassays were kept under observation, and the results were as expected, since 100% mortality was observed (egg rafts from both pots).

As previously mentioned, the implementation of the attract-and-kill strategy against mosquitoes has never been applied before. This strategy is already used against insecticide-resistant Lepidoptera[4] and is suggested as a new low-emission way to control insect pests.[25] Even though the results were encouraging, further research is needed in order to evaluate the bioactivity of the MSP with other killing agents. Furthermore, it is important to appreciate the need for field experiments (in particular, taking into consideration the climatic conditions and the quality of the water of the tested area) in order to verify these initial highly promising results.

In conclusion, from these preliminary studies it is clear that polyurea microcapsules could be used as release carriers for pheromones. When used in combination with the appropriate killing agent, high efficacy can be achieved when an attract-and-kill strategy is implemented. The advantages are almost self-evident: the method is highly selective, and the amount of larvicide used is much lower than in conventional larvicidal applications. However, the effectiveness of the MSP is separate from the oviposition pattern of the killing agent[26–28] and additional knowledge on the mode of action of such combinations would allow for effective low-input pest management strategies. Thus, further research is needed in order to evaluate the effectiveness and to establish the utility of MSP in field applications. Another application could be in surveillance projects, using MSP as an attractant in oviposition traps.[29] Taking into account the many advantages of the use of the urea polymer (non-toxic, physical and chemical stability, slow biodegradation), the current delivery system provides a good candidate for the use of polyurea microcapsules in the implementation of the attract-and-kill strategy.

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