PN-ABC-873

AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D.C. 20523

DATE: 8/31/88

MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18

FROM: AID/SCI, Victoria Ose

SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. C5-142
PR #2 (Jan - June 88)

Attachment

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AGENCY FOR INTERNATIONAL DEVELOPMENT
UNITED STATES-ISRAEL PROGRAM, PROJECT C5-142
Grant No. DPE-5544-G-SS-7014-00

BIOLOGICAL CONTROL OF MOSQUITO LARVAE BY MEANS OF WATER-DWELLING BACTERIA EXPRESSING CLONED δ-ENDOTOXIN GENES OF *Bacillus thuringiensis* var. *israelensis* (BTI)

Progress Report No. 4 (January - June, 1989)

presented by Prof. Arieh Zaritsky, Dr. Ze'ev Barak and Prof. David M. Chipman
Department of Biology, Ben Gurion University of the Negev
P.O. Box 653, Beer Sheva, Israel 84105

During the last six months, we have made significant progress in two projects, namely:

(A) Dr. S. Ranaweera (from Sri Jayewardenepura University) gained much experience in recombinant DNA techniques. While spending three months at Ben Gurion University, he characterized the largest plasmid of BTI, as follows:

(a) Nine fragments were generated by restricting it with *Bam*HI, the sizes of which (2.5, 7.5, 8.0, 12.5, 15, 19, 19.5, 21 and 25 kb long) sum up to about 130 kb, 10 - 15% longer than previously described in the literature. (A manuscript reporting these observations has recently been submitted for publication.)

(b) About half of this plasmid has already been partially mapped using libraries of *Bam*HI and other rare-cutting restriction enzymes (e.g., *Eco*RI, *Hin*dIII, *Sal*I, *Xho*I, *Sma*I). This sub-project is now being pursued here, with the intention of preparing a manuscript for submission soon.

Upon returning home, and in spite of the political unrest in Sri Lanka, both Dr. Ranaweera and Prof. H.G. Nanadadasa are now purchasing the appropriate equipment, which will enable them to extend their studies and instruct graduate students. Dr. Preeni Tissera is the next trainee in our laboratory from S.-J. University, who just arrived here for a three months period.

(B) Meanwhile, our ecological studies expanded by an M.Sc. student (Mr. Eitan Ben-Dov) under the instruction of a senior Ph.D. student (Mr. Kamal Khawaled). They succeeded to encapsulate BTI toxicity in carcasses of *Aedes aegypti* pupae. Mosquito pupae do not ingest particles, and are thus known to be unaffected by BTI powder. BTI-killed pupae were however obtained, by treating synchronized fourth instar larvae with a low dose of BTI powder (8,000 spores/ml). Increase or decrease of spore concentrations reduced the fraction of dead pupae: Increased proportion of dead larvae were obtained at higher concentrations whereas lower concentrations resulted in more living pupae.

Multiplication of BTI is not restricted to BTI-killed larvae only, but is shown to occur also in dead pupae. The number of spores in each pupal carcass followed a similar kinetics as in larval carcasses, but the final yield was ten fold higher. This difference seem to be a consequence of differeneces between the composition of the two mosquito developmental stages. The larvicidal activity developed in dead pupae was similar to that of BTI powder, with an LC_{50} of 650 - 1,550 spores/ml.

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C5-142

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Progress Report No. 3 (July - December, 1988)

presented by Prof. Arieh Zaritsky, Dr. Ze'ev Barak and Prof. David M. Chipman
Department of Biology, Ben Gurion University of the Negev
P.O. Box 653, Beer Sheva, Israel 84105

The beginning of the second year saw substantial progress in several lines:

(A) A study was initiated to describe ingestibility and digestibility by mosquito larvae of algal species found in mosquito breeding sources. The information obtained is included in the enclosed manuscript, which has recently been submitted for publication.

(B) Attempts were made to characterize the various plasmids of BTI and to construct a shuttle vector able to replicate in both *Escherichia coli* and BTI, as follows:

(1) Strain 4Q2-72 obtained from the *Bacillus* Genetic Stock Center in Columbus, Ohio, was cured of its only (112 kb) plasmid by growth at 37°C with 50 μg/ml acridine orange. The cured strain (BTI-AO-50) has lost its parent's toxicity to *Aedes aegypti* larvae, as expected. It is being used for transformation with the shuttle vector (see (3)).

(2) The pattern of plasmids in wild-type BTI (strain # 576) was looked at after chloramphenicol treatment. A plasmid which can be amplified (the replication control of which is relaxed) would be most suitable for a proper shuttle vector. Four out of the nine plasmids present were readily amplified.

(3) The two smallest plasmids of BTI strain # 576 were purified and partially mapped by restriction analysis. They were opened in their *HindIII* sites for ligation into pBR322 which had been cut with *HindIII* and dephosphorylated.Transformation into *E.coli* HB101 resulted in 10 ampicillin-resistant clones. The resident plasmid of each will now be checked for ability to transform BTI-AO-50 (see (1)).

(4) Professor H.G. Nandadasa, from Sri-Jayewardenepura University, spent almost three months at our laboratories, studying BTI plasmids while refreshing and updating himself with recombinant DNA techniques. Prof. Nandadasa returned home in the beginning of November with defined plans to establish an appropriate laboratory and to continue his studies on mosquito control, initiated at Ben Gurion University. A young microbiologist, lecturer at Sri Jayewardenepura University, is planning his visit here during 1989, to gain experience in genetic engineering. Resources from the A.I.D. grant, transferred to Sri Lanka, are now being invested in purchasing equipment necessary for such studies.

(C) A novel encapsulation system was designed for efficient delivery of the δ-endotoxin of BTI to larvae of *A.aegypti*, using characteristics of the infusorian *Tetrahymena pyriformis*.

Cells of *T.pyriformis* were firstly shown to be ingested and effectively digested by *A.aegypti* larvae. The bioencapsulation system devised afterwards is purely biological, exploiting the natural interactions between its constituents, and is based on the following facts:

(1) The toxic crystal is stable at pH lower than 9;
(2) The pH of food vacuoles in ciliates is significantly lower than 9;
(3) *T.pyriformis* feeds on bacteria;
(4) The natural habitat of *T.pyriformis* is fresh water and, at least in laboratory conditions, it tends to accumulate at the air/water interface, thus sharing the natural zone of surface feeding mosquito larvae;
(5) Mosquito larvae prey efficiently on unicellular microorganisms.

Larvicidal effect of BTI would thus be increased, provided mosquito larvae prey on *T.pyriformis* fed with settling BTI spores. It would also be enhanced due to high concentration of crystals in the ciliates.

A manuscript describing in detail the findings is now being prepared.

A reprint of the first paper, acknowledging the financial support by US-AID, is enclosed herein.

AGENCY FOR INTERNATIONAL DEVELOPMENT
UNITED STATES-ISRAEL PROGRAM, PROJECT C5-142
Grant No. DPE-5544-G-SS-7014-00

BIOLOGICAL CONTROL OF MOSQUITO LARVAE BY MEANS OF WATER-DWELLING BACTERIA EXPRESSING CLONED DELTA ENDOTOXIN GENES OF *Bacillus thuringiensis* var. *israelensis*

Progress Report No. 2 (January - June, 1988)

presented by Prof. Arieh Zaritsky, Dr. Ze'ev Barak and Prof. David M. Chipman
Department of Biology, Ben Gurion University of the Negev,
P.O. Box 653, Beer Sheva, Israel 84105

During the second half of the first year, we have made a major breakthrough by isolating the gene for the larvicidal 28 kD protein.

Analysis of the plasmids in the transformed cells (see Progress Report No. 1) was achieved through (A) Autoradiography of DNA, hybridized to nick-translated, [^{32}P]-labelled mixture of fragments of *EcoRI*-cut purified 112 kb plasmid; (B) Partial restriction mapping of positive clones; (C) Hemolytic potential of colonies with subclones.

(A) Purified 112 kb BTI plasmid was restricted by *EcoRI* (fractions I and III) and by *HindIII* (II), the generated libraries were ligated separately to pUC9 (I and II) and pBR322 (III), both cut accordingly and dephosphorylated, and the mixtures were used to transform *Escherichia coli* strains JM83 (I and II) and MM294 (III). Over 10% of apparent transformants (totalling about 4,000; Table 1) contained DNA homologous to the cloned plasmid (Fig. 1).

Table 1

ligation mixture (fraction)	I	II	III	Total
total No. of colonies (Ca.)	1,500	1,500	1,000	4,000
No. of Lac$^-$ colonies (Ca.)	800	800	irrelevant	1,600 (+ 1,000)
No. of positive colonies (Ca.)	150	150	250	550

Figure 1. Autiradiography of 300 transformants (on 6 plates), after hybridization to [^{32}P]-labelled mixture of fragments of *EcoRI*-cut purified 112 kb BTI plasmid.

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(B) Screening positive colonies is performed by partial restriction mapping of their plasmid DNA. One of several suspicious clones (Fig. 2) amongst the 100 screened was demonstrated to contain a 5.4 kb long insert (Fig. 2, ninth lane from right, clone number F9) which yielded fragments identical in size to those expected of its sequence (Ward, E.S. & Ellar, D.J., 1986, J. Mol. Biol. **190**, 1) (Fig. 3, clone F9).

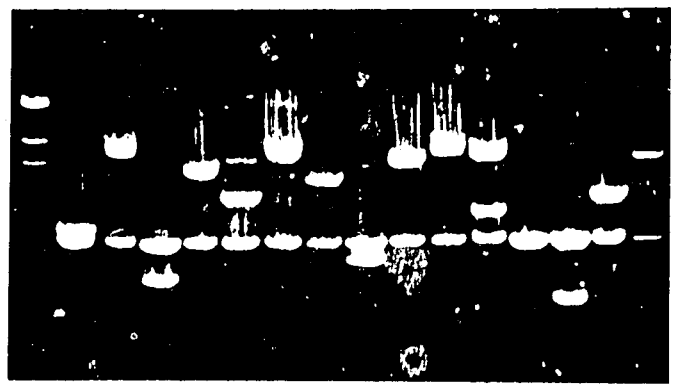


Figure 2. *EcoRI* plasmids from 15 positive recombinant clones.
The leftmost lane contains *HindIII*-cut λ-DNA fragments as markers.
The ninth clone from right (F9) was chosen for further analysis.

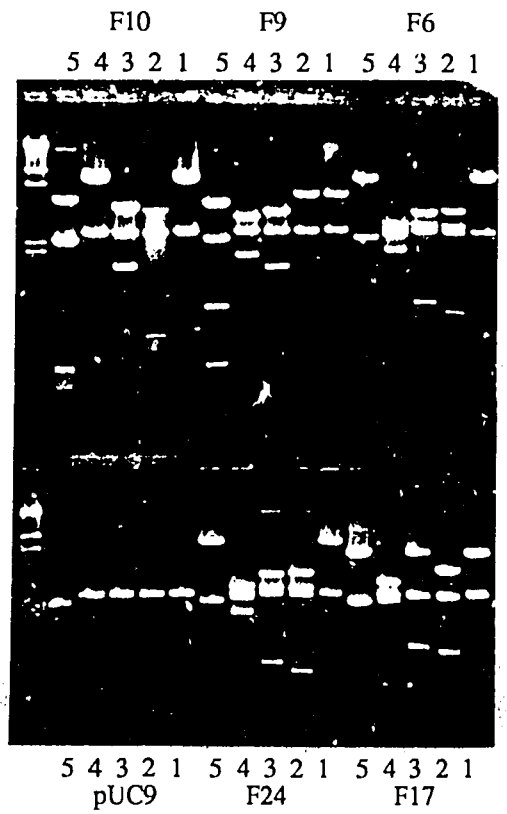


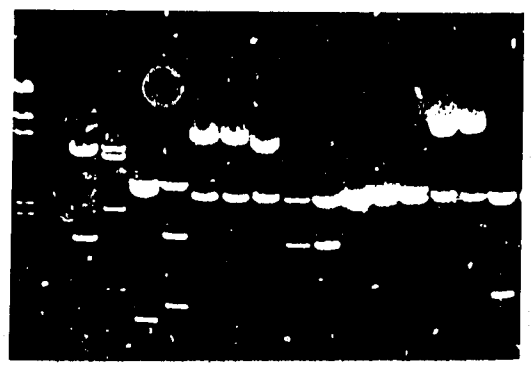
Figure 3.

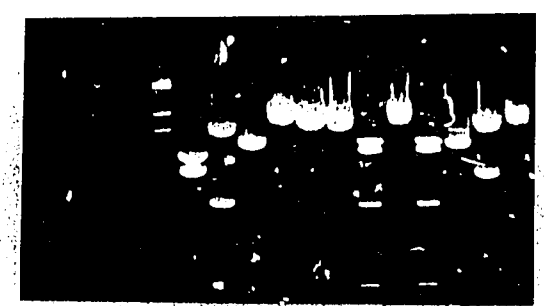
Partial restriction mapping of five clones (F6, F9, F10, F17, F24) and of pUC9 by a series of five enzyme combinations each, with *EcoRI* (1 - 5) and with (2) *HindIII*, (3) *PstI*, (4) *BamHI*, (5) *PvuII*.

In an attempt to isolate the 5'-flanking region of the 28 kD protein gene, which contains an open-reading frame presumed to code for a regulating protein (McLean, K.M. & Whiteley, H.,1987, J. Bact., **169**, 1017), the *HindIII*-containing positive colonies (fraction II; A) were hybridized to [^{32}P]-labelled purified 5.4 kb insert of F9. Twenty one positive colonies were found (Fig. 4), three of which (Fig. 5, lane ; clones F37, F40 and F42) seem promising.

Figure 4. Autoradiography of 250 positive (Fig. 1) transformant colonies (on 5 plates), after hybridization to [^{32}P]-labelled 5.4 kb insert of F9 (Fig. 3).

F37





F42 F40

Figure 5. Plasmids of 28 positive transformant colonies, cut by *EcoRI*. Leftmost lanes contain *HindIII*-cut λ-DNA fragments as markers. F37, F40 and F42 are three clones with the fragment sizes (0.9, 2.0, 2.7, 3.3 kb) as expected (McLean & Whiteley, 1987).

(C) In an attempt to obtain high levels of expression in *E.coli* of the 28 kD protein, the 2 kb *EcoRI-HaeIII* fragment (Fig. 6) of clone F9 containing the coding information for the protein was subcloned by ligation to either *EcoRI-SmaI*-cut or *EcoRI-HincI*-cut pUC9, downstream to its *lac* operator.

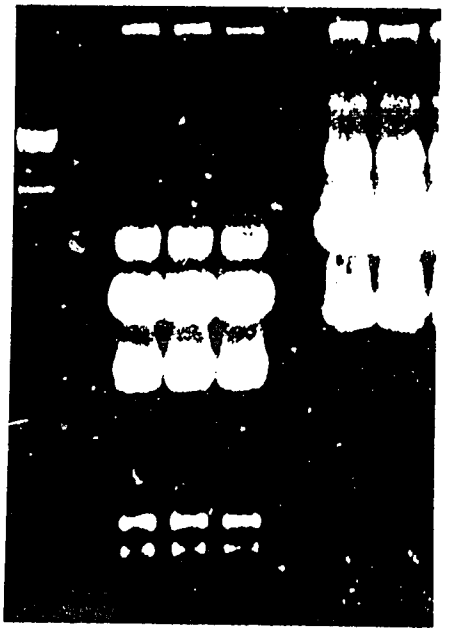
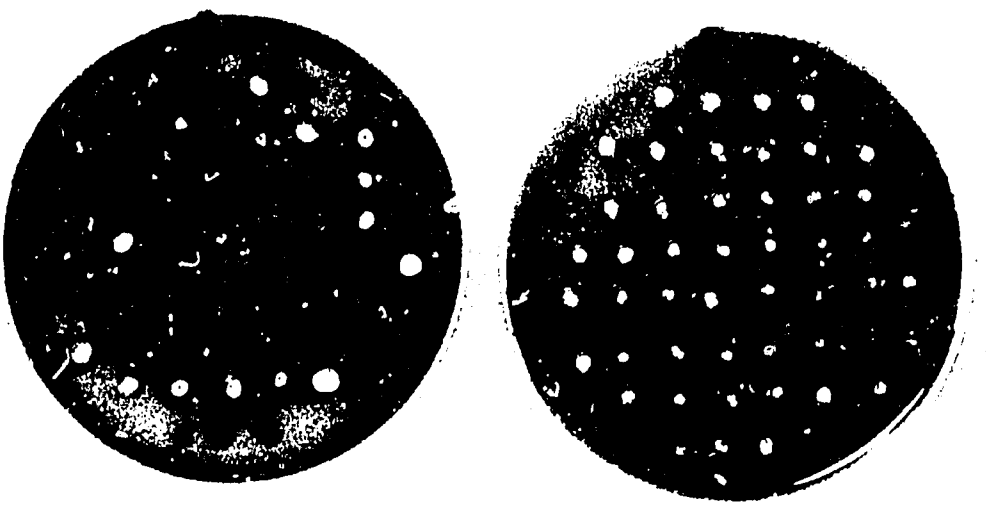


Figure 6.

About sixty white colonies obtained after transformation of *E.coli* strain JM83, were transferred to a blood-agar plate, to test for hemolytic activity of the 28 kD protein, presumably produced in them (Fig. 7). The results were quite surprising, and are to be dealt with at a later stage.



A B

Figure 7. Photographs of a blood-agar plate with 47 white transformant colonies and three controls (F9 and JM83 with and without pUC9).
A - Light transmitted from below;
B - Light projected sidewards.

Progress has also been made in preparing the ground for studies of the activation and breakdown of the δ-endotoxin in the larval gut, using isolated "crystalloid" labelled with [^{35}S] at a very high specific activity. For this purpose we developed a defined medium for the growth of BTI, with special attention to minimal methionine requirement and to the levels of sporulation and toxicity produced. We examined the appropriate times and media for introduction of labelled methionine.

Pulse-chase labelling experiments show that the 28 and 64 kD proteins are synthesized in parallel over the course of spore formation, while there is a sharp peak in synthesis of the 130 kD protein about 6 - 8 hours after onset of sporulation. This promises to allow us to produce toxin with one or another peptide specifically enriched in radioactive label.

The dependence of the specific activity of the labelled toxin peptides formed, on the amount of labelled methionine in the medium (Fig. 8), indicates that the sporulating bacteria dilute the methionine (either by turnover of proteins of the vegetative cells or by *de novo* synthesis) with an endogenous pool. The estimation of this pool size (~10 μg/ml of cells at sporulation density, ca. $5 \cdot 10^9$ cells/ml) makes it possible to optimize the labelling of the cells.

We have worked out conditions for the production and isolation of highly labelled toxin crystalloid, and for SDS-PAGE analysis of peptide content of a sample, and will now proceed with studies of the fate of the various toxin proteins *in vivo* .

Our original plans called for Prof. H.G. Nandadasa of Sri Jayewardenepura University in Sri Lanka working in our laboratories earlier in 1988. However, various political and academic problems in Sri Lanka required a postponement of these plans. Prof. Nandadasa is now expected in Beer Sheva on August 1, and will immediately join in the Genetic Engineering aspects of this project. Funds provided by the CDR have meanwhile been transferred to Sri Jayewardenepura University, and we hope that this will accelerate work in the Sri Lankan laboratory during comming months.

For: **Bull. Soc. Vector Ecol.**

Please, send galley proof to:
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C5-142

DISTRIBUTION AND ABUNDANCE OF ALGAE IN MOSQUITO DEVELOPMENTAL SITES

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ABSTRACT

Bacillus thuringiensis var. *israelensis* is highly toxic to mosquito and blackfly larvae but its larvicidal activity has low persistence under field conditions. Expression in algae of cloned genes which encode its endotoxic polypeptides is one approach to enhance control of the target organisms. To determine species composition, abundance and distribution, algal fauna was studied in various mosquito breeding sources. Clean water ponds supported development of numerous species of unicellular algae without any marked influence of *Bacillus sphaericus* or *Gambusia affinis* on parameters of algal fauna. Algal density increased during the first month post-flooding (April, 1988) and decreased to very low levels later (May, 1988). The low densities observed during June - July and August - September, 1988 (less than one thousand cells/ml), could be a result of an increase in water temperature during this period. Dairy wastewater lagoons supported development of algal species other than found in the clean water ponds, and at higher densities. Periodicities of algal species in the lagoons, but not in the clean water ponds, varied during the study period.

Larvae of *Culex quinquefasciatus* were shown to ingest four different species of unicellular algae, but did not degrade chlorophyll of *Synechocystis* sp..

KEY WORDS

Algae, Mosquito larvae, Biological control, Larvicidal genes, Ingestion rates, Chlorophyll digestion.

INTRODUCTION

The high larvicidal activity of *Bacillus thuringiensis* var. *israelensis* (BTI) against mosquito larvae (Lacey and Undeen 1986, Mulla 1985) disappears within 24 - 48 hrs under field conditions (Margalit et al. 1983, Silapanuntakul et al. 1983). Much effort is being expended around the world to increase persistence of BTI in habitats of mosquito larvae. One approach is to encapsulate it in semipermeable or degradable polymers (Lacey et al. 1984, Margalit et al. 1984, Cheung and Hammock 1985), another is to express its cloned genes (McLean and Whiteley 1987, Sekar and Carlton 1985, Ward et al. 1984, 1986) into organisms which could multiply in nature and be ingestible as well as digestible by the target organisms. Algae seem to be excellent candidates (WHO 1987) because many algal species are found in clean as well as in polluted waters (Palmer 1962, 1964) and some species have already been genetically manipulated (Golden and Sherman 1983, Sherman and van de Putte 1982, van den Handel et al. 1980). In addition, algae are reported to be ingested by mosquito larvae (Gophen and Gophen 1986, Howland 1930, Marten 1986, Pucat 1965), which are known to be filter feeders (Aly and Mulla 1986, Dadd 1968, Wallace and Merritt 1980).

The study reported here was initiated to assess species composition, abundance and distribution of planktonic algae in various mosquito developmental sites, and to identify such species having an ecological potential for carrying larvicidal genes. This was accompanied by quantitating the rate of ingestion by mosquito larvae of local algae.

METHODS AND MATERIALS

Mosquito developmental sites.

This study was carried out in two habitats as follows:

(1) Experimental ponds in the Coachella Valley of Southern California, the surface area of each was 35 m^2. These were supplied with clean water, at a level (30 cm) kept constant by float valves, provided from an artesian well through underground pipelines (Mulla et al.

1982). The vegetation cover in the ponds was mowed before flooding to keep its hight at 10 - 15 cm.

(2) Dairy wastewater lagoons at Lakerkirk and Kasbergen Dairies in Riverside and San Bernardino Counties, California, respectively. Those at Kasbergen Dairy were square-shaped with surface areas between 0.25 - 0.3 acre (1010 - 1350 m^2). At Lakerkirk Dairy, they were rectangular with surface areas between 0.15 - 0.25 acre (675 - 1010 m^2) (Mulla and Darwazeh 1988). All lagoons were in an uncontrolled flooding situation: they were dried and reflooded at varying intervals during the study, but sampling for algae continued to follow the successional trends in the algal populations.

Impact of larval control.

To determine the impact of various mosquito larval control measures on algal abundance and distribution in April - May, 1988, ponds in the Coachella Valley were treated at ten days post flooding (A) with *Bacillus sphaericus* 2362 (ABG-6184) (0.11 kg/ha), (B) with *Gambusia affinis* (1.1 kg/ha, two males and four females per pond), (C) with a combination of fish and bacteria [(A) + (B)], and (D) without any treatment as checks.

Ponds were reflooded in June and August because algal densities in all treatments decreased to very low levels at seventy days post-flooding, and algal prevalence was followed in these until September, 1988.

Algal counting and identification.

Five parallel dips (400 ml each) per pond were taken at intervals after each treatment and mixed in a plastic bag, from which 200 ml were removed into vials, transported in an ice chest to the laboratory. These samples were filtered through Whatman filter paper #1 to remove large particles, and the algae in the filtrate were concentrated by centrifugation (6,000 rpm x 10 min at 5°C, with an HS-4 rotor of Sorval centrifuge) and identified according to the keys of Forest (1954) and Prescott (1970). Cells were counted in a Bright-

Line hemacytometer under a compound microscope (Dhillon and Mulla 1982). The algal density data were subjected to ANOVA analysis (Sokal and Rohlf 1981).

Samples from the dairy wastewater lagoons were filtered through 100-mesh screens (200 μm pore size) to avoid filter clogging due to high algal density. When necessary, the filtrate was diluted with tap water.

Minnow traps (0.25 inch-mesh screens) were used for monitoring the population of *Gambusia affinis* (Reed and Bryant 1974) during the first month of the experiment. One trap was placed in each pond for 24 hours, after which time all the captured fish were counted and returned into the ponds.

Algal ingestion rates.

Twenty five fourth instar larvae of *Culex quinquefasciatus* Say were introduced into varying concentrations of a filtered sample from the dairy lagoons (50 ml), and incubated at 25 ± 2°C. The larvae were washed with tap water at different times and the green gut segments (filled with algae) were counted (Dadd 1968). To preserve the larvae for later counting, they were fixed with formaldehyde (4%).

Chlorophyll digestion studies.

In these experiments, 20 larvae were introduced into 50 ml, and incubated under dark conditions at 27 ± 2°C. Two groups of twenty larvae each were removed, washed with tap water and homogenized in 5.7 ml acetone (90%). The homogenate was incubated overnight in the refrigerator, centrifuged (10,000 rpm x 10 min, as before) and optical density of the supernatant was determined at 665 nm, as a measure for the amount of intact chlorophyll (Parson et al. 1985). Algal suspensions without larvae were used as controls.

RESULTS

Species composition, abundance and distribution.

1. Clear-water ponds.

The planktonic algae found at different sampling times in the Coachella Valley ponds were identified at least to the generic level and the percentage composition of each

species was calculated (Table 1). Two species, *Synedra* sp. and *Tetradriella* sp., appeared in negligible densities (< 1,200 cells/ml) during the whole study period (April - May, 1988). Six other species were found in significant numbers: *Closterium* sp., *Scenedesmus* sp., *Dictyosphaerium* sp., *Chlorella pyrenoidosa, Nannochloris* sp. and *Chlorococcum* sp.. The first four species were found in ponds of all four treatment groups while the latter two (*Nannochloris* sp. and *Chlorococcum* sp.) occured in ponds with all three treatments, but not in the check ponds.

Table 1

A notable heterogeneity in algal distribution was observed among ponds with the same treatment. For example, during the first month, one *B.sphaericus*-treated pond contained *Closterium* sp. and *Dictyosphaerium* sp. while another contained *Scenedesmus* sp. and *Synedra* sp.. Ponds stocked with fish revealed similar variability: *Scenedesmus* sp. and *Dictyosphaerium* sp. occured in one pond during the first month while *Closterium* sp. and *Chlorella pyrenoidosa* were found in the second pond during the same period. The control ponds also experienced similar heterogeneity in algal distribution: *Closterium* sp., *Scenedesmus* sp., *Dictyosphaerium* sp. and *Chlorella pyrenoidosa* were found in one pond while the second supported no algal growth at all during the first month. The second pond was therefore not sampled later.

The statistical ANOVA analysis of the data presented in Table 1 revealed no significant differences in algal densities between the treatments. Thus, the average density in all six ponds sampled was plotted as a function of time post-flooding (Fig. 1). Flooding the ponds supported algal growth to reach a maximum density of $2x10^5$ cells/ml at 35 days (April, 1988). However, a decrease to a very low level was noted at seventy days post-flooding (May, 1988).

Figure 1

Eight ponds were flooded in June and August, 1988, and the influence of a hot weather (Fig. 2) on algal densities was determined during June - July and August - September, respectively. Eight species appeared in these ponds: *Closterium* sp., *Scenedesmus* spp., *Dictyosphaerium* sp., *Synedra* sp., *Navicula* sp., *Phacus* sp.,

Figure 2

Tetraedron sp. and *Tetradriella* sp.. The densities of all these species were exceedingly low (several hundred cells/ml; data not shown).

Fish multiplication (a 4.5 ± 1 fold increase in density after one month; data not shown) was observed in the stocked ponds, with or without *B.sphaericus* .

2. Dairy lagoons.

In the Dairy lagoon habitats, ten species showed different periodicities during the sampling period (Tables 2 & 3): *Chlamydomonas* sp., *Ourococcus* sp. and *Scenedesmus* sp. were abundant in April; *Chlamydomonas* sp., *Phacus* sp., *Chlorella pyrenoidosa* and *Scenedesmus* sp., in May; *Euglena* sp., *Synechocystis* sp., *Clorogonium* sp. and *Phacus* sp., in June; *Closterium* sp., *Chlorella pyrenoidosa* and *Synechocystis* sp., in July; *Synechocystis* sp., in August and *Synechocystis* sp., *Chlorella pyrenoidosa* and *Scenedesmus* sp., in September.

Table 2

Table 3

Overall density in these lagoons varied from zero to 2 x 10^9 cells/ml.

Ingestion rates.

A filtered sample from a Dairy lagoon, containing *Chlamydomonas* sp., *Chlorella pyrenoidosa* and *Clorogonium* sp., was used for ingestion studies with early fourth instar larvae of *Cx. quinquefasciatus*. Larval gut was filled after 30 min incubation with the undiluted sample (containing 6 x 10^6 cells/ml (Fig. 3A)). The time required for filling the gut increased upon diluting the sample (100 min at 6 x 10^5 cells/ml (Fig. 3A)). The ingestion rate at each concentration was calculated and found to be directly related to algal concentration (Fig. 3B). However, thirty fold decrease in algal concentration resulted in seven fold decrease only in the ingestion rate.

Figure 3

Digestion studies.

Chlorophyll degradation was looked at, using the working hypothesis that algal digestion will lead to a decrease in the optical density of the chlorophyll extracted from the larval guts at different times during incubation in the algal suspension, because:

1. The proportion of the digested algae in the larval guts will increase with time since the

larvae also ingest their fecal pellets;

2. The absorption coefficients at 665 nm of the digestion products (phaeo-pigments) are lower than the parent chlorophyll (Parson et al. 1985).

Incubation of early fourth instar *Cx.quinquefasciatus* larvae with a suspension of *Synechocystis* sp., which resulted in filled guts within 30 min (Fig. 4A), was not accompanied by a reduction in the optical density of chlorophyll extracted from the guts for 10 hr (Fig. 4B).

Figure 4

DISCUSSION

Algal productivity in the Coachella Valley ponds (Fig. 1) and the abundance of each indigenous species (Table 1) were found to be exceedingly variable between and within the treatments. Total algal densities showed however no significant differences between the treatments. Thus, *B.sphaericus* (0.11 kg/ha) or *G.affinis* (1.1 kg/ha) had no marked influence on algal abundance and distribution. Reduction of algal densities during May - September could be caused by an increase of about 10°C in water temperature (Fig. 2) or depletion of nutrient contents during this period.

In the Dairy wastewater lagoons, the variations in successional trends of algal species and in their abundance during the study period (April - September, 1988; Tables 2 and 3) could have been caused by differences in nutrient contents as well. These lagoons supported appearance of species other than those which were found in the Coachella Valley ponds. The 360-fold higher mean cell density here ($2.5 \pm 1.3 \times 10^7$ cells/ml compared to $6.9 \pm 3.5 \times 10^4$ cells/ml in the Coachella Valley ponds), could have also resulted of higher levels of organic nutrients.

Mosquito larvae were shown in this study to ingest different species of unicellular algae at a rate directly proportional to algal density (Fig. 3A). A thirty-fold decrease in algal concentration led to a seven-fold decrease only in ingestion rate, implying increased larval filtering rate upon decreased algal concentration (Fig. 3B). This could result of a feedback

mechanism responding to decreased net energy gained at low food supplies (Khawaled et al. 1988).

Chlorophyll was shown to pass intact through the larval gut (Fig. 4B): optical density of the suspension at 665 nm remained constant for 10 hr, during which time all the suspension was ingested at least once (the calculation was based on gut volume of 2 - 4 µl, cell volume of *Synechocystis* sp. (Desikachary 1959), ingestion rate and algal concentration (Fig. 4A)). This however does not preclude the possibility that mosquito larvae do digest ingested algae, but are unable to degrade chlorophyll. Degradation of algal components other than chlorophyll should be examined after being ingested by mosquito larvae. This could be achieved using radiolabeling techniques, for example.

The results demonstrate that different algal species varying in abundance and periodicities prevail in different mosquito breeding sources. Distribution of algae in additional mosquito developmental sites, as well as ingestibility and digestibility by larvae feeding in different trophic levels (bottom and surface feeders (Harbach 1977)), should be looked at further in order to determine which algae are the most suitable candidates for carrying larvicidal genes.

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LEGENDS TO FIGURES

Figure 1 Algal abundance in clear-water ponds in the Coachella Valley.
The average density is plotted as a function of time post-flooding.
Flags represent standard errors.

Figure 2 Recorded temperatures during the study period.
Water temperature was monitored with a Mini-Max thermometer at about 15 cm below water surface.

Figure 3 Algal ingestion by mosquito larvae.
(A), Gut segments filled with algae of at least five larvae were counted each time during incubation in suspensions of 6×10^6 cells/ml (■), and 6×10^5 (□) cells/ml;
(B), Calculated ingestion rates as a function of algal concentration.
Flags represent standard errors.

Figure 4 Ingestion of *Synechocystis* sp. and chlorophyll digestion by mosquito larvae.
(A), Green gut segments of at least five larvae were counted at each time during incubation in a suspension of 3.6×10^7 cells/ml.
(B), Optical density (665 nm) of chlorophyll extracted from mosquito larvae-ingested algae.
Flags represent standard errors.

Table 1. Algal abundance and species composition in ponds (Coachella Valley) treated with *Bacillus sphaericus* (B.s.) and *Gambusia affinis* (G.a.).

Days post flooding	algal density (cells/ml) and species composition (%) in					
	Control	Control	B.s.	B.s.	G.a.	G.a.	B.S.+G.a.
11	2.4×10^4 a/ Clost. b/ 21 c/ Scen. 4 Chl. p. 75	0	— d/	—	3.4×10^3 Scen.	3.7×10^4 Chl. p.	—
24	3.9×10^5 Clost. 80 Scen. 2 Dicty. 10 Chl. p. 8	0	1.6×10^5 Clost. 10 Dicty. 90	1.6×10^3 Scen. 25 Syne. 75	1.1×10^5 Scen. 70 Dicty. 30	1.7×10^3 Clost. 53 Ch. p. 47	2.2×10^4 Clost. 40 Scen. 40 Tetr. 1 Chl. p. 19
39	6×10^4 Clost. 14 Dicty. 83 Chl. p. 2 Syne. <0.7	0	6×10^2 Clost. 78 Dicty. 22	5×10^4 Syne. <0.1 Scen. 62 Dicty. 38	9×10^4 Scen. 98 Dicty. 2	1.1×10^6 Clost. 0.5 Chl. p. 2 Dicty. 41.5 Nann. 56	8.3×10^4 Clost. 2.5 Scen. 27 Tetr. 0.5 Dicty. 70
53	2.3×10^3 Clost. 24 Scen. 47 Dicty. 29	—	1.6×10^3 Clost. 82 Dicty. 6 Scen. 6 Chl. p. 6	1.8×10^4 Scen. 98 Tetr. 2	1.6×10^4 Scen. 69 Dicty. 3 Chl. p. 27 Syne. <0.7	2.6×10^4 Clost. 0.5 Scen. 4 Chloroc. 95.5	2.9×10^4 Scen. 95 Tetr. 4 Syne. 1
62	0	—	0	0	3.4×10^4 Scen. 3 Dicty. 97	8×10^2 Scen.	7×10^2 Tetr. 54 Dicty. 46
68	0	—	1.4×10^3 Syne.	0	3×10^3 Dicty.	0	3×10^2 Tetr.

a/ Algal density (cells/ml).
b/ Clost.: *Closterium*; Scen.: *Scendesmus*; Dicty.: *Dictyosphaerium*; Chl. p.: *Chlorella pyrenoidosa*; Tetr.: *Tetraedriella*; Syn.: *Synedra*; Nann.: *Nannochloris*; Chloroc.: *Chlorococcum*.
c/ Species composition (%).
d/ Not determined.

Table 2. Algal abundance and species composition in dairy wastewater lagoons at Lakerkirk Dairies during the study period (1988)

Sampling period	algal density (cells/ml)		species composition (%)			
	Pond A	Pond B	Pond A		Pond B	
22 April	0	$2.8 \pm 0.1 \times 10^6$	—		Chlam.[a]	1.5±0.3
					Ouro.	1.1±0.2
					Chloroc.	97 ±0.4
9 May	$1.2 \pm 0.1 \times 10^7$	Dry	Cnlam.	62±2	—	
			Chl.	30±2		
			Chlorg.	8±0.7		
2 June	Dry	Dry	—		—	
13 June	2.6×10^5	Dry	*Euglena*	92	—	
			Phacus	8		
23 June	Dry	$3.4 \pm 0.5 \times 10^5$	—		Chlorg.	
1 July	Dry	0	—		—	
7 July	Dry	0	—		—	
15 July	Dry	$3.4 \pm 0.1 \times 10^7$	—		Clost.	
25 July	$7.9 \pm 2.2 \times 10^5$	$4.9 \pm 0.3 \times 10^8$	Syne.		Clost.	23 ±3
					Syne.	77 ±3
9 Aug.	—	0	—		—	
7 Sept.	$5.0 \pm 0.1 \times 10^6$	0	Syne.	60±2	—	
			Chl. p.	40±2		
22 Sept.	$1.6 \pm 0.3 \times 10^7$	0	Scen.	3±0.6	—	
			Chl. p.	97±1.9		

[a] As in Table 1 and Chlam.: *Chlamydomonas*; Chlorg.: *Chlorogonium*; Chloroc.: *Chlorococcum*; Ouro.: *Ourococcus*; Syne.: *Synechocystis*.

Table 3. Algal abundance and species composition in dairy wastewater lagoons at Kasbergen dairies during the study period (1988).

Sampling period	Algal density (cells/ml)		species composition (%)			
	Pond C	Pond M	Pond C		Pond M	
22 April	$1.6 \pm 0.3 \times 10^6$	Dry	Chlam.[a/] Chloroc. Scen.	65 ±15 35 ±15 0.3± 0.2	—	
9 April	0	$1.6 \pm 0.1 \times 10^6$	—		Chlam. Scen. Phacus	92 ±1 7.7±1 0.3±0.07
2 June	Dry	$7.5 \pm 0.4 \times 10^7$	—		Syne.	
13 June	Dry	$3.4 \pm 0.1 \times 10^8$	—		Syne.	
23 June	0	10^5	—		Syne.	
1 July	0	$1.3 \pm 0.2 \times 10^8$	—		Syne.	
7 July	0	$7.3 \pm 0.3 \times 10^7$	—		Syne.	
15 July	0	$4.0 \pm 0.7 \times 10^7$	—		Syne.	
25 July	0	$5.4 \pm 0.5 \times 10^4$	—		Chl. p.	
9 Aug.	0	$9.7 \pm 0.2 \times 10^7$	—		Syne.	
7 Sept.	0	$2 \pm 0.1 \times 10^9$	—		Syne. Chl. p.	99.8 0.2
22 Sept.	0	Dry	—		—	

a/ As in Tables 1 and 2.

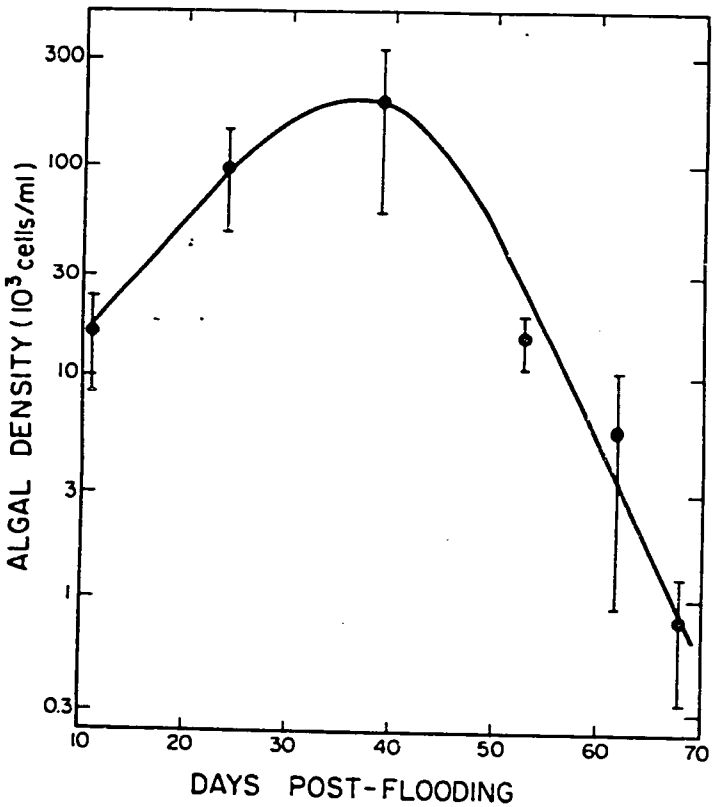
ALGAL DENSITY (10^3 cells/ml)
300
100
30
10
3
1
0.3
10
20
30
40
50
60
70
DAYS POST-FLOODING

Fig. 1

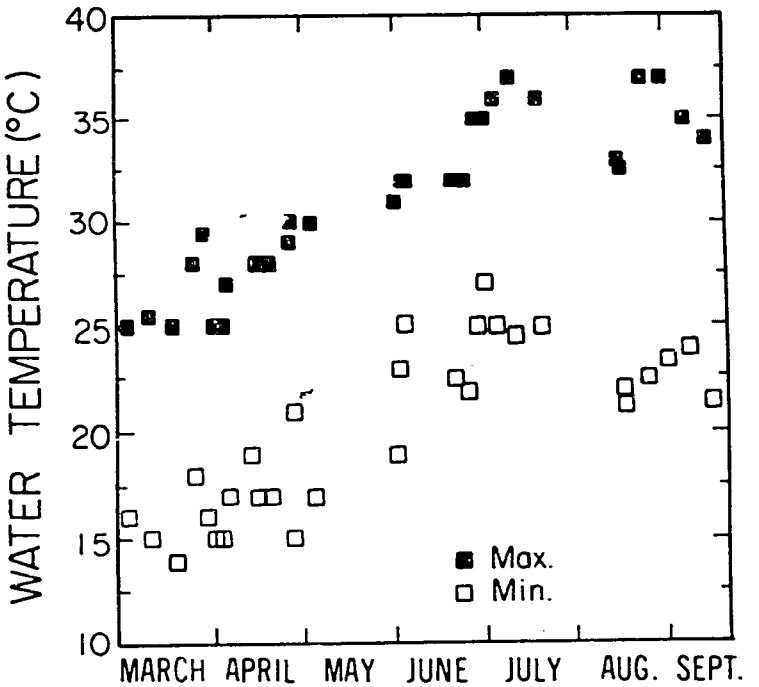
WATER TEMPERATURE (°C)
40
35
30
25
20
15
10
Max.
Min.
MARCH
APRIL
MAY
JUNE
JULY
AUG.
SEPT.

Fig. 2

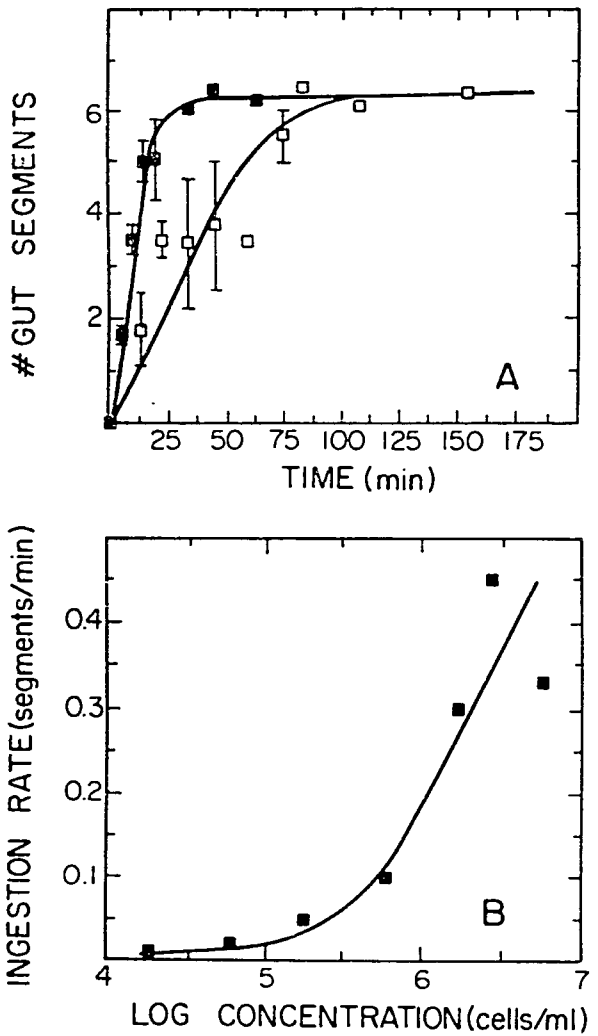
GUT SEGMENTS
6
4
2
A
25 50 75 100 125 150 175
TIME (min)
INGESTION RATE (segments/min)
0.4
0.3
0.2
0.1
B
4 5 6 7
LOG CONCENTRATION (cells/ml)

Fig. 3

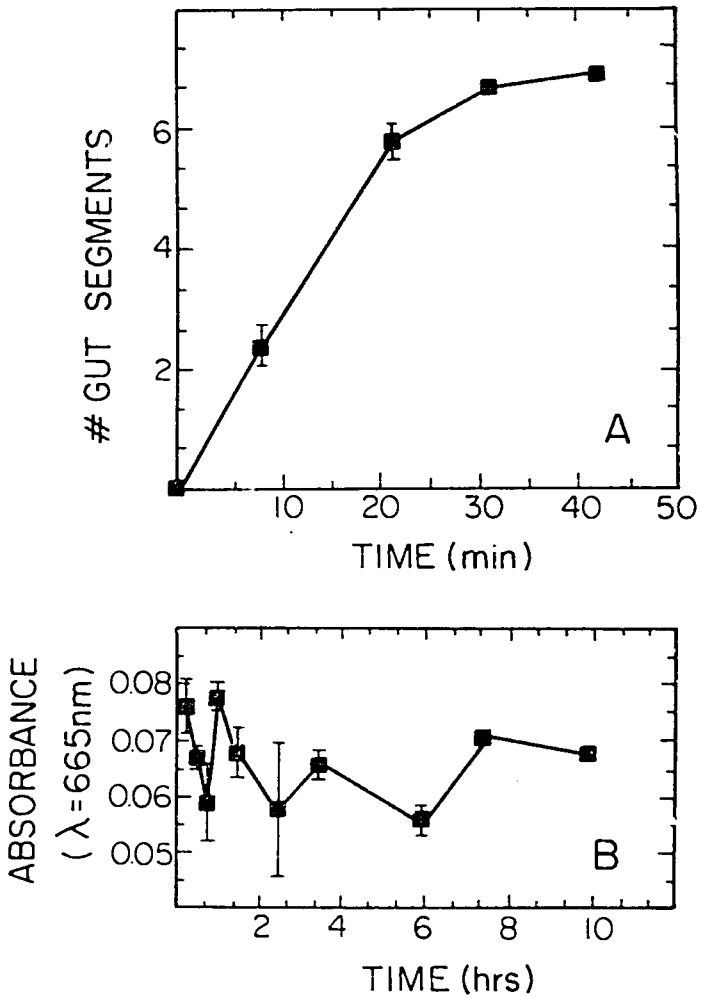
GUT SEGMENTS
6
4
2
A
10 20 30 40 50
TIME (min)
ABSORBANCE
(λ=665nm)
0.08
0.07
0.06
0.05
B
2 4 6 8 10
TIME (hrs)

Fig. 4