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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.

INTRODUCTION

The high larvicidal activity of *Bacillus thuringiensis* var. *israelensis (Bti)* against mosquito larvae (Lacey and Undeen 1986, Mulla 1985) disappears within 24 to 48 hours 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) and 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 quantitfying 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 30 to 35 m². 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 height at 10 to 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 and 0.3 acre (1010 to 1350 m²). At Lakerkirk Dairy, they were rectangular with surface areas between 0.15 and

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0.25 acre (675 to 1010 m²) (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 70 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, and 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 minutes 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 to 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 20 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 minutes, as before), and optical density of the supernatant was determined at 665 nm, as a measure for the amount of intact chlorophyll (Parsons 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 *Tetraedriella* sp., appeared in negligible densities (<1,200 cells/ml) during the whole study period (April - May, 1988). Six other species were found in significant number: *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.) occurred in ponds with all three treatments but not in the check ponds.

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. occurred 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.

TABLE 1. Algal abundance and species composition in ponds (Coachella Valley) treated with *Bacillus sphaericus* and *Gambusia affinis*.

	Algal Density (cells/ml) and Species Composition (%) Days Post-Treatment					
	11	24	39	53	62	68
Control	$2.4x10^{4a}$	$3.9x10^{5}$	$6x10^{4}$	$2.3x10^{3}$	0	0
Closterium	21[b]	80	14	24		
Scenedesmus	4	2		47		
Dictyosphaerium		10	83	29		
Chlorella pyrenoidosa	75	8	2			
Synedra			<0.7			
Control	0	0	0	--	--	--
Bacillus sphaericus	--[c]	$1.6x10^{5}$	$6x10^{2}$	$1.6x10^{3}$	0	$1.4x10^{3}$
Closterium		10	78	82		
Scenedesmus				6		
Dictyosphaerium		90	22	6		
Chlorella pyrenoidosa				6		
Synedra						100
Bacillus sphaericus	--	$1.6x10^{3}$	$5x10^{4}$	$1.8x10^{4}$	0	0
Scenedesmus		25	62	98		
Synedra		75	<0.1			
Dictyosphaerium			38			
Tetraedriella				2		
Gambusia affinis	$3.4x10^{3}$	$1.1x10^{5}$	$9x10^{4}$	$1.6x10^{4}$	$3.4x10^{4}$	$3x10^{3}$
Scenedesmus	100	70	98	69	3	
Dictyosphaerium		30	2	3	97	100
Chlorella pyrenoidosa				27		
Synedra				<0.7		
Gambusia affinis	$3.7x10^{4}$	$1.7x10^{3}$	$1.1x10^{6}$	$2.6x10^{4}$	$8x10^{2}$	0
Chlorella pyrenoidosa	100	47	2			
Closterium		53	0.5	0.5		
Dictyosphaerium			41.5			
Nannochloris			56			
Scenedesmus				4	100	
Chlorococcum				95.5		
Bacillus sphaericus and *Gambusia affinis*	--	$2.2x10^{4}$	$8.3x10^{4}$	$2.9x10^{4}$	$7x10^{2}$	$3x10^{2}$
Closterium		40	2.5			
Scenedesmus		40	27	95		
Tetraedriella		1	0.5	4	54	100
Chlorella pyrenoidosa		19				
Dictyosphaerium			70		46	
Synedra				1		

[a]Algal density (cells/ml).

[b]Species composition (%).

[c]Not determined.

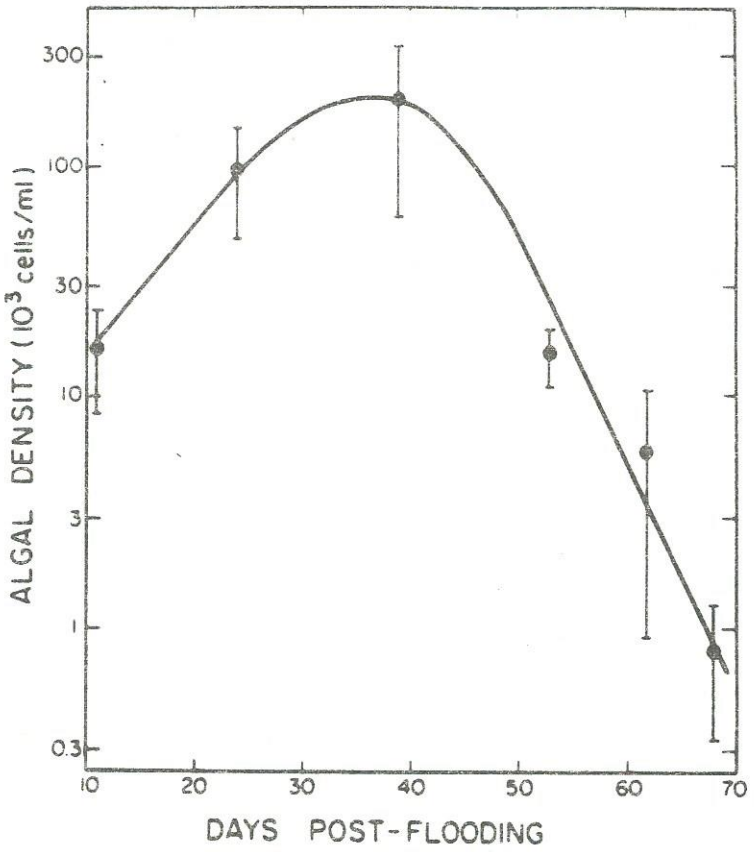

Figure 1. Algal abundance in clear-water ponds in the Coachella Valley. The average density is plotted as a function of time post-flooding. Flags represent standard errors.

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 2×10^5 cells/ml at 35 days (April, 1988). However, a decrease to a very low level was noted at 70 days post-flooding (May, 1988).

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., *Tetraedron* sp., and *Tetraedriella* 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 and 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., *Chlorogonium* 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.

Overall density in these lagoons varied from 0 to 2 x 10^9 cells/ml.

Ingestion Rates

A filtered sample from a dairy lagoon containing *Chlamydomonas* sp., *Chlorella pyrenoidosa*, and

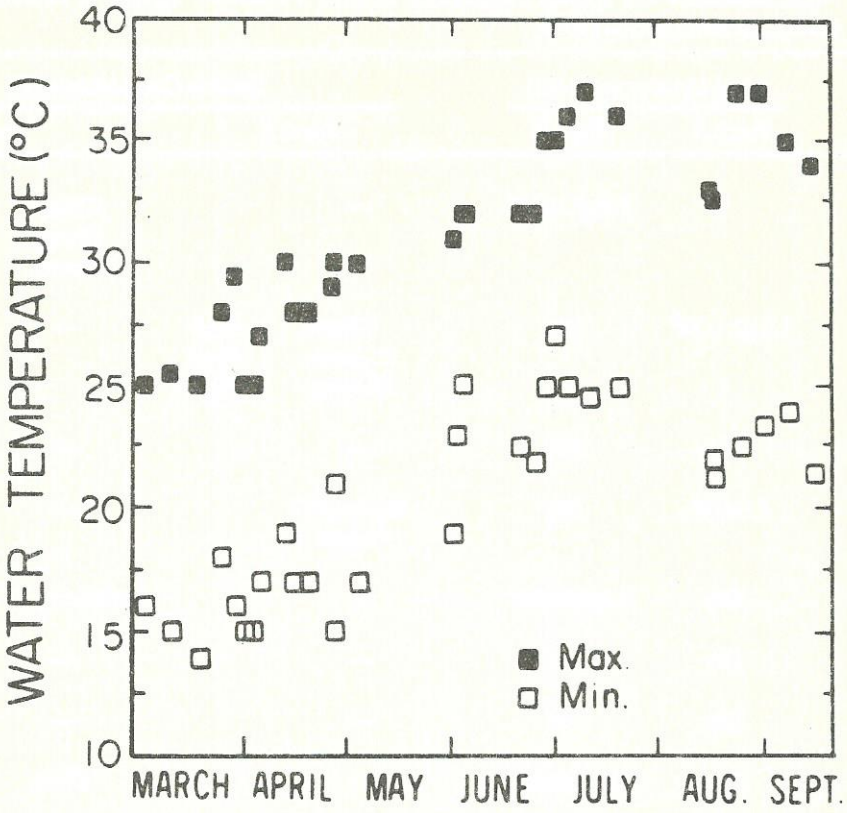

Figure 2. Recorded temperatures during the study period. Water temperature was monitored with a Mini-Max thermometer at about 15 cm below water surface.

Chlorogonium sp. was used for ingestion studies with early fourth instar larvae of *Cx. quinquefasciatus*. Larval gut was filled after 30 minutes incubation with the undiluted sample [containing 6×10^6 cells/ml (Fig. 3A)]. The time required for filling the gut increased upon diluting the sample [100 minutes at 6×10^5 cells/ml (Fig. 3A)]. The ingestion rate at each concentration was calculated and found to be directly related to algal concentration (Fig. 3B). However, 30-fold decrease in algal concentration resulted in 7-fold decrease only in the ingestion rate.

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 minutes (Fig. 4A), was not accompanied by a reduction in the optical density of chlorophyll extracted from the guts for 10 hours (Fig. 4B).

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.*

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

Sampling Date	Algal Density (cells/ml)		Species Composition (%)			
	Pond A	Pond B	Pond A		Pond B	
April 22	0	$2.8\pm0.1\times10^6$	—		*Chlam.*[a] *Ouroc.* *Chloroc.*	1.5 ± 0.3 1.1 ± 0.2 97 ± 0.4
May 9	$1.2\pm0.1\times10^7$	Dry	*Chlam.* *C. pyren.* *Chlorog.*	62 ± 2 30 ± 2 8 ± 0.7	—	
June 2	Dry	Dry	—		—	
June 13	2.6×10^5	Dry	*Euglena* 92 *Phacus* 8		—	
June 23	Dry	$3.4\pm0.5\times10^5$	—		*Chlorog.*	100
July 1	Dry	0	—		—	
July 7	Dry	0	—		—	
July 15	Dry	$3.4\pm0.1\times10^7$	—		*Clost.*	100
July 25	$7.9\pm2.2\times10^5$	$4.9\pm0.3\times10^8$	*Syne.*		*Clost.* *Syne.*	23 ± 3 77 ± 3
August 9	—	0	—		—	
September 7	$5.0\pm0.1\times10^6$	0	*Syne.* *C. pyren.*	60 ± 2 40 ± 2	—	
September 22	$1.6\pm0.3\times10^7$	0	*Scen.* *C. pyren.*	3 ± 0.6 97 ± 1.9	—	

[a]*Chlam. = Chlamydomonas; C. pyren. = Chlorella pyrenoidosa; Chloroc. = Chlorococcum; Chlorog. = Chlorogonium; Clost. = Closterium; Ouroc. = Ourococcus; Scen. = Scenedesmus; Syne. = Synechocystis.*

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 ($2.5\pm1.3\times10^7$ cells/ml compared to $6.9\pm3.5\times10^4$ 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 30-fold decrease in algal concentration led to a 7-fold decrease

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

Sampling Date	Algal Density (cells/ml)		Species Composition (%)			
	Pond C	Pond M	Pond C		Pond M	
April 22	$1.6\pm0.3\times10^6$	Dry	*Chlam.*[a] *Chloroc.* *Scen.*	65±15 35±15 0.3±0.2	—	
May 9	0	$1.6\pm0.1\times10^6$	—		*Chlam.* *Scen.* *Phacus*	92±1 7.7±1 0.3±0.07
June 2	Dry	$7.5\pm0.4\times10^7$	—		*Syne.*	100
June 13	Dry	$3.4\pm0.1\times10^8$	—		*Syne.*	100
June 23	0	10^5	—		*Syne.*	100
July 1	0	$1.3\pm0.2\times10^8$	—		*Syne.*	100
July 7	0	$7.3\pm0.3\times10^7$	—		*Syne.*	100
July 15	0	$4.0\pm0.7\times10^7$	—		*Syne.*	100
July 25	0	$5.4\pm0.5\times10^4$	—		*C. pyren.*	100
August 9	0	$9.7\pm0.2\times10^7$	—		*Syne.*	100
September 7	0	$2.0\pm0.1\times10^9$	—		*Syne.* *C. pyren.*	99.8 0.2
September 22	0	Dry	—		—	

[a]*Chlam. = Chlamydomonas; C. pyren. = Chlorella pyrenoidosa; Chloroc. = Chlorococcum; Scen. = Scenedesmus; Syne. = Synechocystis.*

only in ingestion rate, implying increased larval filtering rate upon decreased algal concentration (Fig. 3B). This could result from 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 hours, 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 then 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

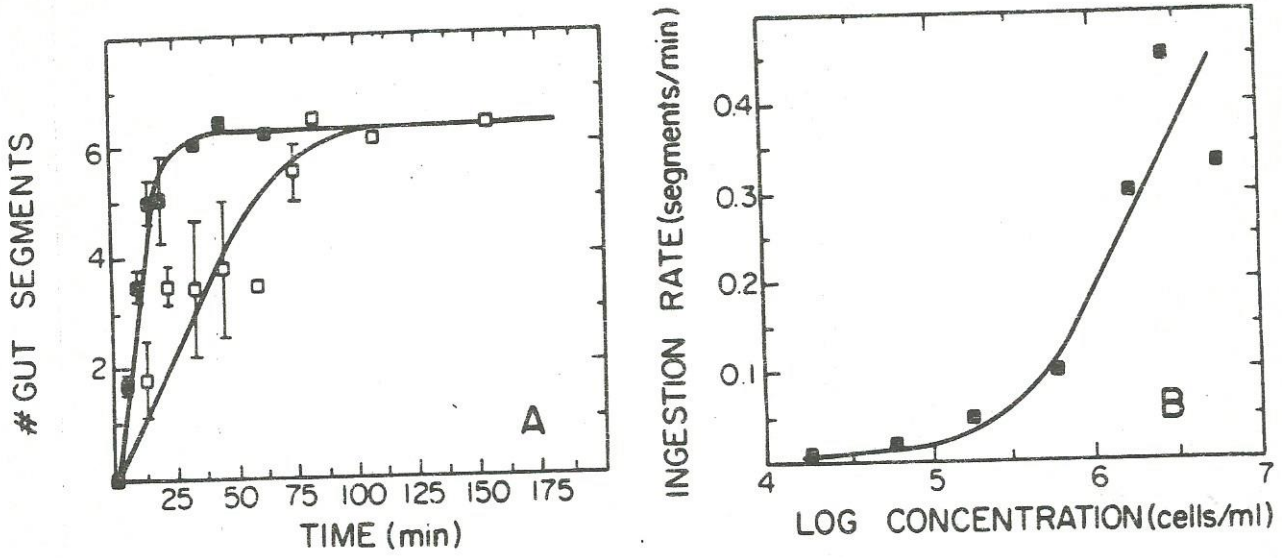

Figure 3. Algal ingestion by mosquito larvae. (A) Gut segments filled with algae of at least five larvae were counted each time during incubation in suspensions of 6 x 10^6 cells/ml (■), and 6 x 10^5 (□) cells/ml; (B) Calculated ingestion rates as a function of algal concentration. Flags represent standard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

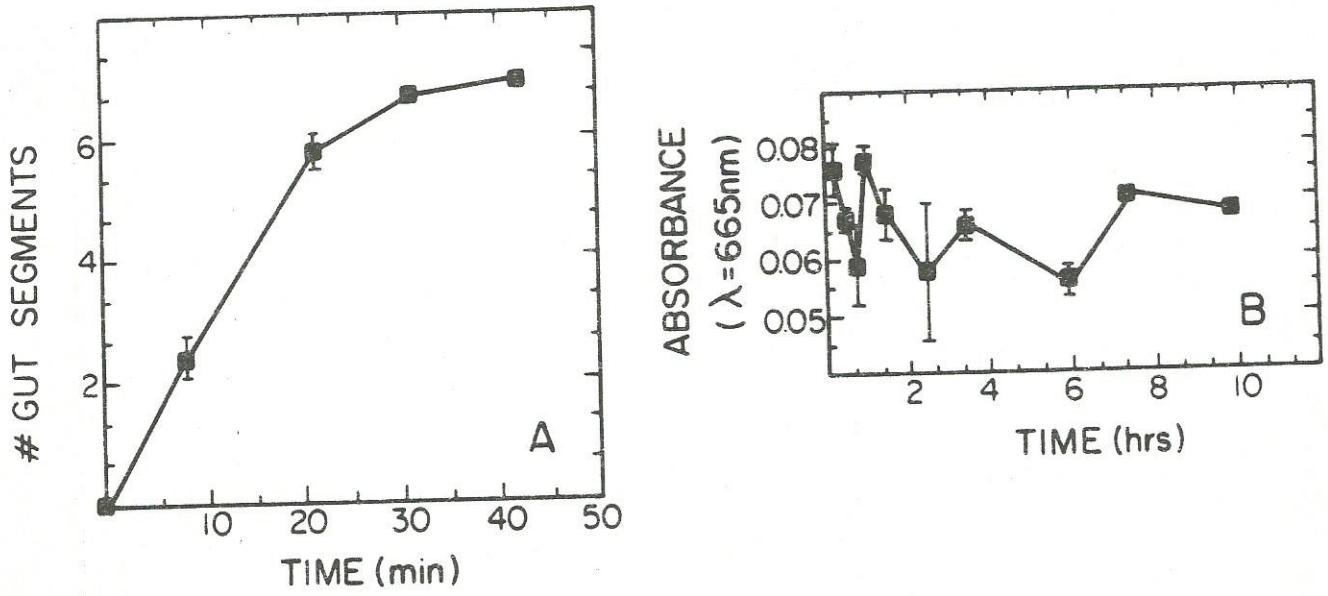

Figure 4. Ingestion of *Synechocystis* sp. and chlorophyll digestion by mosquito larvae. (A) Green gut segments of at least five larvae were counted each time during incubation in a suspension of 3.6 x 10^7 cells/ml; (B) Optical density (665 nm) of chlorophyll extracted from mosquito larvae-ingested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

determine which algae are the most suitable candidates for carrying larvicidal genes.

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