TEMPERATURE AND WATER QUALITY EFFECTS IN SIMULATED WOODLAND POOLS ON THE INFECTION OF *CULEX* MOSQUITO LARVAE BY *LAGENIDIUM GIGANTEUM* (OOMYCETES: LAGENIDIALES) IN NORTH CAROLINA[1]

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ABSTRACT. Asexual stages of the California (CA) isolate of *Lagenidium giganteum* cultured on sunflower seed extract (SFE)-agar, were applied to outdoor pools containing *Culex* larvae near Raleigh, NC in August and September 1984. Infection rates among the larvae ranged from 19 to 74% at 2–4 days posttreatment and subsequent epizootics eliminated most of the newly hatched larvae for at least 10 days posttreatment. Substantial reductions in numbers of larvae and adult emergence were achieved from a single application of the fungus. Water quality and temperature data are presented. From laboratory assays of organically polluted water, the percent infection of *Culex quinquefasciatus* by the fungus was correlated with water quality and temperature. A logistic model of water quality (COD and NH_3-N) effects on infectivity rates by the CA isolate is described.

INTRODUCTION

The entomogenous fungus, *Lagenidium giganteum* Couch has been the subject of investigations to determine its biology and potential for biological control of mosquito larvae (reviews: Lacey and Undeen 1986, McCray 1985.). Various environmental and biological factors have been shown to limit the performance of this fungus against mosquito larvae. Merriam and Axtell (1982) reported inhibition of mycelial growth and zoosporogenesis of two *L. giganteum* isolates, North Carolina (NC) and Louisiana (LA), with an increase in water salinity levels. Jaronski and Axtell (1982) presented data on the effects of organic water pollution on the infection of *Culex quinquefasciatus* Say by the NC isolate, and proposed a quantitative equation for prediction of percent infection based on various water quality indicators. The effects of temperature on growth and zoosporogenesis of the LA and NC isolates have also been described (Jaronski and Axtell 1983a, Jaronski et al. 1983). Fetter-Lasko and Washino (1983) reported that *L. giganteum* persisted in seepage ditches associated with rice fields through several consecutive seasons. The sites had been previously inoculated by McCray et al. (1973) with material isolated by Umphlett and Huang (1972) in North Carolina. In field trials in North Carolina (Jaronski and Axtell 1983b), the NC and LA isolates of *L. giganteum* infected larvae of the genera *Aedes*, *Culex*, *Anopheles* and *Psorophora*, and persisted during periods of low host densities and short-term drying of the treated sites.

Our preliminary laboratory studies have indicated differences among the NC, LA and California (CA) isolates of *L. giganteum* in their infectivity to mosquito larvae and the CA isolate appears to be the most virulent. Differences in zoosporogenesis among these isolates were noted by Lord and Roberts (1985a). These differences could be due to variations in laboratory culture as well as the inherent biological characteristics of the isolates (Lord and Roberts 1986). The CA isolate has not been evaluated under field conditions in North Carolina although it has been evaluated extensively in riceland conditions in California (Kerwin and Washino 1986). In addition, few data on the effects of water quality on the CA isolate have been published. Therefore, the CA isolate of *L. giganteum* was evaluated outdoors during August and September in North Carolina against mixed species of *Culex* mosquito larvae in simulated woodland pools and a laboratory experiment conducted to determine the short term effects of water quality (organic pollution) and temperature on the ability of the isolate to infect mosquito larvae.

MATERIALS AND METHODS

The California (CA) isolate used in these experiments was obtained from J. Lord, Boyce Thompson Institute, Ithaca, NY who obtained it from J. Kerwin, University of California, Davis, CA. The CA isolate is maintained by the American Type Culture Collection (ATCC, 12301 Parklawn Drive, Rockville, MD 20852) as Accession No. 52675. The isolate was cultured on sunflower seed extract (SFE) as described by Jaronski and Axtell (1984) and Guzman and Axtell (1986). For the field experiments, SFE-

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agar culture plates (100 mm diam. petri-dishes containing 10 ml of culture) were used and the culture material was minced, mixed with water, and then applied to the experimental sites as a slurry.

Field experiments. Experiments were conducted in August and September 1984 in the vicinity of Raleigh, NC using plastic wading pools (1.2 m^2 area × 0.2 m deep) containing water (100–130 liters per pool) from a nearby pond, and a substrate of sand and mixed deciduous forest litter (2 cm deep) to simulate natural woodland pools. The water temperature was measured with submerged maximum-minimum thermometers in 4 pools in each experiment. Water samples were taken from each pool at the beginning and end of each experiment and analyzed for chemical oxygen demand (COD), total Kjeldahl nitrogen (TKN), total phosphorus (TP) and ammonium nitrogen (NH_3-N) by the Water Quality Laboratory, NCSU Department of Biological and Agricultural Engineering, using standard methods (American Public Health Association 1976). Those water quality parameters have been shown to be correlated with the efficacy of other isolates of *Lagenidium giganteum* for mosquito control (Jaronski and Axtell 1982). The pH and conductivity of the water were recorded at the site.

The first experiment consisted of 12 pools located in a deciduous woods. The mosquito larvae in the pools resulted from natural oviposition and the supplemental addition of egg rafts (12 per pool per day) collected from other nearby pools, which were free of *Lagenidium giganteum* (based on frequent monitoring and microscopic inspection of larvae). The species were mostly *Culex quinquefasciatus* and *Culex restuans* Theobald along with lesser numbers of *Culex territans* Walker. The fungus was added to the pools on August 3 with 8 pools treated (4 each treated with 1 SFE-agar plate, 4 each treated with 3 SFE-agar plates) and 4 pools left untreated. Microscopic examination of the culture plates indicated that zoospore production was ca. 5.5 × 10^6 zoospores per plate; therefore, the estimated zoospore doses were 55 and 165 zoospores per ml of pool water.

The second experiment consisted of 8 pools which were each enclosed in a 5.8 m^3 screen cage (20 mesh) to allow counts of emerged adult mosquitoes and to prevent invasion of the pools by mosquitoes and other organisms. The mosquito larvae in the pools were *Cx. quinquefasciatus* resulting from the addition of egg rafts (24 per pool per week) from a laboratory culture previously established from the same area. The fungus was added to the pools on September 12 with 6 pools treated (3 each treated with 0.33 SFE-agar plate, 3 each treated with 1 SFE-agar plate) and 2 pools left untreated. Based on subsamples of the inoculum, the doses were ca. 17 and 51 zoospores per ml of pool water.

In both experiments the effect of the fungus introduction on the population of *Culex* mosquitoes in each pool was measured by determining the mean number of larvae retrieved with a 350 ml dipper (6 dips per pool) before treatment and at 2–3 day intervals after treatment. In the second experiment the total number of adults resting on the sides and top of the screen cages were counted at the same intervals. Relative activity of the zoospores in the water was measured by determining the percentage infection of sentinel larvae. Sentinel larvae were second- and third-instars of *Cx. quinquefasciatus* which were confined in screen-sided plastic cups (30 larvae per cup, 2 cups per pool) in each pool for 24–48 hours. The larvae were removed from the pools after this exposure period, then held in the laboratory and examined microscopically for infection at intervals of 8 hr or less for 2 days. Zoospore activity was also measured by determining microscopically the percent infection in samples of larvae collected from the pools and held in the laboratory.

Laboratory experiment. The effects of water quality and temperature on the infectivity of the CA isolate of the fungus were examined in an experiment using larvae of *Cx. quinquefasciatus* in water of varying degrees of organic pollution held at three temperatures (16, 22 and 28°C). The polluted water was prepared (Jaronski and Axtell 1982) by mixing fresh poultry manure with well water at rates of 0.7, 1.4, 2.8 and 5.7 ml/liter in 8-liter plastic containers and then holding the water for 2 weeks at ca. 25°C. After this period, a 1-liter aliquot was removed from each container and filtered to eliminate macroscopic particles. The resultant filtrates were each diluted with deionized water at rates of 1:19, 3:17, 5:15 and 7:13 to obtain a series of 16 pollution levels. Aliquots (120 ml) of each were removed and analyzed for COD, TKN, TP and NH_3-N, while the remaining water was used for the experiments. Nine aliquots (100 ml) of each of the 16 sets of polluted water were placed in 175-ml plastic cups (9 cm diam. × 5 cm height), and 25 second-instar larvae of *Cx. quinquefasciatus* added. Three cups were placed at each of the 3 temperatures after adding SFE-agar culture disks of the fungus to each. These disks (20 mm^2 × 2 mm thick) were removed from 100 mm diameter culture plates using a cork borer, and randomly distributed into the containers at 1, 3 or 9 disks per container at each temperature. Dead larvae and pupae were removed daily, counted and inspected microscopically for fungal infection. To each cup, 0.25 ml of liver pow-

der mixture (35 mg/ml H_2O) was added daily during the bioassay to assure adequate nutrients for the larvae. This small amount of nutrients did not significantly alter the water quality during the experiment.

Analyses of variance (ANOVA) were performed on the data from the field experiments to determine if there were significant differences ($P < 0.05$) among treatments. The laboratory data were analyzed by ANOVA and regression analysis using SAS procedures (SAS Institute 1982). A logistic regression model (Draper and Smith 1981) was used to fit the data obtained at 22° and 28°C which are temperatures in the range commonly occurring in habitats producing mosquitoes. The percent infection (p) for each water pollution level was transformed to: Ln [1/p) − 1]. Simple linear regression analyses were conducted on the transformed values versus the COD, NH_3-N, TKN, and TP data. The slopes and intercepts of the regression equations were used to construct logistic equations for NH_3-N and COD relating percent infection of the larvae to the individual water quality parameters. The equation has the form:

$$\% \text{ infection} = \frac{100}{1 + e^{(a + bx)}}$$

where a is the intercept and b the slope of the linear regression equations.

RESULTS

In the first field experiment using unscreened pools, the water temperature ranged from 23.2 to 26.2°C and the organic pollution was low (Table 1). The mean number of larvae per dip per pool was reduced in the treated pools compared to the untreated after addition of the SFE-agar culture slurry (Table 2). At 3 and 5 days posttreatment, the reduction was 86 and 87%, respectively with 3 plates per pool and 73 and 85%, respectively with one plate per pool. Concurrently, 65–74% of resident *Culex* larvae which were collected were infected with the fungus at 3 days posttreatment. At 5 days posttreatment, most of the resident larvae collected from the untreated pools were infected which indicated an unexplained accidental introduction of the fungus. Sentinel larvae exposed in screen cages in the pools were 50–54% infected at 3 days posttreatment. At 5 days posttreatment, the sentinel larvae were 23–31% infected in the treated pools but infection also occurred in larvae exposed in the untreated pools. Due to the contamination of the untreated pools the experiment was terminated.

Table 1. Water analysis and field measurements of pH and conductivity of water in pools at the time of introduction of *Lagenidium giganteum* (CA isolate) in the first field experiment (August 1984).

Water quality parameter	Mean[1] ± SD	Range
COD (mg/liter)	77.7 ± 13.3	52.5–97.0
TKN (mg/liter)	3.4 ± 0.9	2.5–5.8
TP (mg/liter)	0.6 ± 0.1	0.4–0.7
NH_3-N (mg/liter)	0.8 ± 0.4	0.3–1.6
pH	7.1 ± 0.1	6.9–7.3
Conductivity (μmhos/cm)	129.5 ± 20.0	108–170

[1] Means based on 12 water samples (1 sample from each pool).

Table 2. Effect of the introduction of the CA isolate of *Lagenidium giganteum* at 1 or 3 SFE-agar culture plates per pool on mixed populations of *Culex* spp. in pools (4 per treatment) in first field experiment (treated August 3).

Treatment (plates per pool)	Days posttreatment 0	3	5
	Mean no. larvae per dip (n = 6) per pool		
3	22.5	7.2	2.8
1	32.0	13.5	3.1
Untreated	42.5	51.5	21.5
	Percent infection of resident larvae		
3	0	74.8	15.4
1	0	65.8	41.2
Untreated	0	9.2	95.5
	Percent infection of sentinel larvae		
3	0	54.9	23.9
1	0	50.2	31.7
Untreated	0	0	20.8

In the second field experiment, using screened pools, the water temperature ranged from 21.2 to 22.0°C at the time of fungus addition (September 12) and the pollution level was slightly higher than in the first experiment (Table 3). During the 11 days posttreatment (September 12–23) the temperature declined (range = 13–27°C) and the water pollution, as measured by COD, increased. The differences in the effects of the treatments with 1 and 0.33 plates per pool (Table 4) on the mean number of larvae per dip per pool, and the percent infection of resident larvae at 2, 4, 7, 9 and 11 days posttreatment, were variable and not significant ($P < 0.05$). The number of larvae per dip was significantly higher in the untreated pools than in the treated at 4, 7, 9 and 11 days posttreatment.

The reduction in the numbers of larvae and the increase in the percent infection of resident larvae occurred at a slower rate in the September experiment than in the August experiment probably due to the lower temperatures. After 4 days

Table 3. Water analysis and field measurements of pH and conductivity of water in pools at the time of introduction (0 day) of *Lagenidium giganteum* (CA isolate) and at 9 and 16 days posttreatment in the second field experiment (September 1984).

Water quality parameter	Date Sampled (and days posttreatment)					
	Sept. 12 (0)		Sept. 21 (9)		Sept. 28 (16)	
	Mean ± SD[1]	Range	Mean ± SD	Range	Mean ± SD	Range
COD (mg/liter)	109 ± 19	135–85	149 ± 40	97–214	161 ± 70	85–312
TKN (mg/liter)	3.1 ± 0.7	2.5–4.4	4.2 ± 1.6	2.3–7.0	3.0 ± 0.8	2.2–4.8
NH_3-N (mg/liter)	0.4 ± 0.4	0.2–1.2	0.4 ± 0.8	0–2.5	0.1 ± 0.1	0–0.2
TP (mg/liter)	0.5 ± 0.2	0.2–0.9	0.7 ± 0.3	0.2–1.2	0.5 ± 0.3	0.2–1.1
pH	7.01 ± 0.1	6.9–7.2	7.2 ± 0.1	7.1–7.2	7.1 ± 0.1	7.0–7.3
Conductivity (μmhos/cm)	84 ± 23	113–74	86 ± 28	43–130	70 ± 32	12–112

[1] Means based on 9 water samples taken at each date; one sample from each pool.

Table 4. Effect of the introduction of the CA isolate of *Lagenidium giganteum* at 0.33 or 1 SFE-agar culture plate per pool on populations of *Cx. quinquefasciatus* in screened pools (3 per treatment, 2 per untreated) in second field experiment (treated September 12).

Treatment (plate per pool)	Days posttreatment							
	0	2	4	7	9	11	14	16
	Mean no. larvae per dip (n = 6) per pool							
1	95.7	81.1	40.9	50.0	21.0	5.4	1.2	0.2
0.33	113.6	84.6	26.8	33.1	10.8	2.3	0.6	0.2
Untreated	91.1	73.8	54.5	74.6	50.2	45.6	49.5	39.5
	Percent infection of resident larvae							
1	0	19.8	53.6	65.1	46.6	80.0	—	—
0.33	0	20.8	69.5	94.6	68.0	92.0	—	—
Untreated	0	0	0	0	0	0	—	—

posttreatment there was 25 and 50% reduction and at 11 days 88 and 95% reduction in the number of larvae with the 2 treatments as compared to the untreated. At 14 and 16 days posttreatment larvae were nearly totally absent from all of the treated pools while the mean number of larvae per dip in the untreated pools were 49.5 and 39.5, respectively. The very low numbers of larvae in the treated pools made it impractical to collect larvae for determining percent infection. Although sentinel larvae were exposed in screen cages during this experiment, the percent infection of those larvae was surprisingly 5% or less during the experiment (data not presented).

Since there were no significant differences in the effects of the 2 dosages of fungus in the second experiment the data were pooled for larval and pupal abundances and for adult emergence (Fig. 1). There was a decline in the numbers of adult mosquitoes and immatures (larvae and pupae) per treated pool during the 11 days posttreatment (September 12–23). The number of adults and immatures were substantially greater in the untreated pools. Under these conditions, the fungal treatments effectively eliminated the immature and adult mosquito populations in 11 days even though egg rafts were added. Monitoring of adult emergence was continued through October 13 (data not shown) and during that 11–30 day posttreatment interval the mean number of adults per cage per sampling period (2–3 day interval) was 1 in the treated and 29 in the untreated pools.

Laboratory experiment. The prepared series of organically polluted water had the following ranges of water quality measurements (mg/liter): COD = 101–367, NH_3-N = 3.0–40.3, TKN = 3.1–47.2 and TP = 0.3–12.3. With increasing organic pollution, there was a decrease in the infection of larvae exposed to *L. giganteum* in the water. Infection rates were significantly lower ($P < 0.05$) at 16°C (53.0%) than at 22° (70.2%) and 28° (66.5%) when averaged over water quality and dose levels. There was no significant difference ($P < 0.05$) between the infection rates at 22° and 28°C. Based on the data obtained at 22° and 28°C, over 98% infection occurred in samples with the COD ranging from 101 to 157 mg/liter and NH_3-N levels ranging from 3.0 to 9.2 mg/liter. Infection rates lower than 4% occurred in water samples with

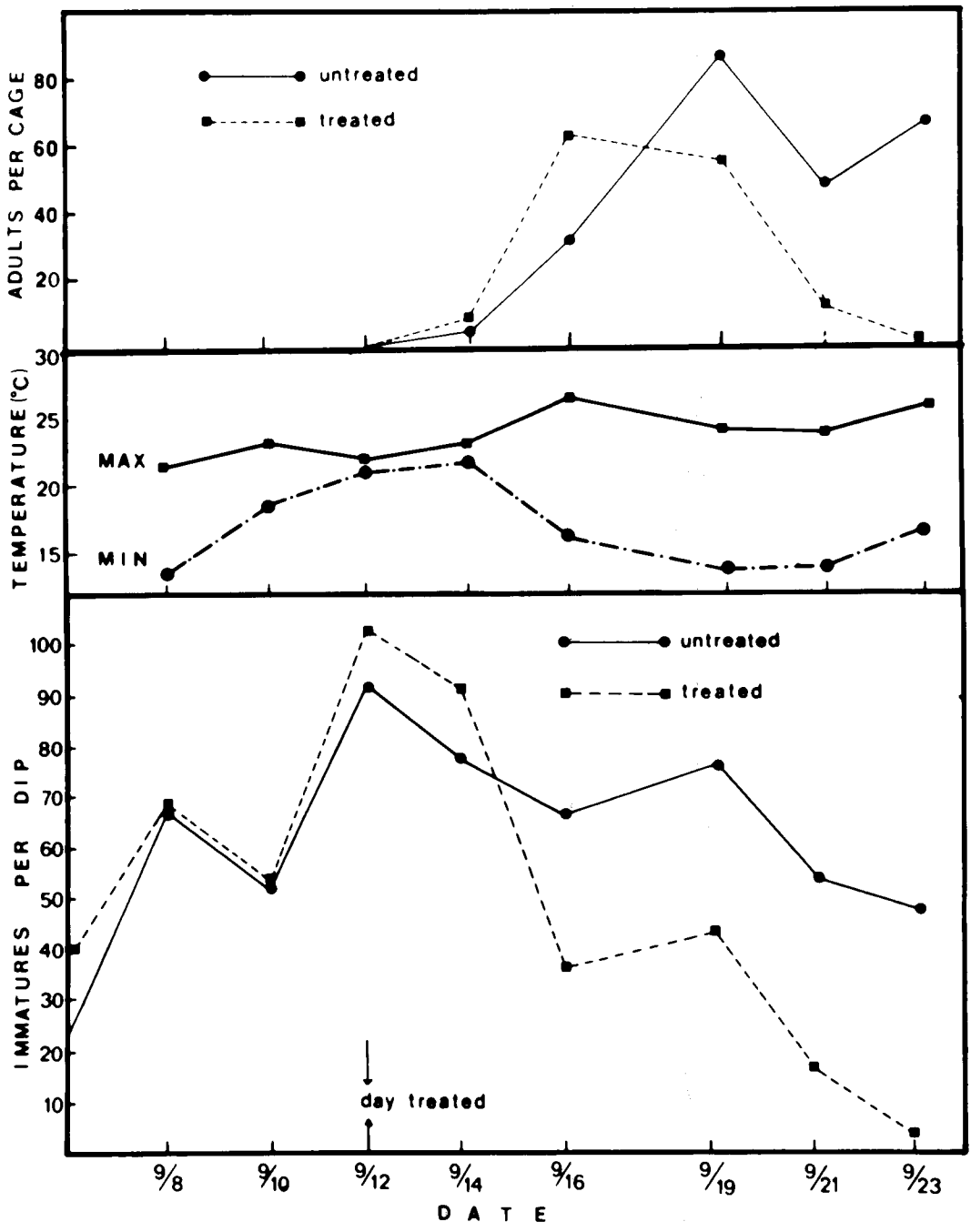

Fig. 1. Relative abundance of adult and immature *Culex quinquefasciatus* (larvae and pupae) before and after treatment with *Lagenidium giganteum* (CA isolate) in September 1984 and maximum-minimum temperatures in the water during the study period. Points are means based on total number of adults resting on sides and top of each cage and 6 dips per pool with 6 caged pools for the treated and 2 caged pools for the untreated.

COD = 229–367 mg/liter and NH_3-N = 23.2–40.3 mg/liter.

Significant regressions were found for the four water quality parameters (Table 5). Higher correlation coefficients resulted from the NH_3-N and COD regressions. From the logistic equations it was calculated that 50% reduction of infection rates by the CA isolate of *L. giganteum*

would be expected to occur in water with the NH_3-N = 15.9 mg/liter or the COD = 221 mg/liter. The logistic relationships between infection rates in *Culex* larvae and water quality (as measured by concentration of NH_3-N or COD) are illustrated in Fig. 2.

Further analysis of the data was conducted to determine the combined effect of temperature (including data obtained at 16°C), dose, and water quality (NH_3-N and COD) on the rates of infection of *Culex* larvae by the fungus. Stepwise multiple regression ($r^2 = 0.710$) indicated significant effect of the 3 parameters but the greatest impact was attributed to water quality ($F = 141.4$, 3 and 58 d.f., $P = 0.0001$ for NH_3-N, and $F = 131.1$ for COD with same d.f. and P). The logistic multiple regression equations for the three parameters were (a = NH_3-N or COD concentration in mg/liter, t = temperature in °C and d = dose in mm^2 agar culture):

For NH_3-N:

$$\% \text{ infection} = \frac{100}{1 + e^{(0.30a - 0.10t - 0.008d - 0.7)}}$$

For COD:

$$\% \text{ infection} = \frac{100}{1 + e^{(0.04a - 0.14t - 0.008d - 4.0)}}$$

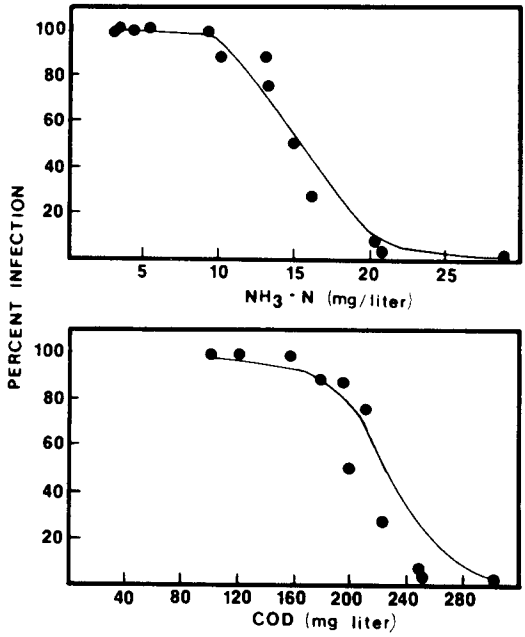

Fig. 2. Percent infection of *Culex quinquefasciatus* larvae and approximate logistic regression curve in relation to NH_3-N and COD concentrations in water samples in laboratory bioassay. Each point is the overall mean of 6 samples of 25 larvae treated at 3 rates of *Lagenidium giganteum* and held at 22 and 28°C.

DISCUSSION

In the first field experiment with uncaged pools, the water quality (relatively unpolluted based on COD and NH_3-N) and temperature (23.2–26.2°C) favored the establishment of *L. giganteum* in the host population. Relatively high larval densities in the untreated pools appeared to favor a rapid increase in epizootic levels after low level accidental introduction of *L. giganteum*. Possibly, the fungus was transported between the unscreened pools by frogs which were abundant in the area; however, attempts to prove this hypothesis were not successful. Infected cadavers apparently accumulated in the untreated pools and zoosporogenesis from these caused a rapid increase in zoospore activity as evidenced by the high infection rates in sentinel larvae. This aborted experiment demonstrated that low levels of *L. giganteum* can lead to high larval mortality in a relatively short period when conditions are favorable. After 5 days, more than 95% of the larval population was infected in these contaminated but untreated pools.

In the second field experiment, the higher COD levels and lower temperatures (mean temperature during the study was 20.6°C), probably accounted for the lower mortality rates observed among the *Culex* larvae. The lower temperatures caused a longer development time of *L. giganteum* in the infected hosts. The prolonged survival of infected larvae under cool conditions can account for the less rapid decline in the number of larvae in the second field experiment relative to the first. This is supported by data from the laboratory experiment in which the LT_{50} values (time posttreatment after which 50% of infected larvae died) at 28, 22, and 16°C were 36, 60, and 110 hours, respectively.

The levels of NH_3-N, TKN and TP were low in both experiments and most likely did not affect the activity of *L. giganteum* in the test pools. One of the treated pools in the second field experiment had a COD level of 214 mg/liter on September 20 and larval abundance was consistently higher in this pool relative to the other treated pools, suggesting some degree of inhibition of infection by organic pollution. From the equation presented, it was calculated that infection of 57% of the larvae (second-instar) could be expected in water with the COD = 214 mg/liter. Applying the equation to the results in the first experiment suggests that water quality had no effect on infection rates in that trial. Substitution of the observed mean values for water quality presented in Table 2 into the equations presented in Table 5, resulted

Table 5. Simple linear regressions of transformed percent infection (Y) of *Culex quinquefasciatus* larvae by *Lagenidium giganteum* (CA isolate) and water quality parameters.

Water quality parameter (X) mg/liter	Linear regression	R^2	F-Value
COD	$Y = -11.01 + 0.05X$	0.907	88.24**
TKN	$Y = -6.19 + 0.35X$	0.873	61.84**
TP	$Y = -4.28 + 0.66X$	0.529	10.09*
NH_3-N	$Y = -6.35 + 0.40X$	0.916	97.86**

* Significant at $P < 0.05$, ** significant at $P < 0.01$.

in calculated infection rates above 99%. Similar analyses for the data in the second experiment (Table 4) suggests only minimal inhibition of infection rates due to high COD levels on September 21 (92% infection) and September 28 (95% infection), and no effect due to the other water quality indicators (>99% infection).

Based on the laboratory experiment, the levels of NH_3-N and COD were the best predictors of percent infection in organically polluted water ($R^2 = 0.916$ and 0.907, respectively). A previous study by Jaronski and Axtell (1982) using the NC isolate indicated a high correlation between ammonia nitrogen concentration and percent infection in organically polluted water. In that study, COD did not significantly affect the percent infection in organically polluted water. The discrepancy between those results and the present data may reflect the bioassay methods employed and the source of water. In our experiment, water from only one source was employed and was filtered to eliminate macroscopic particles prior to the assays, whereas unfiltered water subjected to varying regimes of pollution were used in the previous study. Lord and Roberts (1985a, 1985b) have shown that certain types of bacteria, yeast, organic and inorganic compounds can inhibit or induce zoosporogenesis and can affect zoospore survival and motility. It is possible that other unmeasured factors contributed to inhibition of infection in our experiments, and could have interacted with the parameters that were measured. Although the simple water quality regression models may be useful aids in delineating waters suitable for the fungus, they should be applied with caution. Polluted water is a complex system and water quality parameters are interrelated. Different mosquito habitats (e.g., organic disposal lagoons, woodland pools, rice field habitats) may have widely varying characteristics, which affect fungal activity. It is also important to consider that prediction of infection rates by *L. giganteum* in the field should include the effects of the age-structure of the mosquito larvae population.

Overall, these field and laboratory experiments indicate that the CA isolate is highly effective against mosquito larvae in standing pools of water under North Carolina conditions. The CA isolate appears to be affected by water quality (organic pollution) and temperature in a manner similar to the NC and LA isolates.

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