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SALINITY TOLERANCE OF TWO ISOLATES OF *LAGENIDIUM GIGANTEUM* (OOMYCETES: LAGENIDIALES), A FUNGAL PATHOGEN OF MOSQUITO LARVAE[1]

T. L. Merriam and R. C. Axtell[2]

Abstract. Laboratory experiments were conducted to determine the salinity range over which North Carolina (NC) and Louisiana (LA) isolates of the fungus *Lagenidium giganteum* (Couch) grow vegetatively and infect mosquito larvae. The mycelial growth rates of the 2 isolates on nutrient agar were increased with 2.5 and 5.0 parts per thousand (ppt) of NaCl added and reduced with 7.5 ppt or more NaCl. The LA and NC isolates did not grow on agar containing 20 and 30 ppt NaCl, respectively. The concentrations of NaCl for 50% inhibition (IC_{50}) of mycelial growth of the LA and NC isolates (10.9 and 12.0 ppt, respectively) were not significantly different ($P > 0.05$). The ability of the 2 isolates of *L. giganteum* to infect larvae of *Aedes taeniorhynchus* decreased as salinity of the water increased. The IC_{50} values for the inhibition of infection in mosquito larvae by the LA and NC isolates (0.52 and 0.55 ppt NaCl, respectively) were not significantly different ($P > 0.05$). Microscopic examination of the fungus in saline and distilled water showed that NaCl inhibited the production of zoospores. In water containing 1.5 ppt NaCl there was complete inhibition of zoosporogenesis and mosquito infection in each isolate of *L. giganteum*. Zoosporogenesis was ca. 22 times more sensitive to salinity than was mycelial growth.

One of the promising candidates as a biological control agent for mosquitoes is the fungus *Lagenidium giganteum* Couch (Oomycetes: Lagenidiales) (Roberts 1974, Briggs 1975). The fungus was originally described as a saprophyte with the capacity to function as a weak, facultative parasite of mosquito larvae, *Daphnia* and copepods (Couch 1935). A strain of *Lagenidium* isolated in 1969 from infected *Culex* larvae (Umphlett & Huang 1970) and later identified as *L. giganteum* (Umphlett 1973) has been shown to be a virulent pathogen of larvae of several genera of Culicidae (McCray et al. 1973a, b, Umphlett 1975) and of Chaoboridae larvae (Brown & Washino 1977, 1979). The fungus develops rapidly and kills host mosquito larvae, usually within 72 h after infection (Umphlett & Huang 1972). The elucidation of a sterol requirement in *L. giganteum* has made possible the in vitro production of infective zoospores (Domnas et al. 1977). In initial field tests, *L. giganteum* gave variable control of *Culex tarsalis* (Coquillett) larvae in rice fields and drainage ditches and of *Aedes nigromaculis* (Ludlow) larvae in pastures (McCray et al. 1973b, Christensen et al. 1977). It is also apparently safe for nontarget organisms (McCray et al. 1973b, Fetter-Lasko & Washino 1978). The fungus has demonstrated persistence, since it has overwintered in field situations (Washino et al. 1975, 1976, Glenn & Chapman 1978).

Little is known about the environmental limitations that may affect the efficacy of different strains of *L. giganteum* as microbial control agents for mosquitoes. Fetter-Lasko & Washino (1978) tentatively concluded (but presented no data) that persistently high water temperatures or water with a high organic load may limit mosquito infection by *L. giganteum*. Jaronski & Axtell (1982) quantified the detrimental effects of even low levels of organic pollution on the infectivity and survival of *L. giganteum*. McCray et al. (1973b) reported that *L. giganteum* infections in mosquito larvae were reduced in water with high salinity, but Fetter-Lasko & Washino (1977) were unable to verify this. Most of the research conducted on *L. giganteum* has been with the 1969 isolate of Umphlett & Huang. As pointed out by Roberts (1974), different fungal strains may possess different properties (e.g., virulence and host range). Thus, when developing a potential microbial control agent such as *L. giganteum*, other isolates should not be overlooked.

Since some important pestiferous and disease-vectoring *Aedes* mosquito species are produced in brackish water habitats such as salt marshes (O'Meara 1976) and diked, dredged-material disposal sites (Ezell 1978, Scotton & Axtell 1979, Vorgetts et al. 1980), the degree to which *L. giganteum* can be considered part of an integrated pest management program (Axtell 1979) for mosquitoes in coastal areas depends, in part, upon the tolerance of the fungus to salinity. Consequently, the objective of this study was to determine the salinity range over which 2 isolates of *L. giganteum* grow

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[2] Department of Entomology, North Carolina State University, Raleigh, North Carolina 27650, USA.

vegetatively and infect mosquitoes under laboratory conditions.

MATERIALS AND METHODS

Sources and maintenance of fungi

The 2 isolates of *L. giganteum* used in this study were: (1) Louisiana strain (LA) isolated by Glenn & Chapman (1978) from infected *Culex territans* (Walker) in a blackgum swamp near Moss Bluff, Louisiana, USA, and (2) North Carolina strain (NC) isolated by A. L. Knight from infected mosquito larvae in Chapel Hill, North Carolina, USA, in 1979. Stock cultures of both isolates were routinely maintained on PYG agar (0.125% peptone, 0.125% yeast extract, 0.3% glucose and 2.0% agar).

Mycelial growth

The 1st experiment was conducted to study the effects of salinity on mycelial growth of *L. giganteum*. Each isolate was grown on PYG agar and incubated at 25–27 °C. After 5 days, circular plugs of agar (5.0 mm diam) bearing the most recent mycelial growth from the outer edge of the culture were removed with a sterile metal cork borer. The agar plugs were transferred, mycelial growth downward, to the center of sterile, 100-mm diam petri dishes (1 plug per dish). Each dish contained ca. 20 ml PYG agar, with 5 dishes at each of the following NaCl concentrations (parts per thousand, w/v): 0, 2.5, 5.0, 7.5, 10.0, 15.0, 20.0, 25.0 and 30.0. The experiment was repeated 3 times. The surface area of the mycelium growing on the agar in each petri dish was determined using an electronic planimeter at 5 and 7 days postinoculation. After correction for the surface area of the agar plug used for inoculating the petri dishes, the radial mycelial growth rates (RMGR, mm/day) for the fungus in each petri dish during growth period #1 (days 1–5) and growth period #2 (days 6–7) were calculated. A mean RMGR and its standard error were calculated for each isolate at each salinity level using the corresponding RMGR's from the 2 growth periods. Differences in mean RMGR between isolates and salinity concentrations were analyzed using analysis of variance and Duncan's multiple range test (Barr et al. 1979).

Zoospore production and infectivity

The ability of *L. giganteum* to produce viable zoospores and infect mosquito larvae under saline conditions was determined by bioassay using larvae of *Aedes taeniorhynchus* (Wiedemann). These larvae came from a laboratory colony established from pupae collected on 29 May 1979 from pools in diked, dredged-material disposal areas in coastal Onslow Co., North Carolina.

The 2 isolates of *L. giganteum* were cultured following methods similar to those used by Domnas et al. (1974). Flasks containing 75 ml HWYG broth (hemp seed extract at 0.25 mg soluble protein/ml broth, 0.32% wheat germ, 0.14% yeast extract, and 0.12% glucose) were inoculated with agar slices bearing recent mycelial growth from the stock cultures. After 5 days, the fungus was washed twice with sterile distilled water and homogenized briefly to break the mycelia into segments of a few cells each. Approximately 1.5 ml of washed fungus (mean dry weight = 2.4 mg/ml, SE = 0.14) were used to inoculate ca. 20 ml of whole hemp seed extract (WHS) agar (1.0 mg soluble protein/ml) in each of several 100-mm diam petri dishes. The fungus was allowed to grow on the WHS agar for 7 days prior to testing.

Bioassay tests were conducted in enamel-coated steel trays (23 × 36 × 5 cm) filled with 1 litre of distilled water and NaCl at the following concentrations (parts per thousand, w/v): 0, 0.25, 0.5, 0.75, 1.0 and 1.5. There were 3 trays at each salinity level. The experiment was repeated 3 times. Distilled water was added to the water in the trays when necessary to compensate for evaporation and maintain the initial NaCl concentrations. The WHS agar bearing 7-day-old mycelial growth in each petri dish was cut in half and a total of 6 agar sections were placed into the water in each of the trays at random to minimize any intra- or interdish differences in zoospore production. Approximately 100 larvae (48 h old) were added to the water in each tray immediately following fungal inoculation. The mosquito larvae were fed 50 mg ground rabbit chow and brewers yeast per day. The trays were incubated at 25–27 °C in a 12:12 (L:D) photoperiod. The mosquito larvae were removed from the trays after 72 h and examined microscopically for signs of infection by *L. giganteum*. Most of the larvae infected by the fungus were patent at Stage II infection, while a few were at Stage III (Domnas et al. 1974). The criterion for infection was the visual presence of hyphae and sporangia in the head capsules and thoraces of the larvae.

Statistical methods

Data from the mycelial growth experiments and the bioassay experiments were plotted using probit transformations of the growth rates and infectivi-

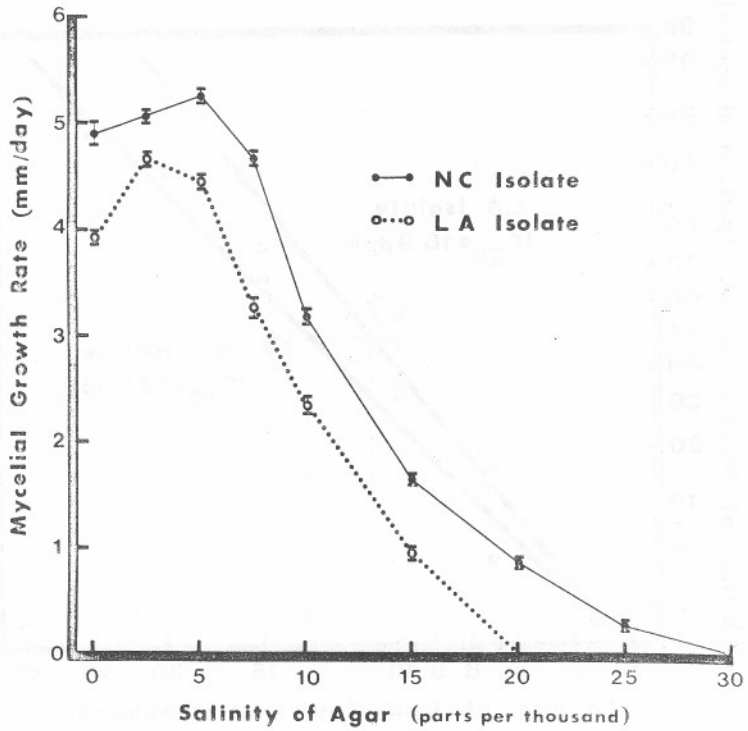

FIG. 1. Mean mycelial growth rates (±SE) of the North Carolina (NC) and Louisiana (LA) isolates of *Lagenidium giganteum* on nutrient (PYG) agar containing various concentrations of NaCl. Each point is based on the mycelial growth in each of 15 petri dishes (5 dishes/experiment, 3 experiments).

ties [each expressed as percentage inhibition (I) compared to the control] and $\log_{10}$ transformations of the NaCl concentrations. The inhibitory concentrations for 50% reduction of mycelial growth rate and percentage infection (IC_{50}), and slope values of the dose-effect lines were calculated for each isolate (Barr et al. 1979). The 95% confidence limits of the IC_{50} values were determined and tests for parallelism of the dose-effect lines were conducted according to the methods of Litchfield & Wilcoxon (1949).

RESULTS

Mycelial growth

The effects of various salinities (NaCl in agar) on the mycelial growth rates of the LA and NC isolates of *L. giganteum* are shown in Fig. 1. The mean mycelial growth rates of the NC isolate were significantly greater ($P < 0.001$) than those of the LA isolate at all salinity levels tested (except 30 ppt), including the control in which the NC isolate grew 1.2× faster than the LA isolate. At the lower salinity levels (2.5 and 5.0 ppt) the mean mycelial growth rates of the 2 isolates were significantly greater ($P < 0.001$) than in the controls. Mycelial growth rates were reduced on agar containing NaCl concentrations of 7.5 ppt or higher. The LA isolate did not grow on agar containing 20 ppt NaCl or higher. The NC isolate grew on agar containing 20 and 25 ppt NaCl, but did not grow on agar containing 30 ppt NaCl.

Based on the transformations of the data to $\log_{10}$ concentration NaCl and probits of the percentage inhibition of mycelial growth, the IC_{50} value (95% confidence limits) for the inhibition of the mycelial growth rate of the LA isolate was 10.9 (9.2 to 13.0) ppt NaCl, while that of the NC isolate was 12.9 (10.9 to 15.3) ppt NaCl (Fig. 2). Overall, the mycelial salinity tolerances of the 2 isolates were similar, since the IC_{50} values and the slopes of the dose-effect lines (LA = 7.1, NC = 5.9) were not significantly different ($P > 0.05$).

Zoospore production and infectivity

The percentage inhibition of infection of *Ae. taeniorhynchus* larvae by the LA and NC isolates of *L. giganteum* in water containing various concentrations of NaCl are shown in Fig. 3. As salinity of the water increased, the ability of each isolate to infect mosquito larvae decreased in relation to the

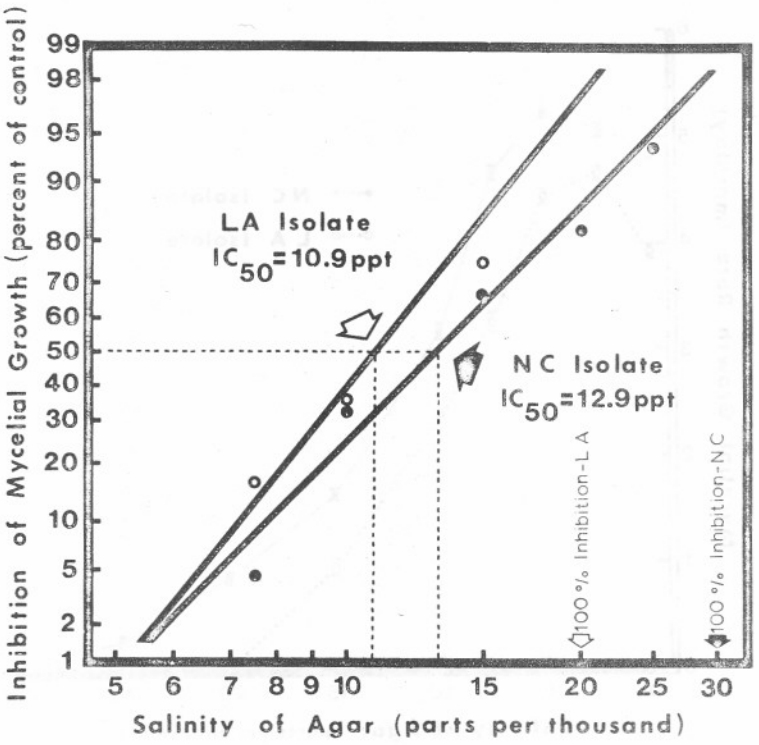

FIG. 2. Percentage inhibition (probit scale) of mycelial growth rates (mm/day) of the North Carolina (NC) and Louisiana (LA) isolates of *Lagenidium giganteum* on nutrient (PYG) agar containing various concentrations of NaCl (log scale). Each point is based on the mean percentage inhibition of mycelial growth (relative to that on nutrient agar containing no NaCl) on each of 15 petri dishes (5 dishes/experiment, 3 experiments).

percentage infection in their respective controls (NC = 92.6 ± 4.0, LA = 49.1 ± 13.7). The IC_{50} values (95% confidence limits) for the inhibition of infection of mosquito larvae by the LA and NC isolates were 0.52 (0.50 to 0.55) and 0.55 (0.53 to 0.58) ppt NaCl, respectively. These IC_{50} values and the slopes of the dose-effect lines (LA = 7.9, NC = 8.1) of the 2 isolates were not significantly different ($P > 0.05$). Microscopic observations of agar sections in the various treatments showed that NaCl adversely affected the production of zoospores. At 18 h postinoculation, agar sections in the control water bearing the NC isolate had a mean of 12.8 ± 0.7 ($n = 20$) vesicles per mm^2 (surface area) as well as many discharge tubes and viable, swimming zoospores. In contrast, vesicles, discharge tubes, and zoospores were not observed on agar slices in water containing NaCl concentrations of 0.5 ppt and above. In repeated microscopic observations of fungus of both strains from these test waters during the experiments, no evidence of zoosporogenesis was found in water containing NaCl concentrations at or above 0.5 ppt. The bioassay with mosquito larvae was more sensitive in detecting zoosporogenesis than direct observation, since some mosquito larvae became infected by *L. giganteum* in water containing NaCl concentrations of 0.5 and 1.0 ppt. At 1.5 ppt NaCl there was complete (100%) inhibition of both zoosporogenesis and infection of mosquito larvae in each isolate of *L. giganteum*.

Comparison of the IC_{50} values of mycelial inhibition (Fig. 2) with those of inhibition of infection (Fig. 3) for *L. giganteum* shows that zoosporogenesis was ca. 22× more sensitive to salinity than was mycelial growth under these test conditions. The slopes of the dose-effect lines for inhibition of mycelial growth (Fig. 2) and for the inhibition of infection (Fig. 3) in each isolate of *L. giganteum* were, however, not significantly different ($P > 0.05$). Thus, because the 2 dose-effect lines of each isolate are parallel, the degree to which mycelial growth and zoosporogenesis are inhibited by salinity is similar within each isolate of *L. giganteum*.

DISCUSSION

These results indicate that moderate NaCl concentrations inhibit *L. giganteum* mycelial growth,

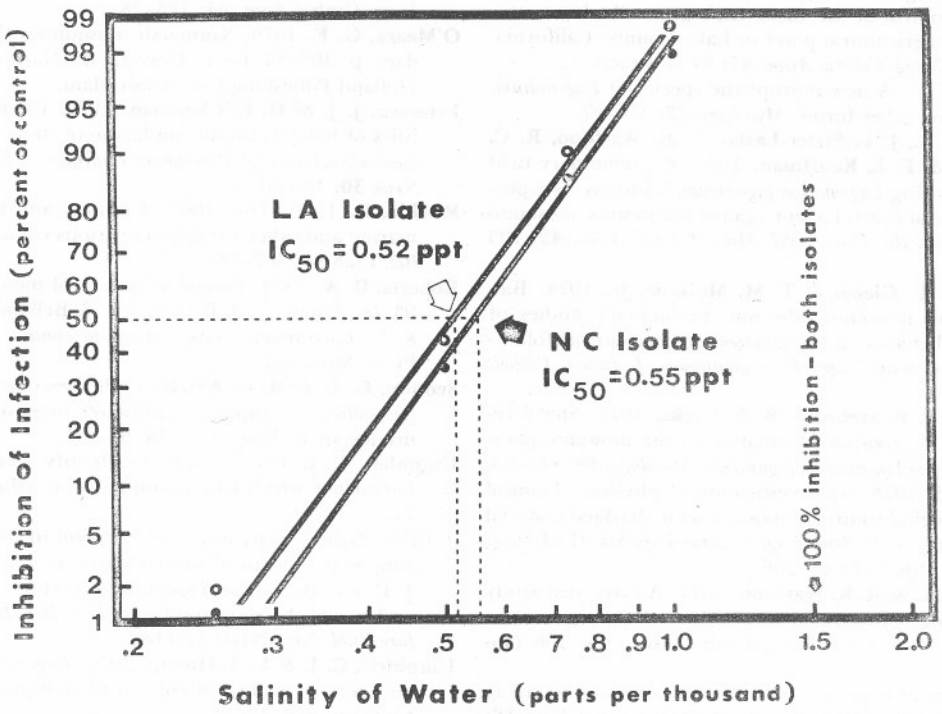

FIG. 3. Percentage inhibition (probit scale) of infection of *Aedes taeniorhynchus* larvae by the North Carolina (NC) and Louisiana (LA) isolates of *Lagenidium giganteum* in water containing various concentrations of NaCl (log scale). Each point represents the mean percentage inhibition of infection (relative to that in distilled water containing no NaCl) of ca. 100 larvae in each of 3 trays, replicated 3 times.

and that low NaCl concentrations inhibit zoosporogenesis and infection in mosquito larvae by *L. giganteum*. In field tests conducted in rice field seepage ditches using *L. giganteum* against *Cx. tarsalis*, McCray et al. (1973b) reported that 100% of the mosquito larvae collected from water with 0.5 ppt NaCl were infected, while 8.8% were infected in water with 7.2 ppt NaCl. McCray et al. (1973b) also reported, however, that according to their laboratory data (unpubl.) the efficacy of *L. giganteum* should have been severely reduced under saline conditions. The results of our study verify this statement, in that given a sufficient inoculum, 100% infectivity could be achieved at 0.5 ppt NaCl; however, the fungus should not have been able to produce zoospores and infect mosquito larvae in water containing 7.2 ppt NaCl. Ritchie (1959) studied the effects of salinity on marine and other fungi and concluded that, in general, the laboratory responses of fungi were correlated with their fitness for their natural habitats. Thus, the inability of *L. giganteum* to infect mosquito larvae under most saline conditions in the laboratory is understandable, since the fungus has only been isolated from freshwater habitats. The mosquito-producing waters of salt marshes can have salinities that range from 0.6 to 20.2 ppt NaCl (Peterson & Chapman 1970), and the water in pools in coastal diked, dredged-material disposal areas can have NaCl concentrations ranging from 10.6 to 43.6 ppt (Ezell 1978). Thus, the 2 strains of *L. giganteum* included in our experiments appear to be unsuitable for use as biocontrol agents for mosquitoes in saline habitats. Their use should not be ruled out completely, however, until field tests have been conducted in various coastal areas. There may be coastal habitats with unusually low salinity levels as a result of periodic flooding with rainwater.

LITERATURE CITED

Axtell, R. C. 1979. Principles of integrated pest management (IPM) in relation to mosquito control. *Mosq. News* **39:** 709–18.

Barr, A. J., J. H. Goodnight, J. P. Sall, W. H. Blair & D. M. Chilko. 1979. *SAS user's guide.* SAS Institute, Raleigh, N.C. 494 p.

Briggs, J. D., ed. 1975. Biological regulation of vectors: the saprophytic and aerobic bacteria and fungi. *U.S. Dep. Health, Educ. Welfare Publ.* No. (NIH) 77-1180. 174 p.

Brown, J. K. & R. K. Washino. 1977. Developments in research with the Clear Lake gnat *Chaoborus astictopus* in relation to the fungus *Lagenidium giganteum*. *Proc. Calif. Mosq. Control Assoc.* **45:** 106 (abstract).

1979. Evaluating the fungus *Lagenidium giganteum* for the biological control of the Clear Lake gnat, *Chaoborus asctictopus,* in an agricultural pond in Lake County, California. *Proc. Calif. Mosq. Control Assoc.* **47:** 37 (abstract).

Couch, J. N. 1935. A new saprophytic species of *Lagenidium,* with notes on other forms. *Mycologia* **27:** 376–87.

Christensen, J. B., J. L. Fetter-Lasko, R. K. Washino, R. C. Husbands & E. E. Kauffman. 1977. A preliminary field study employing *Lagenidium giganteum,* a fungus, as a possible biological control agent against the pasture mosquito *Aedes nigromaculis. Proc. Calif. Mosq. Control Assoc.* **45:** 105 (abstract).

Domnas, A., P. E. Giebel & T. M. McInnis, Jr. 1974. Biochemistry of mosquito infection: preliminary studies of biochemical change in *Culex pipiens quinquefasciatus* following infection with *Lagenidium giganteum. J. Invert. Pathol.* **24:** 293–304.

Domnas, A. J., J. P. Srebro & B. F. Hicks. 1977. Sterol requirement for zoospore formation in the mosquito-parasitizing fungus *Lagenidium giganteum. Mycologia* **69:** 875–86.

Ezell, B. W., ed. 1978. An investigation of physical, chemical and/or biological control of mosquitoes in dredged material disposal areas. *U.S. Army Eng. Waterw. Exp. Stn. (Vicksburg, Miss.) Tech. Rep.* D-78-48. 266 p.

Fetter-Lasko, J. L. & R. K. Washino. 1977. A three year study of the ecology of *Lagenidium* infections of *Culex tarsalis* in California. *Proc. Calif. Mosq. Control Assoc.* **45:** 106 (abstract).

1978. Patterns of experimental infection of mosquito larvae by *Lagenidium giganteum. Proc. Calif. Mosq. Control Assoc.* **46:** 90 (abstract).

Glenn, F. E., Jr & H. C. Chapman. 1978. A natural epizootic of the aquatic fungus *Lagenidium giganteum* in the mosquito *Culex territans. Mosq. News* **38:** 522–24.

Jaronski, S. T. & R. C. Axtell. 1982. Effects of organic water pollution on the infectivity of the fungus *Lagenidium giganteum* (Oomycetes: Lagenidiales) for larvae of *Culex quinquefasciatus* (Diptera: Culicidae): field and laboratory evaluation. *J. Med. Entomol.* **19:** 255–62.

Litchfield, J. T., Jr & F. Wilcoxon. 1949. A simplified method of evaluating dose-effect experiments. *J. Pharmacol. Exp. Ther.* **96:** 99–113.

McCray, E. M., Jr, C. J. Umphlett & R. W. Fay. 1973a. Laboratory studies on a new fungal pathogen of mosquitoes. *Mosq. News* **33:** 54–60.

McCray, E. M., Jr, D. J. Womeldorf, R. C. Husbands & D. A. Eliason. 1973b. Laboratory observations and field tests with *Lagenidium* against California mosquitoes. *Proc. Calif. Mosq. Control Assoc.* **41:** 123–28.

O'Meara, G. F. 1976. Saltmarsh mosquitoes (Diptera: Culicidae). p. 303–33. *In:* L. Cheng, ed., *Marine insects.* North-Holland Publishing Co., Amsterdam.

Petersen, J. J. & H. C. Chapman. 1970. Chemical characteristics of habitats producing larvae of *Aedes sollicitans, Aedes taeniorhynchus,* and *Psorophora confinnis* in Louisiana. *Mosq. News* **30:** 156–61.

Ritchie, D. 1959. The effect of salinity and temperature on marine and other fungi from various climates. *Bull. Torrey Bot. Club* **85:** 367–73.

Roberts, D. A. 1974. Fungal infections of mosquitoes. p. 143–93. *In:* Aubin, A., J. P. Bourassa, S. Belloncik, M. Pellissier & E. Lacoursière, eds., *Mosquito control.* Univ. Quebec Press, Montreal.

Scotton, G. L. & R. C. Axtell. 1979. *Aedes taeniorhynchus* and *Ae. sollicitans* (Diptera: Culicidae) oviposition on coastal dredge spoil. *Mosq. News* **39:** 97–110.

Umphlett, C. J. 1973. A note to identify a certain isolate of *Lagenidium* which kills mosquito larvae. *Mycologia* **65:** 970–71.

1975. Pathogenicity as a consideration in the systematics of fungus pathogens of invertebrates. p. 125–34. *In:* Briggs, J. D. ed., Biological regulation of vectors: the saprophytic and aerobic bacteria and fungi. *U.S. Dep. Health Educ. Welfare Publ.* No. (NIH) 77-1180.

Umphlett, C. J. & C. S. Huang. 1970. *Lagenidium culicidum* as an agent of biological control of mosquitoes. *Bull. Assoc. Southeast. Biol.* **17:** 68.

1972. Experimental infection of mosquito larvae by a species of the aquatic fungus *Lagenidium. J. Invert. Pathol.* **20:** 326–31.

Vorgetts, J., Jr, W. B. Ezell, Jr & J. E. Campbell. 1980. Species composition of mosquitoes produced in dredged material, wildlife management, and natural saltmarsh habitats of the South Carolina coast. *Mosq. News* **40:** 501–06.

Washino, R. K., E. M. McCray, C. J. Umphlett & J. F. Lasko. 1975. Recovery of *Lagenidium giganteum,* an aquatic fungal parasite of mosquitoes, two years after field introduction. *Proc. N.J. Mosq. Extermination Assoc.* **62:** 125 (abstract).

Washino, R. K., J. L. Fetter, C. K. Fukushima & K. Gonot. 1976. The establishment of *Lagenidium giganteum,* an aquatic fungal parasite of mosquitoes, three years after field introduction. *Proc. Calif. Mosq. Control Assoc.* **44:** 52 (abstract).