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Experimental Avian Botulism Studies on Sewage Oxidation Ponds in Utah

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EXPERIMENTAL AVIAN BOTULISM STUDIES ON SEWAGE OXIDATION PONDS IN UTAH

by

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Daniel W. Moulton

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Experimental Avian Botulism Studies on

Sewage Oxidation Ponds in Utah

by

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Utah State University, 1975

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Department: Wildlife Science

The objective of this study was to field-test the "microenvironment concept" of avian botulism epizootiology. The "microenvironment concept" hypothesizes that aquatic invertebrate carcasses may serve both as a substrate for toxin production by Clostridium botulinum type C in nature and as a vehicle for toxin transmission to waterbirds. This concept has become the generally accepted, but inadequately tested, working hypothesis of type C botulism epizootiology.

This study attempted to experimentally induce type C botulism in sentinel flocks of mallard ducks (Anas platyrhynchos) and American coots (Fulica americana) on sewage oxidation ponds in northern Utah. The three experimental oxidation ponds were inoculated with Cl. botulinum type C (strain X220B2) endospores in June, 1974. Aquatic invertebrate populations were monitored throughout the summer. Rotenone was used in August in two of the experimental ponds (one pond served as a control) to kill aquatic invertebrates and thereby

provide a large amount of substrate for clostridial growth and toxin production. No botulism was detected among the sentinel birds even though they routinely ingested invertebrate carcasses. None of the samples of dead invertebrates collected from the experimental ponds contained detectable (in white mice) botulinum toxin.

It was concluded that the "microenvironment concept," as it now stands, cannot always be a sufficient explanation of how type C botulism epizootics are initiated in nature. Microbiological experiments designed to determine why the invertebrate carcasses collected from the study ponds contained no botulinum toxin were started and are now ongoing. Early results indicate that Cl. botulinum (X220B2) cells may not normally be able to effectively compete (at least in terms of toxin production) with other microorganisms present in the sewage ponds. It is suggested that the initiation of a type C botulism epizootic in nature may require the alleviation of the inhibitory effects of other putrefactive microorganisms upon clostridial metabolism and/or toxin. This hypothesis is highly speculative and requires much further experimentation.

(48 pages)

INTRODUCTION

The anaerobic, spore-forming bacillus Clostridium botulinum type C is a saphrophytic soil organism having a nearly ubiquitous distribution in western North America. It was nearly 50 years ago that "western duck sickness" was shown to result from the ingestion of the toxin of Cl. botulinum type C (Giltner and Couch, 1930; Kalmbach, 1930). Avian botulism has been recognized as a significant cause of waterfowl mortality in North America since 1910 when Wetmore (1915) focused attention on the problem in the western U. S. The ecology of the disease, i. e., the natural substrates utilized by the bacterium for growth and toxin production and the natural modes of toxin transmission to birds, has not yet been clearly defined.

Research on avian botulism has been extensively reviewed by several authors. Sciple (1953) summarized information on research up to 1950. Kalmbach, in 1968, provided a historical sketch of the incidence of the disease in wild birds and related research. Enright (1971) is the most recent and comprehensive review of research on type C botulism. Due to the ecological complexity of the problem, studies designed to delineate the epizootiology of type C botulism have been few. In 1934 Kalmbach and Gunderson suggested that aquatic invertebrates might serve both as a substrate for toxin production and as a vehicle for toxin transmission to birds. This hypothesis was supported and elaborated by the "microenvironment concept" of Bell, Sciple and Hubert (1955) which stated: "(1) that Cl. botulinum type C

germinates, reproduces and synthesizes its toxin in small discrete particulate substances, possibly invertebrate carcasses; (2) that the particulate substances are in no wise dependent upon the ambient medium for nurture of the bacteria, but contain all of the requisites within them; and (3) that the toxin is probably in the bacteria which reside in the particulate materials, rather than in the form of soluble, freely diffused toxin." The invertebrate-carcass microenvironment concept has become the generally accepted working hypothesis of type C botulism epizootiology (Enright, 1971). However, as Enright pointed out, the hypothesis has not been verified, or even adequately tested, in nature. Jensen and Allen (1960) demonstrated a possible correlation between the abundance of aquatic invertebrates and botulism epizootics at the Bear River Migratory Bird Refuge (BRBR) in northern Utah. The BRBR study indicated that botulism epizootics were preceded by rapid declines in one or more of the predominant aquatic invertebrate populations, and presumably a concomitant increase in the number of invertebrate carcasses available as a substrate for clostridial growth and toxin production. Hunter (1970) has emphasized the possible importance of drowned terrestrial invertebrates associated with flooded land, and maggots associated with vertebrate carcasses as additional major sources of toxin in nature.

The objective of this study was to field-test the invertebrate-carcass microenvironment hypothesis of type C botulism epizootiology. An attempt was made to induce botulism experimentally in sentinel flocks of mallard ducks

(Anas platyrhynchos) and American coots (Fulica americana) on sewage oxidation ponds in northern Utah.

STUDY AREA

The study was conducted during the summers of 1973-74 on the Richmond City sewage oxidation ponds in northern Utah. The system consists of four ponds with maximum surface areas ranging from 2.2 to 3.8 ha (Fig. 1). Pond 1 is the primary pond, pond 2 the secondary, pond 3 the tertiary, and pond 4 the quaternary pond. All ponds, except pond 4, have outflow gates with openings at 0.9 and 1.8 m. The system is closed and produces no effluent. Water loss by evapotranspiration exceeds inflow into the system in the summer months. The system began operation in 1973 and only pond 1 had water in it during that summer. The preliminary study of pond 1 in 1973 was to determine the feasibility of the experiments which were conducted on ponds 2, 3, and 4, in 1974. Water levels in the ponds during the 1974 study period are shown in Fig. 2.

In 1974 pond 2 supported no submerged or emergent vegetation. The dominant primary producers were green (Chlorophyta) and blue-green (Cyanophyta) algae. The Chlorophyte genera Scenedesmus, Uva, Pediastrum, Ankistrodesmus, Pandorina, and Closteridium; and the Cyanophyte genera Microcystis, Oscillatoria, and Spirulina were present. Euglenoids (Euglenophyta) and diatoms (Chrysophyta) were also present. Pond 3 was characterized by the submerged genus Potamogeton (pondweeds); the emergent genera Sparganium (bur-reed), Juncus (wire grass), and Typha (cattails); and green

Figure 1. The Richmond sewage oxidation ponds in northern Utah used for botulism study, 1973-74.

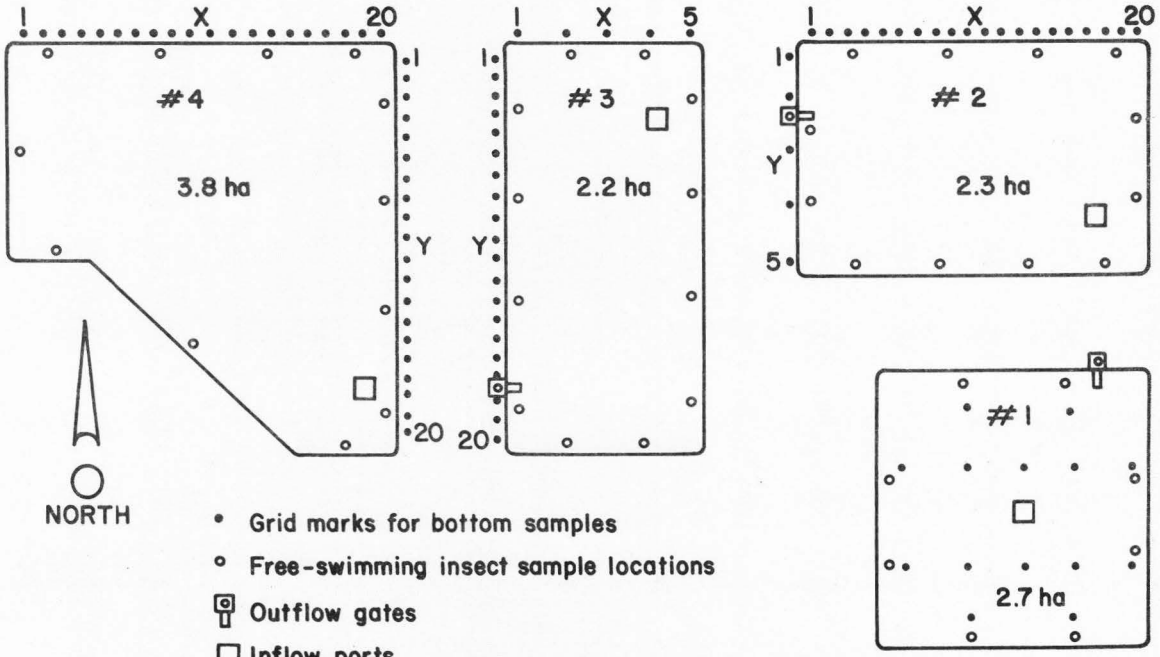
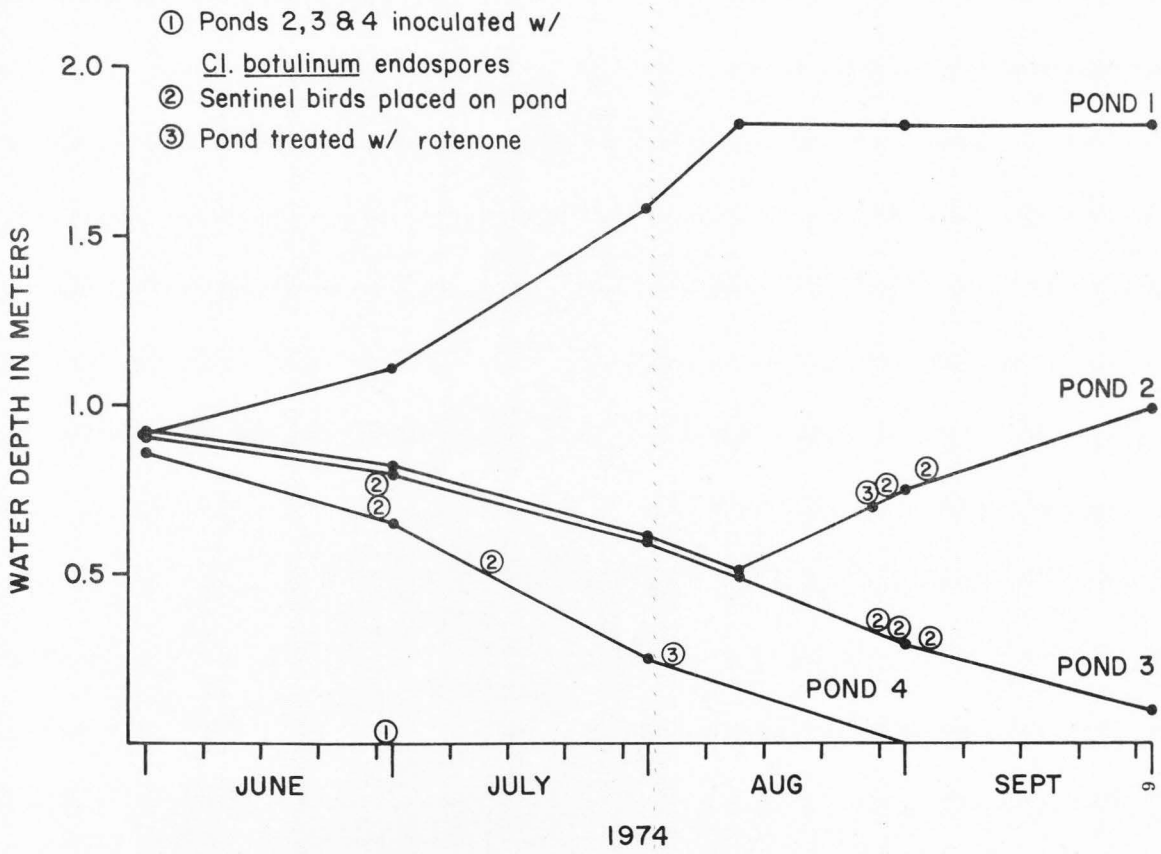

1
X
20
#4
3.8 ha
Y
1
20
NORTH
1
X
5
#3
2.2 ha
Y
1
20
1
X
20
#2
2.3 ha
Y
1
5
#1
2.7 ha
• Grid marks for bottom samples
o Free-swimming insect sample locations
Outflow gates
Inflow ports

Figure 2. Water levels in the Richmond sewage oxidation ponds during the 1974 botulism study.


① Ponds 2,3 & 4 inoculated w/ Cl. botulinum endospores
② Sentinel birds placed on pond
③ Pond treated w/ rotenone
WATER DEPTH IN METERS
2.0
1.5
1.0
0.5
POND 1
POND 2
POND 3
POND 4
JUNE
JULY
AUG
SEPT
1974

filamentous algae of the genus Cladophora. Pond 4 was largely dominated by green filamentous algae of the genus Cladophora.

These ponds were chosen for study primarily because they represented newly-created aquatic habitats in which we expected to find abundant aquatic invertebrate populations. In Utah marshes, the inundation of land which has been dry for a year or more can produce large numbers of low trophic-level aquatic insects, especially Chironomidae and Corixidae, during the first summer after reflooding (McKnight and Low, 1969). This phenomenon of aquatic insect abundance appears also to be true of new sewage oxidation ponds in Utah (Dodge, 1972; Kemmerer, 1970). Also, type C botulism epizootics in waterbirds have occurred on sewage oxidation ponds in Logan, Utah, in 1969-71 (Dodge, 1972), the Sacramento (Hunter, 1970) and San Joaquin (Jensen, 1975) Valleys of California, and in Auckland, New Zealand, in 1971-72 (Martinovich et al., 1972). The Utah epizootics occurred on the Logan City oxidation ponds 19 km south of the Richmond ponds. The most serious epizootic on the Logan ponds (ca. 2000 birds lost) occurred in the late summer and fall of 1969 when the system was new.

MATERIALS AND METHODS

Water Quality

The physicochemical parameters measured were: pH; temperature; dissolved oxygen concentration (ppm); and turbidity (Jackson Turbidity Units). These parameters were decided upon as the ones most likely to affect clostridial growth and toxin production and/or stability. Water samples were taken with a Kemmerer water bottle[1] lowered from a boat. All measurements were made in the field with a portable water testing kit.[2] Measurements were made at irregular intervals over the period of study.

Endospores

Ponds 2, 3 and 4 were inoculated with type C endospores on 6/29/74. The strain of Cl. botulinum from which the spores were derived was X220B2 type C_{alpha}, a strain originally isolated from the liver of a BRBR duck. The toxicity of the cultures used was about 2×10^5 mouse intraperitoneal (i.p.) $LD_{50's}$/ml. The bacteria were cultured for 5 days at 37 C in 32 l of Lactalysate-Yeast Autolysate (LYA) broth.[3] The suspension was centrifuged

[1] Wildlife Supply Co., Saginaw, MI. 48602.

[2] Model DR-B, Hach Chemical Co., Ames, Iowa 50010.

[3] Lactalysate (Baltimore Biological Laboratories), 3.0%; Yeast Autolysate (Albimi Laboratories, Flushing, N.Y.), 2.0%; Glucose, 0.5%; Na Citrate, 0.35%; pH adjusted to 7.3-7.5.

to concentrate vegetative cells and endospores. The yield of 800 ml was heat shocked at 80 C for 10 min to destroy vegetative cells and residual toxin. The endospore suspension contained about 4050 spores/ml, i.e., the number of spores which germinated, when cultured. Pond 4 received 300 ml of suspension and ponds 2 and 3 received 250 ml each. The spores were spread over the entire surfaces of the ponds but the edges were covered especially well because invertebrate carcasses tend to accumulate there.

The inoculation of ponds 2, 3 and 4 with X220B2 endospores was done primarily to insure that a negative result, i.e., no botulism in the sentinel birds, could not be attributed to an absence of the etiologic agent. However, it seems likely that any aquatic habitat in northern Utah, an area where type C botulism is enzootic, would have a natural population of Cl. botulinum type C.

Invertebrate Populations

Benthic

In 1973 bottom sampling locations on pond 1 were approximately as shown in Fig. 1. One set of samples, one sample near each of the 14 locations, was taken each week during the study. In 1974 the sampling procedure for ponds 2, 3 and 4 (1 was not studied) was altered considerably. Grid marks were placed along two edges of each pond (Fig. 1). These marks formed X-Y grids of 100 possible sampling points (X-Y coordinates) for ponds 2 and 3, and 263 possible sampling points for pond 4. A computer program was used to randomly select 20 sampling points from the possible points formed by the

grids. Sets of 20 bottom samples were taken from each pond at approximately weekly intervals. The computer program was used to generate new sets of 20 randomly selected X-Y coordinates for each set of samples taken. The irregular shape of pond 4 was programmed into the computer resulting in 263 possible sampling points on the 20 x 20 point X-Y grid.

Bottom samples were taken from a boat with a standard Ekman dredge.[4] This device takes a 232 cm^2 (6 in^2) sample of the bottom substrate. Samples were placed in a wire-bottomed wash bucket[5] and washed to remove mud. Invertebrates were placed in 100 ml plastic specimen cups[6] and preserved with 70% ethanol or 4% formalin. In samples that contained large amounts of plant material the preservative was 4% formalin mixed with the red dye Phloxine B.[7] This dye causes animal tissue to become stained bright red whereas plant tissues do not absorb the dye. This was helpful in separating the animal from the undesired plant material. The invertebrates were classified, counted, oven dried at 70 C for 48 h, and weighed to the nearest 0.1 mg on an electronic analytical balance.[8] Invertebrate sample means and 90% confidence intervals (based on t statistic) were computer generated.

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[4]Wildlife Supply Co., Saginaw, MI. 48602.

[5]Wildlife Supply Co., Saginaw, MI. 48602.

[6]Falcon Plastics, Oxnard, CA. 93030.

[7]J. T. Baker Chemical Co., Phillipsburg, N.J. 08865.

[8]Sartorius Model 2500.

Free-swimming

Samples of free-swimming invertebrates were taken only around the edges of the ponds. The approximate sampling locations are shown in Fig. 1. In 1973 sample sets (N = 8) were taken at approximately weekly intervals from pond 1. In 1974 sample sets (N = 12) were taken from each pond (2, 3 and 4) at approximately weekly intervals.

Samples were taken by pushing a plastic bucket rapidly into the water to create a suction which drew in a water sample of constant volume (2 U.S. gallons or 7.57 l). The samples were poured through a standard Wisconsin plankton net[9] and the invertebrates preserved in 70% ethanol.

The invertebrates were classified and counted in 1973 but not counted in 1974 due to the large numbers of small Corixidae in many samples. All samples were dried at 70 C for 48 h and weighed to the nearest 0.1 mg on an electronic analytical balance.

Rotenone Treatment

Rotenone (5%) was used to kill invertebrates in ponds 2 and 4 in 1974. In both cases preliminary tests were run in order to determine what concentration of rotenone would be required to cause high mortality in invertebrate populations. These tests consisted of six jars each containing 3500 ml of water from the pond to be treated. Approximately equal numbers of live invertebrates,

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[9]Wildlife Supply Co., Saginaw, MI. 48602.

both benthic and free-swimming forms, from the pond to be treated were placed in each jar. One jar served as control and received no treatment. Each of the other five jars received one of the following rotenone treatments: 0.007 ml = 2 ppm; 0.0175 ml = 5 ppm; 0.035 = 10 ppm; 0.0525 = 15 ppm; or 0.07 ml = 20 ppm. The results indicated that a rotenone concentration $\geq$ 5 ppm would cause high mortality in both benthic and free-swimming invertebrate populations within 24 h. Rotenone was used because of its low oral and dermal toxicity for vertebrates, viz., sentinel birds and investigators, and because it does not persist for extended periods in the environment.

Pond 4 was treated with 113.55 l (30 gallons) of rotenone (Chem Fish Regular)[10] on 8/3/74. At that time pond 4 held approximately 12,353 m^3 of water and the rotenone concentration attained was about 9 ppm. Pond 2 was treated with 132.5 l (35 gallons) of rotenone (30 gallons of Noxfish Fish Toxicant[11] and 5 gallons of Chem Fish Regular) on 8/27/74. At that time pond 2 held approximately 13,573 m^3 of water and the rotenone concentration attained was about 9.5 ppm. Pond 3 served as a control. Rotenone was applied to the ponds with a pump powered by a small gasoline engine. The pump was placed in a boat and the intake hose placed in a plastic tub in which rotenone was mixed with pond water before being sprayed onto the surface of the ponds. The boat was pulled about manually on pond 4, but a small outboard engine was used on pond 2 and this aided in mixing the rotenone into the pond.

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[10] Chemical Insecticide Corp., Edison, N.J. 08817.

[11] S. B. Penick and Co., New York, N. Y. 10007.

Sentinel Birds

Mallard ducks and American coots were placed on pond 1 in 1973 and ponds 2, 3 and 4 in 1974. This insured the continual presence of waterfowl at risk of ingesting botulinum toxin. The sentinel flock on pond 1 consisted of 78 mallards and 24 coots as of 8/25/73. This flock was intended to: (1) determine if the pond would sustain the birds; and (2) monitor the pond for a possible spontaneous botulism epizootic such as occurred on the Logan ponds in 1969. Mallards were from the experimental flock maintained at the Bear River Research Station (BRRS). An airboat was used to capture wild coots on the BRBR. All birds were rendered flightless by clipping the primary flight feathers of one wing. All birds were banded. A chicken-wire fence about 1 m high was erected around pond 3 in 1974 to prevent birds from walking between control and treatment ponds. The exact numbers and dates of bird releases are given in Table 1.

Three mallards were sacrificed in 1974 to check on food habits. Two birds were taken from the pond 2 flock, one on 9/10/74 and one on 10/3/74, and one from pond 3 on 9/10/74.

Microbiology

Samples of dead invertebrates (mixed with other materials) were collected from pond 2 at various times after they were killed by the rotenone treatment of 8/27/74 (Table 2). Samples were placed in 100 ml plastic

Table 1. Release of sentinel birds on Richmond sewage oxidation ponds, 1973-74 botulism study

Pond No.	Release Date	Mallards	Am. Coots
		1973	
1	8/6		12[a]
	7		8[a]
	9	20	
	13	18	
	20		2
	23	40	
	24		1
	25		21
		—	—
Totals		78	44
		1974	
2	8/28	12	8[a]
	29	11	
	9/3	1 (from #4)	
		—	—
Totals		24	8
3	6/28	13	
	8/28		20
	30	12 (from #4)	
	9/3	7 (from #4)	
		—	—
Totals		32	20
4	6/28	12	
	7/12	12	
	13		14[a]
		—	—
Totals		24	14

[a]These birds walked away from the ponds immediately after release.

Table 2. Samples of invertebrates killed by rotenone in Richmond sewage oxidation pond 2, 1974 botulism study

Sample No.	Sample Frozen	Sample Location	Date Collected
1	+	X1 Y1, Surface	9/9/74
2	+	X2 Y1, Bottom	9/9/74
3	+	X10 Y1, Surface	9/9/74
4	+	X10 Y1, Bottom	9/9/74
5	+	X20 Y1, Surface	9/9/74
6	+	X20 Y1, Bottom	9/9/74
7	+	X1 Y1, Bottom	9/16/74
8	+	X10 Y1, Bottom	9/16/74
9	+	X20 Y1, Bottom	9/16/74
10	+	X1 Y1, Bottom	10/2/74
11	+	X20 Y1, Bottom	10/2/74
12	-	X1 Y1 (A), Bottom	11/19/74
13	-	X1 Y1 (B), Bottom	11/19/74
14	-	X1 Y1 (C), Bottom	11/19/74

specimen cups and all but those collected on 11/19/74 were frozen. The procedures for the experiments done with these samples are outlined below.

Experiment 1 (Toxicity of sewage pond samples)

(1) Samples were gently stirred (two or three rotary motions to avoid introducing air) with a stainless steel spatula. After allowing several seconds for the larger particles to settle, 2.0 ml volumes of suspension were removed with a pipette. If invertebrate carcasses (usually corixids) were visible in the

sample, several of them were added to the 2.0 ml sample and crushed in a small mortar with a minimum of stirring.

(2) One-half ml volumes of the resultant suspension were inoculated into two tubes of LYA broth. LYA broth had been autoclaved for 20 min at 15 lb/in^2, tubed in 15 ml volumes, and placed in a boiling waterbath for 10 min just prior to use. After inoculation the tubes were incubated at 34 C for 5 days and tested for toxicity.

(3) The remainder of the suspension was diluted 1:5 in gelatin-phosphate buffer (GPB)[12] prior to toxicity tests.

(4) Toxicity tests were run after the dilutions (1:10 for cultures and 1:5 for unincubated material because toxin levels might be low) were made. Volumes of 0.1 ml were injected i.p. into two 15-20 g white mice (U. S. P. H. S. Rocky Mt. Laboratory strain), one of which was protected with a 0.1 ml (5 IU) i.p. dose of type C antitoxin.[13] In all experiments all mice, except those injected only with X220B2 cultures, were protected from infection with a 0.5 mg i.p. dose of Liquamycin.[14] Mice were observed for 5 days for signs of intoxication or death.

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[12] Bacto Gelatin (Difco Laboratories, Detroit, Mich.), 0.2%; dibasic Na phosphate, 0.4%; pH adjusted to 6.2 with HCl.

[13] Ft. Dodge Laboratories, Ft. Dodge, Iowa 50501 (equine origin).

[14] Chas. Pfizer & Co., Inc., New York, N. Y. 10017 (oxytetracycline hydrochloride).

Experiment 2 (Toxicity of X220B2 cultures grown with sewage pond microbes)

(1) On 1/10/75 0.5 ml volumes of suspension from samples 1, 8, 9, 12, 13 and 14 (Table 2) were each inoculated into two tubes of LYA broth.

(2) One tube of each pair was also inoculated with 0.5 ml of 48-h LYA culture of X220B2. Two tubes of LYA broth were inoculated with 0.5 ml of X220B2 alone.

(3) All tubes were incubated at 34 C for 3 days.

(4) On 1/13/75 toxicity tests were made by i.p. injection of 0.1 ml of each culture into two mice, one protected with antitoxin and one unprotected.

(5) On 1/14/75 subcultures (0.5 ml into 15 ml LYA) were made from all tubes described in (1) and (2). Subcultures were incubated until 1/17/75 and then tested for toxicity.

(6) On 1/17/75 subcultures were made from the 1/14/75 subcultures. These were incubated until 1/21/75 and then tested for toxicity.

(7) On 1/21/75 subcultures were made from the 1/17/75 subcultures. These were incubated until 1/24/75 and then tested for toxicity.

(8) The control for this experiment was X220B2 subcultured alone in LYA broth.

Experiment 3 (Toxin levels of actively growing X220B2 cells grown alone and with sewage pond microbes)

(1) On 3/25/75 four 9.5 ml tubes of LYA broth were each inoculated with 0.5 ml of suspension from sample 12 at 9:00 a.m. and placed in a 34 C incubator.

(2) At 4:00 p.m. two of the tubes and two fresh tubes of LYA broth were each inoculated with 0.5 ml of a 24-h culture of X220B2. This resulted in two tubes of sample 12 alone, two of X220B2 alone, and two with both. The delay in the second inoculation (from 9:00 a.m. to 4:00 p.m.) was to permit the organisms in the refrigerated sewage pond sample to catch up metabolically with the actively growing X220B2 cells.

(3) All tubes were incubated at 34 C for 3 days.

(4) On 3/28/75 toxicity was tested by making serial dilutions of one tube of each culture in GPB. Mice were injected i.p. with 0.1 ml of each dilution. All mice injected with sample 12 were given Liquamycin 1/2 h beforehand.

Experiment 4 (Toxin levels of inactive X220B2 culture grown alone and with sewage pond microbes)

In this experiment an inactive inoculum (X220B2 culture stored at -20 C for 6 years) was used instead of an actively growing culture of X220B2. This procedure was based upon the speculation that the Cl. botulinum cells that initiate an epizootic might be spores rather than actively reproducing vegetative cells.

(1) On 3/27/75 four 9.5 ml tubes of LYA broth were each inoculated with 0.5 ml of suspension from sample 12 at 11:30 a.m. and incubated at 34 C until 4:00 p.m. Then two of the tubes and two fresh tubes of LYA broth were each inoculated with 0.5 ml of a culture of X220B2 that had been inoculated on 4/29/69, incubated until 4/30/69, and then frozen. The culture was thawed just prior to use in this experiment.

(2) On 3/31/75 serial dilutions were made and injections given to mice as in Experiment 3.

RESULTS

Water Quality

The significance of physicochemical features of the ambient medium to botulinum toxin production is not known. In fact, the onset of botulism epizootics has never been clearly correlated with any environmental factor (Jensen and Allen, 1960). Dodge (1972) was unable to find a relationship between the initiation of botulism epizootics and physicochemical parameters of the Logan, Utah, oxidation ponds. Had botulism occurred on one of the treatment ponds, a between-pond comparison of these data (Table 3) might have been useful.

Invertebrate Populations

Benthic

Bottom fauna in the ponds consisted almost entirely of Chironomidae (midge) larvae. Caoborinae larvae were present in small numbers. Fig. 3 shows the chironomid larvae population estimates, in terms of numbers and dry weight, for pond 1 in 1973. Sample size was 14 for all points, and the 90% confidence interval is shown for each mean. The maximum mean number of larvae observed was 462 ± 74 (339 ± 60.3 mg)/232 cm^2 in late summer. That estimate represents a dense chrionomid larvae population compared with populations observed in some Utah marshes (Jensen and Allen, 1960; McKnight and Low, 1969) and oxidation ponds (Dodge, 1972; Kemmerer, 1970).

Table 3. Physicochemical parameters for Richmond sewage oxidation ponds, 1973-74 botulism study

Pond #	Date	Temp. C[a]	pH	O_2 (ppm)	Turbidity (JTU's)
			1973		
1	7/9	24	8.3	2	25
	23	24	8.3	4	20
	8/17	23	8.6	7.5	28
	23	22.5	8.7	7	36
	29	19.5	9.05	13	27
	9/6	19	9.05	11	22
	13	18.5	9.25	13	38
	19	18	9.85	9	22
			1974		
2	6/27	26	8.4	--[b]	78
3		23.5	8.3	--	35
4		23	9.0	--	28
2	7/17	22.5	8.9	9	65
3		23	8.6	2	22
4		22.5	9.0	9	15
2	8/14	17	8.7	< 1	115
3		17	8.8	7	25
4		--	9.7	--	--
2	9/16	19	9.1	11	96
3		17	9.85	12	22
2	9/18	16.5	9.2	15	56
3		17	10.0	12	12

[a] Temp. near surface.
[b] -- = not measured.

Figure 3. Chironomid larvae population estimates for Richmond sewage oxidation pond 1, 1973. N = 14 for all points; 90% confidence interval shown for each $\overline{X}$.

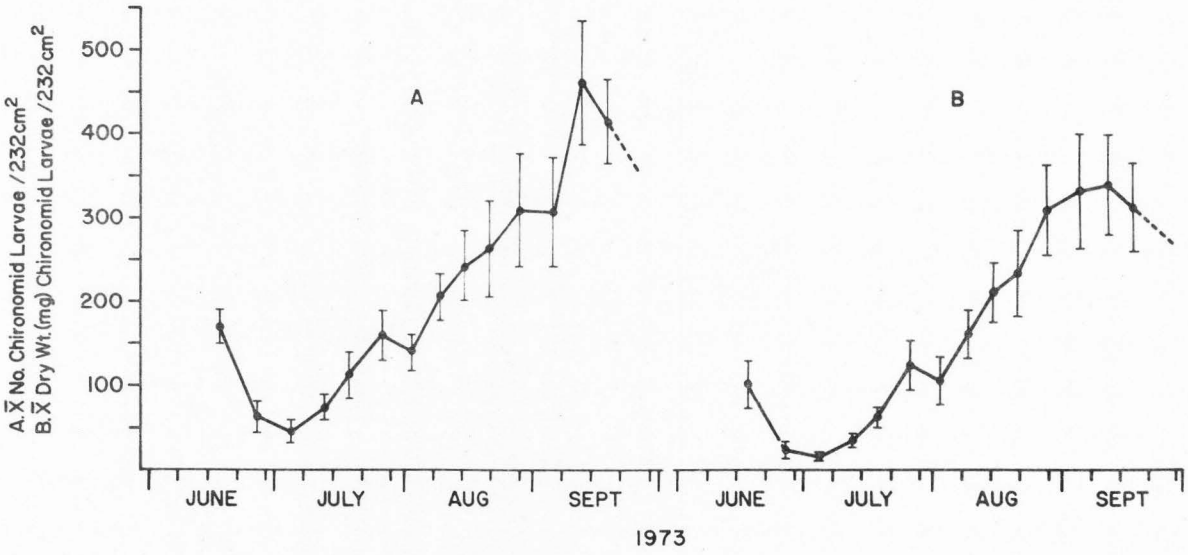

A. X̄ No. Chironomid Larvae /232cm²
B. X̄ Dry Wt.(mg) Chironomid Larvae /232 cm²
500
400
300
200
100
A
B
JUNE
JULY
AUG
SEPT
JUNE
JULY
AUG
SEPT
1973

Figure 4. Chironomid larvae population estimates for Richmond sewage oxidation ponds 2, 3 and 4, 1974. N = 20 for all points; 90% confidence interval shown for each $\overline{X}$.

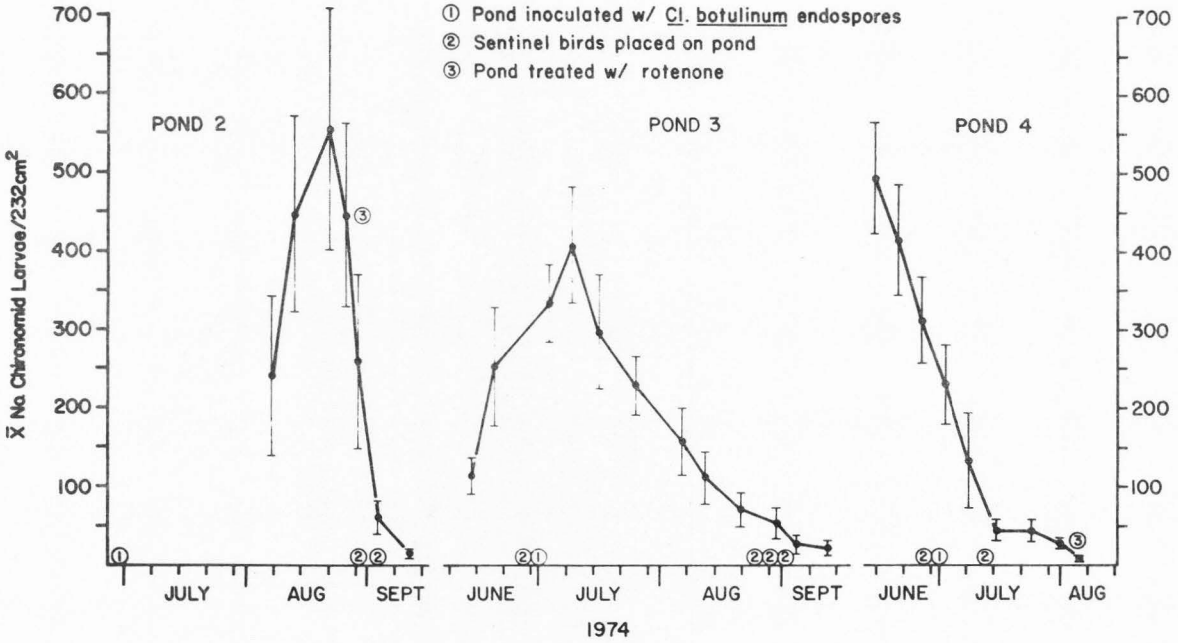
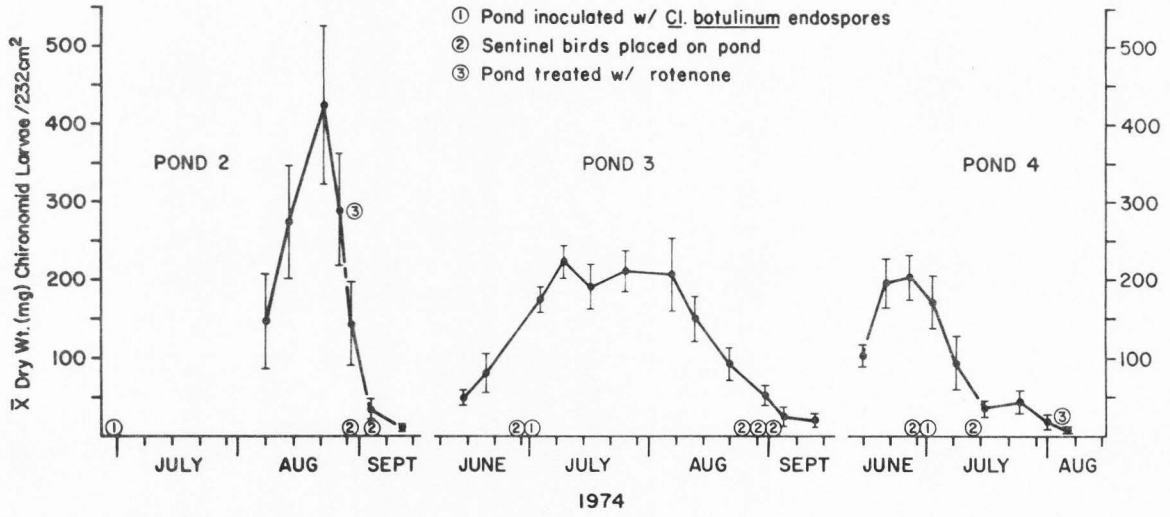

① Pond inoculated w/ Cl. botulinum endospores
② Sentinel birds placed on pond
③ Pond treated w/ rotenone
POND 2
POND 3
POND 4
X̄ No. Chironomid Larvae/232cm²
700
600
500
400
300
200
100
JULY
AUG
SEPT
JUNE
JULY
AUG
SEPT
JUNE
JULY
AUG
1974

Figure 5. Chironomid larvae biomass estimates for Richmond sewage oxidation ponds 2, 3 and 4, 1974. N = 20 for all points; 90% confidence interval shown for each $\overline{X}$.

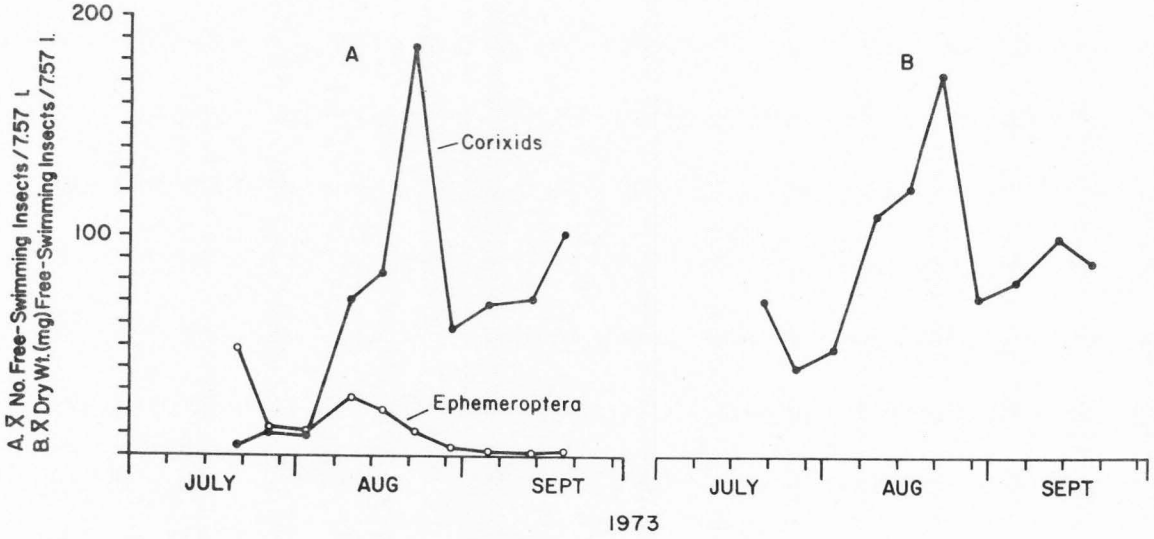

① Pond inoculated w/ Cl. botulinum endospores
② Sentinel birds placed on pond
③ Pond treated w/ rotenone
X̄ Dry Wt. (mg) Chironomid Larvae /232cm²
500
400
300
200
100
POND 2
POND 3
POND 4
JULY
AUG
SEPT
JUNE
JULY
AUG
SEPT
JUNE
JULY
AUG
1974

The chironomid larvae population estimates for ponds 2, 3 and 4 in 1974 are shown in Figs. 4 (numbers) and 5 (dry weight). Sample size was 20 for all points, and the 90% confidence interval is shown for each mean. All three of the new ponds supported abundant chironomid larvae populations at some point during the summer. The maximum mean number of larvae observed in pond 4 was 494 $\pm$ 71 (103.8 $\pm$ 14.2 mg)/232 cm^2 in mid-June. The mean number of larvae in pond 4 decreased steadily as water depth decreased (Fig. 2). The mean number of larvae in pond 3 reached a maximum of 406 $\pm$ 74 (223.6 $\pm$ 21.5 mg)/232 cm^2 in early July and then declined, as in pond 4, as water depth decreased. As the water in ponds 3 and 4 evaporated the pH increased and suspended solids (turbidity) decreased (Table 2). The larval population in pond 2 reached a very high mean density of 554 $\pm$ 154 (424 $\pm$ 101.8 mg)/232 cm^2 in late August. The larvae were not distributed as uniformly over the bottom in pond 2 as in the other two ponds. This resulted in very broad confidence intervals on population estimates.

Free-swimming

The free-swimming invertebrate populations of the ponds were dominated by aquatic Hemiptera. The Corixidae (water boatmen) were by far the most abundant group, and the Notonectidae (back swimmers) were second in abundance. Ephemeroptera (mayfly nymphs) were numerous in pond 1 in 1973 (Fig. 6) and in ponds 3 and 4 in 1974. Aquatic Coleoptera were present but not abundant. Dytiscidae (predaceous water beetles), Hydrophilidae (water

Figure 6. Free-swimming insect population indices for Richmond sewage oxidation pond 1, 1973. N = 8 for all points. B graph is combined dry weight of both groups shown in A.


A. X No. Free-Swimming Insects / 7.57 L
B. X Dry Wt. (mg) Free-Swimming Insects / 7.57 L.
200
100
A
B
Corixids
Ephemeroptera
JULY
AUG
SEPT
JULY
AUG
SEPT
1973

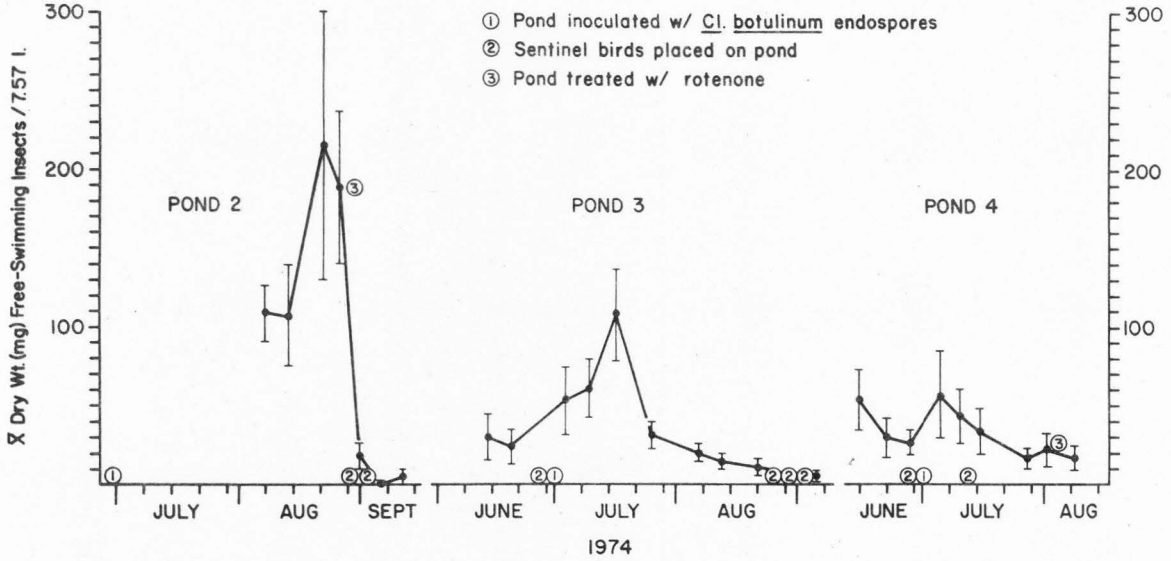
scavenger beetles) and Gyrinidae (whirligig beetles) were present in pond 1 in 1973 and ponds 3 and 4 in 1974. A few Odonata (dragonflies and damselflies) nymphs were taken from ponds 3 and 4. The tiny Cladoceran Daphnia was present in large numbers in certain ponds early in both summers.

The graphs of Figs. 6 and 7 are not population estimates because only the edges of the ponds were sampled. However, these data do provide population indices that show major trends in abundance. Free-swimming insects were counted in 1973 (Fig. 6) but not in 1974 because of the large numbers of small corixids present in all samples from pond 2 prior to the rotenone treatment. Fig. 7 shows the mean dry weights of free-swimming insect samples from ponds 2, 3 and 4 with 90% confidence intervals. Pond 2 supported an abundant population of insects comprised almost entirely of corixids. Ponds 3 and 4 had more varied but less abundant free-swimming insect populations.

Rotenone Treatment

The effects of the rotenone application (8/3/74) on invertebrates in pond 4 are shown in Figs. 4, 5 and 7. Both benthic and free-swimming insect populations were at low densities at the time of treatment. The ratio of dead/living larvae in the last set of bottom samples taken on pond 4 (8/5/74) indicated that approximately 30% of the chironomid larvae were killed by rotenone. The effect of the rotenone upon free-swimming insects, if any, was not obvious. The filamentous algae that choked pond 4 tended to hold the rotenone in localized pockets.

Figure 7. Free-swimming insect biomass indices for Richmond sewage oxidation ponds 2, 3 and 4, 1974. N = 12 for all points; 90% confidence interval shown for each $\overline{X}$.


① Pond inoculated w/ Cl. botulinum endospores
② Sentinel birds placed on pond
③ Pond treated w/ rotenone
X Dry Wt.(mg) Free-Swimming Insects / 7.57 l.
300
200
100
POND 2
POND 3
POND 4
JULY
AUG
SEPT
JUNE
JULY
AUG
JUNE
JULY
AUG
1974

Pond 2 was treated on 8/27/74. Both benthic and free-swimming insect populations were at very high densities at that time (Figs. 4, 5 and 7). The effects of the rotenone treatment were immediate and dramatic. The sharp declines in insect populations in pond 2 shown in Figs. 4, 5 and 7 are the result of a massive die-off. On 8/26/74 the mean number of chironomid larvae in pond 2 was 444 $\pm$ 117 (289.8 $\pm$ 72 mg)/232 cm^2. On 8/29/74, 2 days after the rotenone treatment, the mean number of larvae was 258 $\pm$ 111 (143.9 $\pm$ 53.8 mg)/ 232 cm^2. On 9/3/74, 1 week after rotenone treatment, the mean number of larvae had dropped to 60 $\pm$ 21 (33.8 $\pm$ 12.7 mg)/ 232 cm^2. The declines in numbers of chironomid larvae in ponds 3 and 4 (Fig. 4) do not represent the death of the larvae but rather their removal due to metamorphosis and emergence. This is true also of certain free-swimming forms, e.g., mayfly nymphs. The free-swimming insect population of pond 2 consisted almost entirely of Corixidae and these were virtually wiped out by the rotenone On 8/26/74 the mean dry weight of free-swimming insects was 188.4 $\pm$ 48.6 mg/sample. On 8/31/74, 4 days after rotenone treatment, the mean dry weight of free-swimming insects had dropped to 17.9 $\pm$8.8 mg/sample.

Sentinel Birds

The sentinel flock placed on pond 1 in 1973 demonstrated that a considerable number of waterfowl could be supported by that pond. No botulism was detected in that flock.

In 1974 the sentinel flocks were as follows (Table 1): 24 mallards on pond 2 as of 9/3/74, 23 of which were released on the first 2 days following the rotenone treatment of 8/27/74; 32 mallards and 20 coots on pond 3 as of 9/3/74; and 24 mallards on pond 4 as of 7/12/74. All mallards on pond 4 were moved to the other ponds when pond 4 dried up about the end of August.

It is doubtful that ducks on pond 4 had much opportunity to ingest invertebrate carcasses. However, the 24 mallards on pond 2 were directly observed feeding upon the massive quantities of invertebrate carcasses that were made available by the rotenone treatment. The ducks continued to feed upon invertebrate carcasses as long as they were available. As the water depth increased in pond 2 (Fig. 2) the invertebrate carcasses eventually sank and became unavailable to the ducks. In order to make this material available to the ducks a small anchor was dragged along the bottom around the edges of the pond. This brought large amounts of invertebrate carcasses mixed with bottom sediments to the surface. This was done on 9/16/74 and 10/2/74 and made large quantities of invertebrate carcasses from the bottom available to the ducks. The ducks began to feed upon this material as soon as it became available. The two mallards sacrificed on pond 2, one on 9/10/74 and one on 10/3/74, confirmed the observations that the ducks were ingesting invertebrate carcasses. The esophagi of both ducks contained the cases of dead chironomid larvae and fragments of corixid wings and exoskeletons. The mallard sacrificed on pond 3 on 9/10/74 was full of sago pondweed (Potamogeton pectinatus).

No botulism was observed in any of the birds on any pond in 1974.

Microbiology

Experiment 1

None of the 14 samples of dead invertebrates from pond 2 was toxic for mice either before or after incubation. This accounts for the fact that no ducks contracted botulism even though they ingested invertebrate carcasses.

Experiment 2

Data on the influence of sewage pond organisms on the toxicity of X220B2 cultures are given in Table 4. This experiment indicates that actively growing X220B2 cells (even large numbers of cells under ideal growth conditions) did not continue to produce toxin for long when subcultured in combination with sewage pond organisms. Presumably the X220B2 cells either did not reproduce and the toxin in the original culture was diluted out in the subcultures, or the X220B2 cells did reproduce but could not effectively compete (at least in terms of toxin production) with the other sewage pond organisms. The possibility that the loss of toxigenicity was due to the loss of the specific bacteriophage (CE_{β}) (Eklund et al., 1971; Eklund et al., 1974; Inoue and Iida, 1971; Vinet, Berthiaume and Fredette, 1968) which is involved in toxin production cannot be ruled out. However, the control culture (X220B2 alone) maintained its toxigenicity in all subcultures.

Experiment 3

Table 5 gives the results of the toxicity titrations for actively growing X220B2 cells cultured alone and with sewage pond sample 12. The X220B2

Table 4. The influence of sewage pond microbes on the toxicity (in mice) of Cl. botulinum (X220B2) cultures. The X220B2 inoculum was actively growing cells.

Sample No.		ORIGINAL CULTURE (1/10/75) TOXICITY 1st passage		1ST SUBCULTURE (1/14/75) TOXICITY 2nd passage		2ND SUBCULTURE (1/17/75) TOXICITY 3rd passage		3RD SUBCULTURE (1/21/75) TOXICITY	
		Sample Alone	Sample + X220B2	Sample Alone	Sample + X220B2	Sample Alone	Sample + X220B2	Sample Alone	Sample + X220B2
		(Dead/Injected) [a]		(Dead/Injected)		(Dead/Injected)		(Dead/Injected)	
1	P[b]	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
	U	0/1	1/1	0/1	1/1	0/1	0/1	0/1	0/1
8	P	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
	U	0/1	1/1	0/1	1/1	0/1	0/1	0/1	0/1
9	P	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
	U	0/1	1/1	0/1	1/1	0/1	0/1	0/1	0/1
12	P	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
	U	0/1	1/1	0/1	1/1	0/1	0/1	0/1	0/1
13	P	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
	U	0/1	1/1	0/1	1/1	0/1	0/1	0/1	0/1
14	P	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
	U	0/1	1/1	0/1	1/1	0/1	1/1	0/1	0/1
Control (X220B2 alone)		2/2	1st passage	2/2	2nd passage	2/2	3rd passage	2/2	

[a] No. of mice which died/No. injected.

[b] P = mouse protected with 0.1 ml type C antitoxin; U = unprotected.

cells cultured alone produced eight times as much toxin as the X220B2 cells cultured with sample 12.

Table 5. A comparison of toxin levels of Cl. botulinum (X220B2) in pure culture and with sewage pond microbes. The X220B2 inoculum was actively growing cells.

	TOXICITY IN MICE		
Dilution	X220B2	Sample 12	X220B2 + 12
1:1,000	2/2[a]	0/2	2/2
1:10,000	2/2	0/2	2/2
1:20,000	2/2	0/2	2/2
1:40,000	2/2	-	1/2
1:80,000	2/2	-	0/2
1:160,000	2/2	-	0/2
1:320,000	2/2	-	0/2
1:640,000	0/2[b]	-	0/2

[a] No. dead/No. injected.
[b] These mice showed signs of intoxication.

Experiment 4

Table 6 gives the results of the toxicity titrations for the inactive (frozen for 6 years at -20 C) X220B2 inoculum cultured alone and with sewage pond sample 12. The inactive inoculum cultured alone produced about 16 times as much toxin as the inactive inoculum cultured with sample 12. A titration of the inactive X220B2 inoculum showed that it contained about 0.2 mouse MLD (minimum lethal doses)/ml at the 1:10,000 dilution. The X220B2 + sample 12

culture contained at least 1.0 mouse MLD/ml at the 1:10,000 dilution. That is five times more toxin than would be expected in the X220B2 + sample 12 culture if the toxin were simply residual, i.e., added with the inactive X220B2 inoculum. Therefore, some toxin was produced by actively growing X220B2 cells (perhaps originating from spores) in the X220B2 + sample 12 culture.

Table 6. A comparison of toxin levels of Cl. botulinum (X220B2) in pure culture and with sewage pond microbes. The X220B2 inoculum was from an inactive (frozen for 6 years at -20 C) culture.

	TOXICITY IN MICE		
Dilution	X220B2	Sample 12	X220B2 + 12
1:100	2/2[a]	0/2	2/2
1:1,000	2/2	0/2	2/2
1:10,000	2/2	-	2/2
1:20,000	2/2	-	0/2
1:40,000	2/2	-	0/2
1:80,000	2/2	-	0/2
1:160,000	2/2	-	0/2
1:320,000	0/2[b]	-	0/2
1:640,000	0/2	-	0/2

[a] No. dead/No. injected.
[b] These mice showed signs of intoxication.

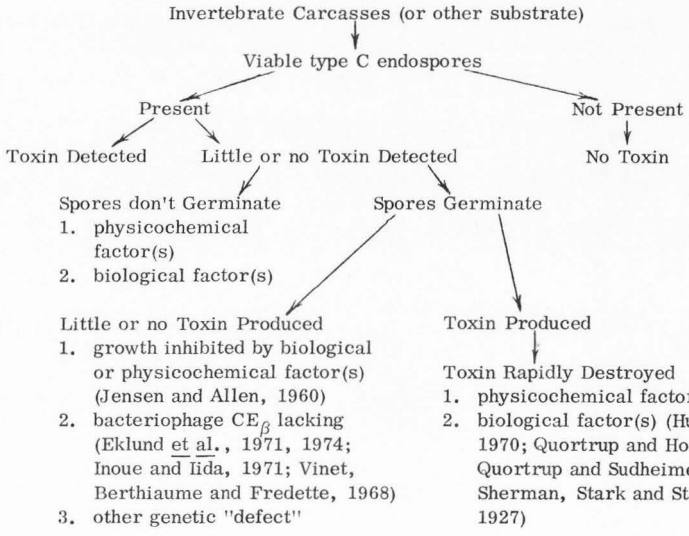
DISCUSSION

The preliminary study of pond 1 in 1973 demonstrated the feasibility of testing the invertebrate-carcass microenvironment hypothesis of type C botulism epizootiology on the Richmond sewage oxidation ponds. The 1973 study showed: (1) that ponds 2, 3 and 4 could be expected to support abundant populations of benthic and free-swimming aquatic insects in 1974; (2) the approximate numbers of samples required to effectively monitor invertebrate populations, i.e., so valid statistical comparisons could be made made if required; and (3) that the ponds could be expected to adequately support sentinel waterfowl flocks. The experiments done on ponds 2, 3 and 4 in 1974 constituted the actual field test of the hypothesis.

The microenvironment concept hypothesizes that the following conditions are necessary for the initiation of a type C botulism epizootic in waterbirds: (1) the etiologic agent, i.e., viable, toxigenic type C cells, must be present in the environment; (2) a suitable substrate, e.g., invertebrate carcasses, must be available for clostridial growth and toxin production; and (3) a susceptible population of waterbirds must be present and must ingest the toxin which is probably contained in the bacterial substrate. This study attempted to satisfy these conditions during the critical time of year, late summer and early fall, when botulism epizootics characteristically occur. The experiment on pond 2 demonstrated that even when conditions (1) and (3) were satisfied, the

provision of large numbers of invertebrate carcasses was not sufficient to cause type C botulism in the sentinel flock. This result indicates that although these conditions may be necessary for the initiation of a type C botulism epizootic in Utah marshes, they probably do not represent all necessary conditions. The problem becomes one of attempting to define the additional condition(s) necessary to initiate a type C botulism epizootic.

Experiment 1 proved that the absence of botulism in the sentinel birds on pond 2 was due to the absence of detectable toxin in the invertebrate carcasses ingested by the birds. The other microbiological experiments started at the BRRS were (and are) an attempt to determine why no detectable toxin was present in these carcasses. The problem can be clearly visualized with the aid of the following scheme:



Experiments can be designed to test most of the hypotheses involved at the branch points of this scheme. The preliminary results, i.e., Experiments 2, 3 and 4, tend to indicate that other microorganisms present in the sewage pond samples have an inhibitory effect upon the toxicity of cultures of the highly toxigenic X220B2 cells; even when large numbers of X220B2 cells are involved under ideal growth conditions. If this is true, it is not difficult to imagine how Cl. botulinum cells might be inhibited in their growth and/or production of toxin by other microorganisms in a sewage oxidation pond (or marsh) where smaller numbers of clostridial cells might be involved under less than ideal growth conditions. This could be why botulism epizootics appear to occur infrequently on sewage oxidation ponds.

The concept that other microorganisms may inhibit the survival of clostridial cells and/or toxin is not new. Sherman et al. reported in 1927 that human intestinal bacteria could destroy botulinum toxin. Quortrup and Holt (1941) stated that aerobic bacteria are capable of rapidly destroying preformed toxin, and that aerobic microbes have much stronger detoxifying properties than have anaerobes. Quortrup and Sudheimer (1943) reported that the detoxifying action of E. coli upon botulinum toxin could be overcome by Pseudomonas aeruginosa which could outgrow E. coli and allow Cl. botulinum to produce toxin. Hunter (1970) stated that one possible cause of subsidence of botulism epizootics might be microbial action upon toxin. Jensen and Allen (1960), in a discussion of the evidence required to prove the validity of the microenvironment concept on the BRBR, stated that either

the abundance of invertebrate carcasses must increase or environmental changes favoring an increased rate of toxin production must occur prior to the initiation of a botulism epizootic. Perhaps the environmental change required to favor an increased rate of toxin production in nature is the alleviation of the normal inhibitory effects of other microbes upon clostridial growth and/or toxin.

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