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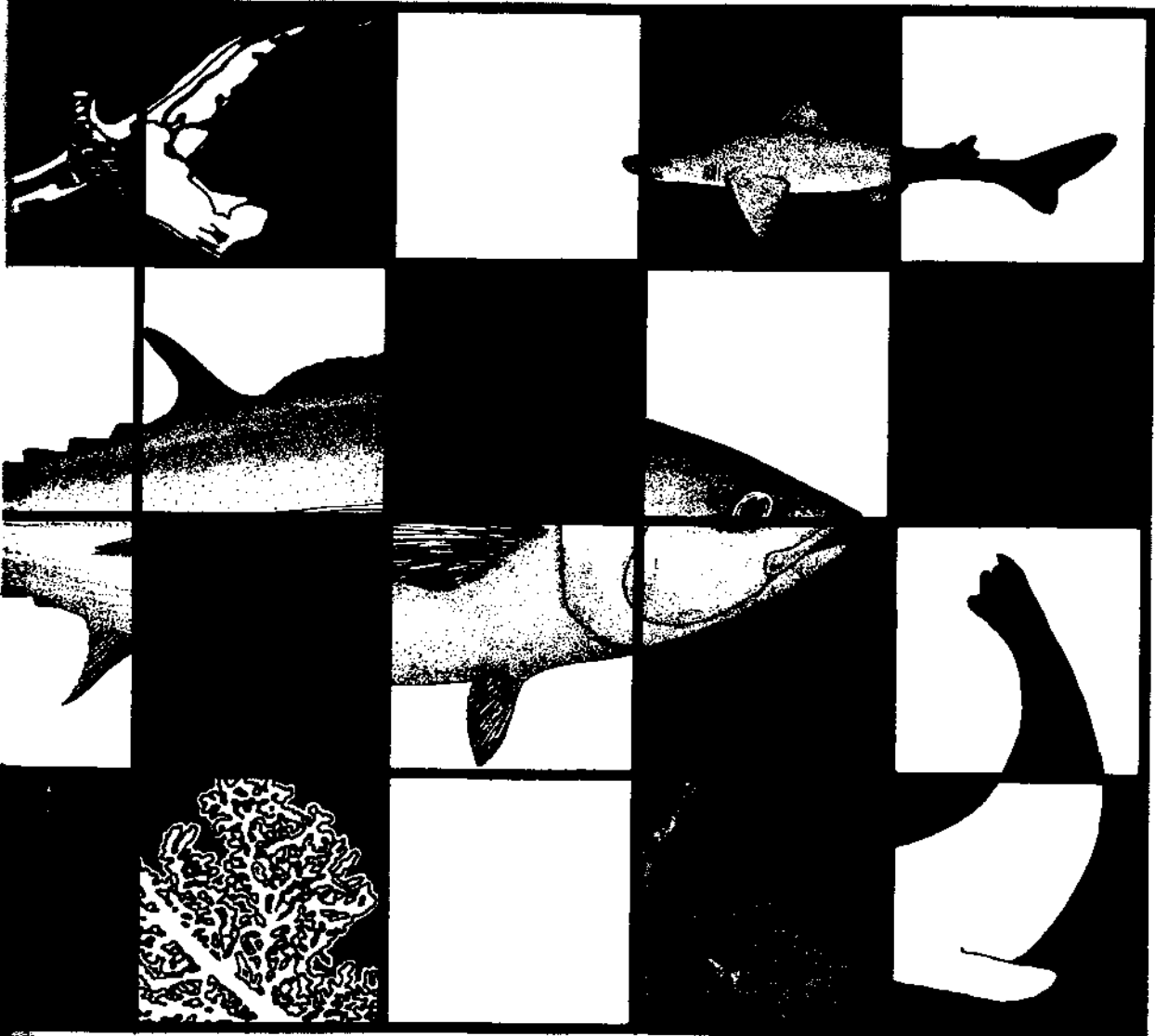
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**CIGUATERA RESEARCH IN THE NORTHWESTERN HAWAIIAN ISLANDS:
LABORATORY AND FIELD STUDIES ON CIGUATOXIGENIC
DINOFLAGELLATES IN THE HAWAIIAN ARCHIPELAGO**

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ABSTRACT

In coral reef ecosystems, blooms of the toxic dinoflagellate, Gambierdiscus toxicus Adachi and Fukuyo, sporadically appear as benthic analogs of toxic planktonic dinoflagellate "red tides." Gambierdiscus toxicus synthesizes toxins which are apparently accumulated through the food web into many species of tropical and subtropical fish, resulting in occasional "ciguatera fish outbreaks" harmful to humans. Little is known about the distribution, growth, and toxin synthesis of this dinoflagellate which was postulated in 1977 to be the primary source of toxin in ciguateric outbreaks. The aim of this research was to learn about the distribution and toxicity of G. toxicus in the NWHI chain. Parallel laboratory studies measured growth and toxin synthesis of the species under controlled conditions.

Results of field studies revealed the widespread yet low-level distribution of G. toxicus (found only on macroalgal substrates) throughout the NWHI: population densities ranged from 3 to 119 dinoflagellate cells per gram weight macroalgal substrate. A marked preference was indicated by the dinoflagellate for specific red and brown algal substrates (especially Dictyota acutiloba, Spyridea filamentosa, and Acanthophora spicifera). Limited "epibenthic blooms" occurred with no seasonal regularity on sites studied on Oahu and at French Frigate Shoals. No specific factors which could trigger a bloom were found; although the data indicate that temperature range and substrate availability may control blooms.

Wild cells collected on Tern Island, Oahu, and Hawaii were always highly toxic as measured by mouse intraperitoneal (i.p.) injection of extracted cells (for wild cells, the LD₅₀ was 750 cells/mouse). In culture, the Kaneohe Bay, Oahu clone synthesized only one-eighth as much toxin as the "wild" parent population (LD₅₀ of 6,200 cells/mouse for cultured cells from dose response studies by mouse i.p.). Both the Kaneohe Bay clone and the Tern Island clone in mass culture (in 200L of F/2-t media plus seaweed extract at 25° to 27°C and 40 microeinstains/m²/sec) yielded consistently large amounts of the potent water soluble toxin "maitotoxin" which appears in many edible fishes. However, little, if any, "ciguatoxin" (compared with reference moray eel ciguatoxin) was detected in these cultured cells. The reason for the reduction in total toxin and apparent absence of ciguatoxin in G. toxicus cultured cells vs collected "wild" cell populations is unknown.

Based on G. toxicus population levels, it is not currently possible to predict ciguatera outbreaks and a reliable test for the detection of ciguatera toxins in fish has not been developed yet. Therefore, a fisheries developed in the NWHI would be subject to the same risk of ciguatera fish poisoning as all other tropical and subtropical fisheries around the world. Fortunately epidemiological studies indicate lower incidence of ciguatera in the Hawaiian islands in comparison with many other Pacific and Caribbean islands.

toxic dinoflagellate	ciguatera
<u>Gambierdiscus toxicus</u>	seaweed
Northwestern Hawaiian Islands	

INTRODUCTION

Ciguatera is a term referring to human intoxications resulting from the ingestion of a variety of tropical reef and inshore fishes which produce diverse but characteristic symptoms, infrequently causing mortality (Banner, 1976; Lewis, 1981; Withers, 1982). The term ciguatera was derived from a name used in the eighteenth century in the Spanish Antilles for intoxication brought about by ingestion of the "cigua" or turban shell Cittarium (Livona or Turbo) pica (Lawrence et al., 1980). Ciguatera was recorded (Gudger, 1930) in the West Indies by the first historian of the Americas, Peter Martyr (1457-1526), and noted in the Pacific as early as 1606 when the sailors with the Spanish explorer de Quiros suffered from ciguatera in the New Hebrides (Banner, 1976). Toxic outbreaks of ciguatera are sporadic and unpredictable, with a patchy distribution in both space and time (Halstead, 1978; Cooper, 1964). The syndrome affects both tropical and subtropical coastal regions of the world, but is largely confined to islands in the true tropics (Banner, 1976). Ciguatera fish poisoning has characteristic gastrointestinal and

neurological symptoms and it is occasionally fatal (Lawrence et al., 1980). In recent surveys (1971-77), ciguatera outranked both scombroid (fish) and paralytic shellfish poisoning as the most frequently reported foodborne disease of chemical nature in the United States, where most of the cases are from southeastern Florida and Hawaii (Hughes, 1979). In the Caribbean and the South Pacific, it is a much more widespread and serious problem, not only as a general health hazard but also because of the restrictions it imposes on the use of fish resources in these tropical island regions, many of which depend upon reef fish as an important protein source (Bagnis, 1973; Dawson, 1977; Morris, 1980). Mortality of an endangered species, Monachus schauinslandi (Hawaiian monk seal) on Laysan Island in the Northwestern Hawaiian Islands may be a result of ciguatera poisoning (Gilmartin et al., 1980).

The liver of the moray eel, Lycondontis javanicus, has been extracted for the major toxin, ciguatoxin, a lipid-soluble, compound of molecular weight 1112, which has been recently crystallized (Scheuer et al., 1967; Nukina et al., in press). Ciguatoxin and other toxins associated with ciguatera (including "ciguaterin" and maitotoxin) arrive in the fish from their diet. A new species and genus of dinoflagellate, the benthic Gambierdiscus toxicus Adachi and Fukuyo, is a suspected source of these toxins (Taylor, 1979). We report here on toxicity of the Hawaiian strain of G. toxicus and its distribution throughout the Northwestern Hawaiian Islands.

MATERIALS AND METHODS

Field Survey

The method of collecting and measuring populations of G. toxicus was a simple procedure slightly modified from that of Yasumoto et al. (1979), which takes advantage of the epiphytic nature of this species. Algae and other substances were collected from selected areas at the 0 to 15 m depth by skin or scuba diving. The algae (or coral debris) were placed in a plastic bag underwater and brought to shore where 50 to 100 g wet weight seaweed (by displacement) were measured out in a 500-ml cylinder filled with filtered seawater. After vigorous shaking 50 times, the suspended material was screened successively through 1-mm, 250- μ , and 75- μ mesh screens. The filtrate was passed through a 36- μ mesh screen, and all the material which was retained was transferred to an 8-ml screw cap tube for immediate microscopic examination, or if no microscope was available, a 3 percent formalin solution was prepared to preserve the specimen for later counting. Table 1 gives the results of G. toxicus sampling in the Hawaiian islands.

TABLE 1. GAMBIERDISCUS TOXICUS SURVEY

Date	Location	No. Samples Collected	No. Samples With <i>G. toxicus</i> Present	No. Cells <i>G. toxicus</i> /100-g Substrate	Substrate
08/78	Hawaii: Keokea Beach Spencer's Beach (Taylor, 1979)		Present in low numbers	Very low numbers	<i>Ceramium</i> , <i>Chondria</i> , <i>Wrangalia</i> , <i>Galidium</i> , <i>Sargassum</i> , <i>Padina</i> , and <i>Turbinaria</i>
01/78	Oahu: Coconut Island	--	+	+	
08/78	Oahu: Checker Reef, Kaneohe Bay Pokai Bay (Taylor, 1979) Lualualei Beach Park Kewalo Basin		+	100 cells/algal thallus Very low numbers Very low numbers Very low numbers	Absent from <i>Ulva</i> and <i>Codium</i>
08/78	Coconut Island water tables		+		<i>Pseudobryopsis</i> sp.
10/10/80	Checker Reef, Kaneohe Bay	--	+	500 cells	<i>Sargassum polyphyllum</i>
09/18/80	Lagoon edge of barrier reef, Kaneohe Bay	--	+	48 cells	<i>Acanthophora spicifera</i>
03/21/80	Kualoa Point (4 locations -- sand, mud, and coral substrate) 0 to 20 ft	21	0	0	<i>Turbinaria</i> , <i>Acanthophora</i> , <i>Sargassum</i> , coral rubble, sand
01/29/81	Checker Reef, Kaneohe Bay 3 to 6 ft	4	2	6 cells	<i>Turbinaria ornata</i> <i>Acanthophora spicifera</i> <i>Dictyosphaeria</i>
01/29/81	Coconut Island, lagoon Kaneohe Bay	1	0	1 cell	<i>Eucheuma</i> sp.
02/09/81	Pokai Bay	1	1	50 cells	<i>Sargassum polyphyllum</i>
11/05/80	Nihoa (west side)	6	Possibly 1	3 cells	<i>Ahnfeltia concinna</i> (red)
11/10/80	French Frigate Shoals a. Tern Island SE reef, inshore b. Whaleskate Island	8 7	4 0	3 to 12, \bar{X} = 7 0	<i>Colpomenia sinuosa</i> , <i>Halimeda opuntia</i> , and <i>Laurencia nidifica</i> <i>Laurencia</i> sp., <i>Microdictyon</i> sp.
11/16/80	Laysan Island a. West reef b. SW reef	5 6	5 6	100 to 400, \bar{X} = 200 12 to 160, \bar{X} = 54	<i>Laurencia</i> sp., <i>Halimeda opuntia</i> , <i>Halimeda discipidea</i> , <i>Chaetomorpha</i> sp., and <i>Dictyota</i> sp. <i>Martenia fragilis</i> , <i>Turbinaria ornata</i> , <i>Dictyota</i> sp., <i>Laurencia</i> sp., <i>Plocosium</i> sp., <i>Halysaeta</i> sp., and <i>Halimeda</i> <i>Sargassum schinocarpum</i>
11/17/80	c. Rock ledges, high wave action	2	0	0	
11/19/80	Pearl and Hermes Atoll a. Seal Island b. SE Island (1) inner reef (2) leeward patch reef - offshore	4 1 2	1 0 2	2 0 3 to 20	On coralline - <i>Porolithon</i> , <i>Jania</i> , none - <i>Microdictyon</i> sp., <i>Cladophora</i> sp., <i>Neomaria</i> sp. Red/bluegreen epiphytes <i>Laurencia nidifica</i> , <i>Trichoglossa requienii</i>
08/07/81	Tern Island NE beach E reef, 8 ft S beach, sand N beach	1 2 1 1	0 1 1 0	0 10 4 0	<i>Lynghya</i> sp. (rose colored) <i>Acanthophora spicifera</i> <i>Acanthophora spicifera</i> <i>Acanthophora spicifera</i>
08/08/81	Tern Island SE end and Open ocean Tern Island W beach, off pier Scuba, 15 ft Scuba, 12 ft Scuba, 12 ft*	3 1 1 1	0 1 1 1	0 200 11,200 10,000	<i>Halimeda</i> , <i>Lobophora</i> <i>variegata</i> on <i>Pocillopora</i> <i>Laurencia</i> , <i>Lynghya</i> <i>confervoides</i> , <i>Dictyota</i> <i>acutiloba</i> <i>Dictyota acutiloba</i> <i>Dictyota acutiloba</i> + <i>Lynghya</i> sp.

*Collected for toxicity testing, mouse intraperitoneal bioassay. Overall toxicity: less than 1,000 cells/mouse unit, Table 2. Clone in culture at HIME (labeled "clone T-39-1").

Cultures

Single cell isolates were made directly from these samples by micropipetting and washing (with serial dilutions) single G. toxicus cells. To reduce diatom contamination, GeO_2 (80 ppm) was added. The cultures were grown in F/2-t media (Guillard, 1975) plus seaweed extract (aqueous autoclaved extract of blended fresh A. spicifera and Sargassum sp. added at 1 ml/l) at 40 micro-einsteins/ m^2 /sec and 25° to 27°C, without aeration, in Fernbach flasks.

Extraction

For the dose response study, the "wild cell" samples of G. toxicus (GT) cells (Kaneohe) mixed with sand and macroalgal fragments were combined and extracted with 150-ml MeOH. After filtration, the residue was re-extracted three times with 100-ml MeOH and mild heating. The methanol extracts were combined and lyophilized to yield a dry weight of 1.24 g which was redissolved in 25-ml methanol. Portions were removed for i.p. injections to estimate toxicity. The highest dose (7 mg extract, dry weight) represents 6.2×10^3 cells of GT extracted. The cultured cells were harvested by centrifugation and immediately extracted with 50-ml MeOH. The extraction procedure was repeated as for the wild cells, and a portion of the methanol extract was used for the dose response curve (maximum dose represents 4×10^4 extracted cultured GT cells).

Fractionation of Extract

The total methanol extracts remaining after the dose response test was evaporated and brought up in 125-ml ether/water, 4/1, v/v. The water phase was re-extracted with ether two times, and the ether fractions combined. The water-soluble material was lyophilized and brought up in MeOH of which portions were used for bioassay. The ether-soluble material was evaporated and brought up in ether, portions of which were tested by bioassay.

Bioassay -- Mouse i.p. Infections

Aliquots of extracts from G. toxicus were transferred in methanol to small tubes, evaporated to dryness under vacuum, weighed, and brought up in 5 percent Tween 80 in distilled water and homogenized with a vortex-mixer. Several controls were used in each experiment with no toxic extract added. The injection volume was 0.5 ml, applied intraperitoneally to ICR female mice, averaging 20 g.

Toxicity

Dose response curves with death as the endpoint were determined for two toxic extracts: the methanol extract (which contains both ether and water-soluble toxins) of GT cells collected

from the reef and GT cells in culture. The LD₅₀ as expressed in equivalent cells or weight from this test was used as a quantitative measurement of toxicity.

In addition, for smaller samples, a "cell/mouse unit" value was determined as a rough estimate of toxicity. A "mouse unit" is the minimal amount of toxin required to kill a 20-g mouse within 48 hours.

RESULTS

Low-level cell counts (0 to 500 cells/100 g algal substrate) of Gambierdiscus toxicus were found in most collection sites in this survey of Oahu and the Northwestern Hawaiian Islands. G. toxicus was present in 45 percent of the samples collected, indicating a widespread, if low-level, distribution of the organism (Table 1). High levels, 400 to 500 cells/100-g substrate, were found on Laysan Island and Checker Reef, Kaneohe Bay in October and November 1980; 250 cells/100-g substrate on Tern Island. Several limited "blooms" (>1,000 cells/100-g substrate) were found around the island of Oahu: at Portlock (maximum 26,400 cells/100 g), Sand Island (11,800 cells/100 g), Kapiolani Park (24,000 cells/100 g), Kalanai Point (15,300 cells/100 g), Waimanalo (10,800 cells/100 g), and all around Kaneohe Bay (maximum 72,500 cells/100 g at Ahuolaka). There was no apparent seasonal periodicity in the appearances of the confined blooms on Oahu. On Tern Island, a confined bloom of 11,200 cells per 100 g of Dictyota acutiloba were collected by scuba. Ostreopsis sp. was also detected (at a level of about 300 cells per 100-g wet weight macroalgal substrate) (Withers, 1981).

Environmental parameters at "bloom" sites (on Tern Island) were standard: pH, 8.0 (night) to 8.25 (day); temperature, 27.5°C (range: 26.5° to 28°C); oxygen, 5.5 ppm (day) to 7.5 ppm (night); CO₂, 0.02 mM/L (calculated); alkalinity, 2.35 mM/L; PO₄, 0 to 0.1 M; and salinity, 35.5 ‰ (range: 35 to 36.5 ‰). No large fluctuations were detected in pH, CO₂, O₂ or PO₄ levels. No specific factors which could trigger a bloom were found, although temperature and macroalgal substrate availability may control blooms.

In the Hawaiian islands, the highest levels of G. toxicus were found on the brown algae, Dictyota acutiloba and Sargassum polyphyllum, and the red algae, Spyridea filamentosa and Martenisia fragilis. Next highest levels were detected on Acanthophora spicifera, Laurencia sp. (rhodophytes); Turbinaria ornata and Dictyota sp. (phaeophytes), and Halimeda opuntia, H. discoidea, and Chaetomorpha sp. (chlorophytes). No G. toxicus cells were seen in the coral rubble or sand particle samples in the present study. Both large benthic dinoflagellates, Ostreopsis sp. and Coolia sp., were occasionally present in the algal samples; Coolia sp. co-occured most frequently with G. toxicus. The Hawaiian strain of G. toxicus ranges from 40 to 100 μ in diameter.

These values approximate the lowest population levels found in the South Pacific by Yasumoto et al. (1979). In that survey, a drop in numbers of *G. toxicus* from 76,500 to 450 cells/100 g of algae occurred on *Hittia* after a 15-month period. This was representative of population changes in the Tahiti region in general. In the Gambier Islands, much higher concentrations of *G. toxicus* -- 17,000 to 31,800,000 cells/100 g algae -- were found. By comparison, in the Caribbean Islands, population densities of *G. toxicus* ranged from 0 to 60,000 cells/100-g substrate which is lower than the South Pacific but still higher than the Hawaiian island populations reported here (F.J.R. Taylor: personal communication; Besada et al., 1982).

Toxicity

The dose response curves are shown for the "wild" and cultured *G. toxicus* cells, crude methanol extract (Figure 1 and Table 2). Table 3 shows that total toxin production on a per cell basis is eight times greater in the "wild" *G. toxicus* cells than in the cultures. The extract from the cultures gave an LD₅₀ of 6200 cells per mouse in contrast with the LD₅₀ of 750 cells for the "wild" GT cells (Figure 1).

Symptoms elicited by the crude extract (total toxins) from *G. toxicus* in "wild" cells included dyspnea, temperature depression, and apparent respiratory failure. For the cultured cells, apnea, temperature depression, and occasional viscous saliva were elicited. There was no severe diarrhea in either wild or cultured cells of *G. toxicus* as noted with eel ciguatoxin. In mice treated with cultured cell extract, the heart was still fibrillating 10 minutes after death. The most significant symptom common to both "wild" and cultured cell extracts upon injection in mice was the extreme depressed body temperature (detected upon touch) (Sawyer et al., in press).

Symptoms elicited by the "ether" extract (so-called ciguatoxin) of the wild cells included convulsions just prior to death, ptalism, and apnea. Symptoms from the methanol fraction (so-called maitotoxin) from the wild GT cells included diarrhea, exophthalmia, and depressed body temperature.

In the cultured cells, most if not all of the toxicity was associated with the water-soluble (a "maitotoxin" fraction) (Table 3). When the ether-soluble fraction of the mass cultured cell extract was re-chromatographed, no substantial toxicity was detected in the fraction corresponding exactly to that of authentic ciguatoxin from *G. javanicus* (Nukina et al., in press). However, both the *G. toxicus* cultured cell extracts and the eel ciguatoxin shared three unique properties in the mouse i.p. bioassay: (1) a very steep dose response curve (Figure 1), (2) heat stability, and (3) temperature depression (Sawyer et al., in press).

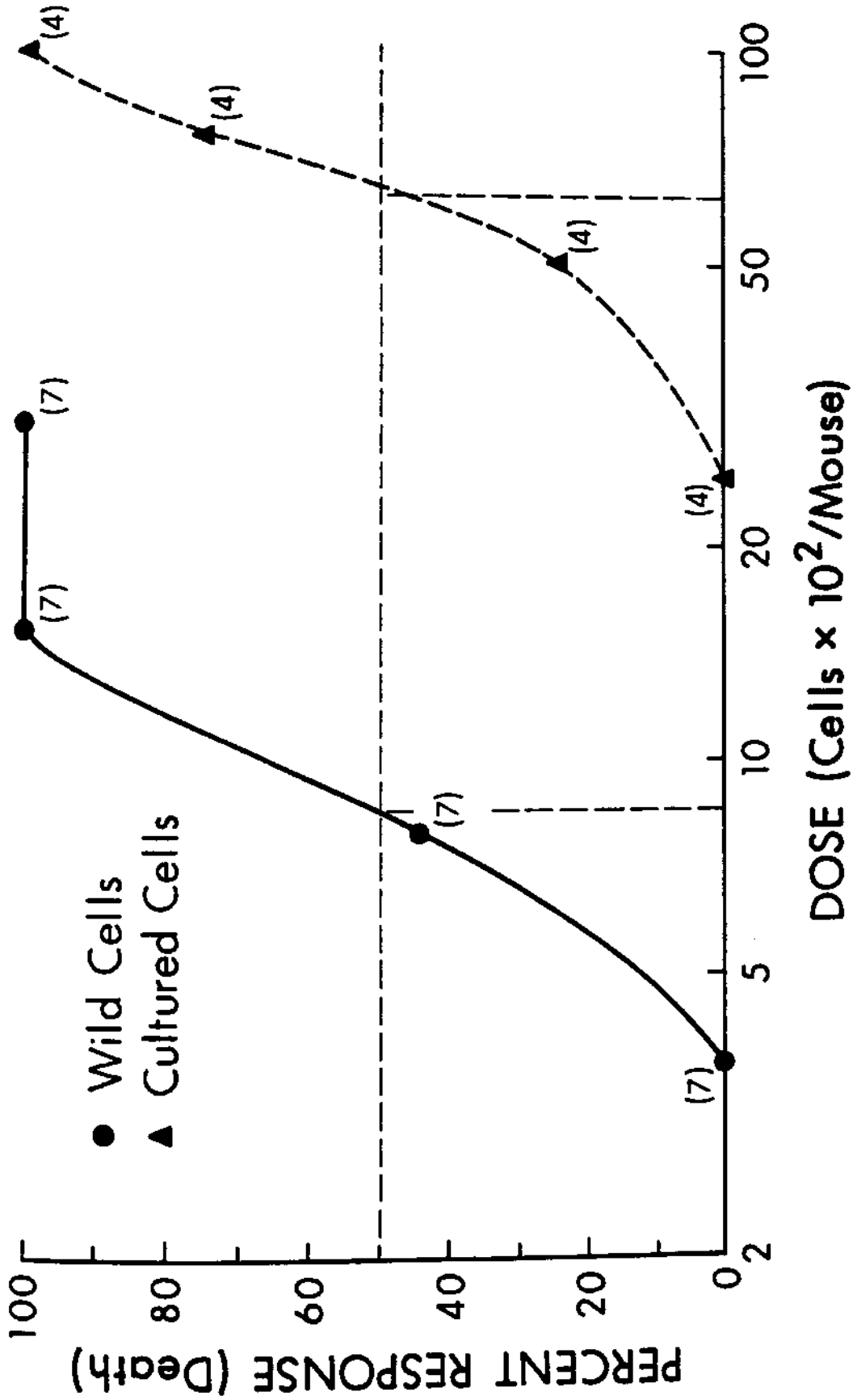


Figure 1. Dose response curves for "wild" and cultured *Gambierdiscus toxicus* cells (Oahu strain), crude methanol extract

TABLE 2. DOSE RESPONSE CURVE -- G. TOXICUS TOTAL TOXINS, MOUSE
INTRAPERITONEAL BIOASSAY

No. of Mice Injected	Amount Crude Toxin (mg)	Equivalent Number Cells Extracted $\times 10^4$	% Response (death)	Death Time $\bar{x} \pm SD$ (min)
Wild cells, Kaneohe Bay (4/81)				
4	0	0	0	0
7	7	6.2	71	156 \pm 47
7	3.5	3.1	100	205 \pm 79
7	1.75	1.55	100	548 \pm 205
7	0.875	0.775	57	1,248 \pm 151
7	0.4875	0.388	0	0
Cultured cells, Kaneohe Bay isolate (8/81) (isolated from "wild cells" above)				
4	--	40	100	150 \pm 18
4	--	20	100	194 \pm 26
4	--	10	100	699 \pm 305
4	--	7.5	75	753 \pm 37
4	--	5.0	25	1,000
4	--	2.5	0	0

TABLE 3. TOXICITY OF GAMBIERDISCUS TOXICUS, HAWAIIAN ISLANDS MOUSE INTRAPERITONEAL BIOASSAY

I. Crude extract, methanol						
Location	Substrate	Equivalent Cell Number Tested (10 ⁶)	Mouse Weight (g)	Death Time (min)	Approximate Cells/Mouse Unit (x)	LD ₅₀ (cells/mouse)
G. toxicus, "wild cells"						
HIMB docks	<i>Spyridia filamentosa</i>	2.5	20	720	<25,000	
HIMB docks	<i>Spyridia, Dictyota</i>	3.86	20	3		
Sand bar	<i>Spyridia, Dictyota</i>	0.62	19	159	1,500	750
Kaneohe Bay	<i>Spyridia, Dictyota</i>	1.8	21	80		
Tern Island pier (Table 1)	<i>Dictyota</i>	0.1	26	1,020	< 1,000	—
G. toxicus, cultured						
HIMB isolate		10.0	20 28	699 ± 305	10,000*	6,200*
II. Fractionated extracts, ether soluble "ciguatoxin" and water soluble "maitotoxin" of "wild" G. toxicus cells						
Location	Equivalent Cell Number Tested (10 ⁶)	Mouse Weight (g)	Death Time (min)	Cells/MU "Ciguatoxin" Fraction (x)	Cells/MU "Maitotoxin" Fraction (x)	
Kaneohe Bay						
Ether soluble	13	18	959			
Ether soluble	11.3	23	48			
Ether soluble	11.3	21	24	<60,000		
	13.5	26	68			
	6.48	24	73			
Water soluble	6.48	20	NE ^a			
Water soluble	11.6	22.5	2			
Water soluble	5.8	21.5	3			11,600
Water soluble	1.16	25.0	1,305			
Water soluble	1.16	265	1,328			
Water soluble	2.32	25.0	301			
Water soluble	3.9	25.0	235			
Kaneohe Bay:				32,000		
Ether soluble				(+/-5,000)		
Water soluble					8,200	
					(+/-800)	

^aCollected for toxicity testing, mouse intraperitoneal bioassay. Overall toxicity: less than 1,000 cells/mouse unit, Table 2. Clone in culture at HIMB (labeled "clone T-39-1"). Shimizu et al., 1982

CONCLUSIONS

A survey through the Northwestern Hawaiian Island chain revealed a widespread low-level population distribution of the epibenthic dinoflagellate Gambierdiscus toxicus. Cell counts normally ranged from 0 to 500 cells/100-g macroalgal substrate. Several epibenthic blooms (1,000 cells/100-g substrate) were found but there was no obvious environmental factor which could explain their appearance. The dinoflagellate appeared most frequently on certain preferred macroalgal "host" species, e.g., Spyridea filamentosa and Dictyota acutiloba.

The dinoflagellate, Gambierdiscus toxicus, Hawaiian strain produces potent toxins, although the cells in culture produce only one-eighth the overall toxicity of the cells collected from the field. Both an "ether-soluble" toxin which may be ciguatoxin and a "water-soluble" toxin are present in the "wild" cells. However, the cultured G. toxicus cells synthesize a "water-soluble" (apparent maitotoxin) fraction; there was little if any toxin which could be identified as ciguatoxin in these cultured cells. However, the three features which are common to both the G. toxicus toxin and eel ciguatoxin (temperature depression in mice, heat stability, and a steep dose response curve) suggest the possibility of bioconversion of the G. toxicus toxin to ciguatoxin in the coral reef food chain. The reasons for the discrepancy in toxin production by G. toxicus cells in the "wild" vs in culture are not known and will be the subject of future study.

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