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DINOFLAGELLATE TOXINS RESPONSIBLE FOR CIGUATERA FOOD POISONING

Annual Summary Report

Donald M. Miller

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During the second year significant quantities of *Gambierdiscus toxicus* was grown in large-scale culture. Gram levels of cells and toxic extracts were processed in an effort to improve existing purification procedures and to develop new procedures for purification and assay. Also numerous physiological (growth) studies were completed in an effort to improve toxin production by *G. toxicus*. Crude and semi-purified toxins were delivered.

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SUMMARY

Ciguatera is a syndrome occurring in humans who have become intoxicated from eating poison fish. Fish spontaneously accumulate the toxin through the food chain or directly from eating toxic dinoflagellates. Previous research points to the presence of multiple toxin involvement. In addition to the establishment of facilities, this contract requires the growth of sufficient quantities of three different species of dinoflagellates to allow purification of milligram quantities of toxins for delivery to the U.S. Army Medical Research and Development Command. In this second year of the contract, growth of the dinoflagellate *Gambierdiscus toxicus* in mass culture has been very successful. Purification of the products of these cultures is in progress. Gram levels of cells and toxic extracts were processed in an effort to improve existing purification procedures and to develop new procedures for purification and assay. Also numerous physiological (growth) studies were completed in an effort to improve toxin production by *G. toxicus*. Crude and semi-purified toxins were delivered.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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BODY OF THE REPORT

STATEMENT OF THE PROBLEM

Ciguatera is one of several forms of food poisoning which occurs in humans resulting from the ingestion of toxic fish. The "ciguatera syndrome" is the result of several toxins accumulated by fish through the food chain from dinoflagellate progenitors. Dinoflagellates produce a variety of toxins, some of which are ion channel inhibitors. In the case of ciguatera, the toxins can be accumulated through the food chain and stored by fish which are eventually consumed by humans. The ingestion of toxic fish produces a variety of digestive and neurological symptoms and sometimes death. At the present time, there is no adequate assay system for the detection and identification of the toxins. The structure and mode of action of these toxins is unknown. However, published results on crude toxins have described them as sodium and calcium channel inhibitors (**1,2**). There is at present no known prophylactic or ameliorating treatment for ciguatera intoxication, notwithstanding a recent popular report of mannitol therapy (**3**).

The acquisition of reasonable amounts of purified toxins would help to unravel the molecular structure of the toxins, their physiological actions and help to develop effective prophylactic treatment and effective countermeasures against the actions of the toxins. Our previous work on "ciguatera" (funded by the U.S. Food and Drug Administration, College Sea Grant Program and Myron Hokin) resulted in the establishment of the SIU culture collection of toxic dinoflagellates. We proposed to grow three species of these toxic dinoflagellates in mass culture for an extended period of time. After the cultures reached maximum growth they would be harvested and crude extracts made. The crude extracts would be assayed by mouse (LD_{50}) and an isolated bioassay preparation. Crude extracts would be purified by HPLC. The purified toxins would be assayed the same as the crude and samples of purified toxin delivered to the US Army Medical Research Institute of Infectious Diseases.

BACKGROUND INFORMATION

Ciguatera poisoning

Ciguatera poisoning is a syndrome which occurs following the ingestion of certain tropical marine reef-fishes that sporadically

acquire toxicity. It is one of nine known forms of ichthyosarcotoxism: poisoning of humans resulting from eating fishes which contain poison within their musculature, viscera or skin (**4**). Halstead (**5**) has implicated over 400 species of marine fishes as carriers, most, if not all, of which are an integral part of the food web of coral reefs associated with oceanic islands within a circum-global belt from 35°N to 34°S (**5, 6**).

The ciguatera syndrome

The symptoms that occur after eating toxic fish typically includes both gastrointestinal and neurological manifestations. Typical symptomatology in humans has been reviewed and summarized by several authors (**7, 8,** and **9**). Earliest symptoms of intoxication usually include gastro-intestinal upset, which may last for several hours or weeks. Moderate to severe intoxications usually produce neurological symptoms which may last weeks to months. Thus, it is quite clear that intoxication affects the nervous system for extended periods of time. In some cases of severe intoxication symptoms have persisted for 25 years (**10**): death may result and usually occurs after several days. In a few cases, death has occurred within ten minutes and the fatality rate has been approximated as 12% (**5**) and 3% (**8**). It is suspected that a large number of ciguatera intoxications, some from eating frozen fish, are not recognized as such (**11**).

Multi-toxin involvement

A few early researchers suggested that the great variety of symptoms displayed by patients suffering from ciguatera and their inconsistent responses to certain clinical treatments indicated that there was more than one primary toxin causing the ciguatera syndrome (**12, 13**), while Banner et al., (**14**) argued that ciguatoxin was the principal factor. Nevertheless, later studies coupled with the variability in results from testing of extracted fish tissues on a variety of preparations have emphasized the occurrence and importance of secondary toxins (**15, 16, 17, 18, 19** and **20**). Yasumoto et al., (**17**) provided evidence that ciguatoxin was of exogenous origin and was not a metabolic product of primary consumers. These authors reported that an analysis of gut contents of *Ctenochaetus striatus* (a detrital feeder, exclusively) revealed a portion, designated as unidentified particles, containing a high concentration of "ciguatoxin".

Dinoflagellate toxins affecting ion channels

The use of ion channel toxins has been crucial to uncovering the mechanisms of how ion channels work. It is well documented that two other toxins from dinoflagellates, saxitoxin and gonyautoxins are inhibitors of sodium channels. Brevetoxins have been found to affect sodium ion channels (**1, 21**). The current literature indicates that ciguatera toxins act on sodium channels or on calcium channels or both (**1, 2**).

Dinoflagellate involvement in ciguatera

That dinoflagellates are the source of ciguatera-toxins has been well documented. In the Pacific, Yasumoto and others (**22, 23**) obtained significant quantities of ciguatoxin from samples of detritus collected from dead coral near the Gambier Islands. The most toxic fraction of the detritus contained large numbers of a dinoflagellate, which he tentatively identified as "*Diplopsalis* sp.". Subsequently, Adachi and Fukuyo (**24**) named the organism *Gambierdiscus toxicus*. Yasumoto et al., (**16**) connected the production of toxin with both the dinoflagellate and toxic effects in mice (**25**). The dinoflagellate, *G. toxicus* has subsequently been isolated from ciguatera prevalent areas near Japan (**25, 26**), and Hawaii (**27, 28**). McFarren and others (**29**) have provided accounts of ciguatera-like poisoning (*G. breve* ?) from shellfish collected from the west coast of Florida. Other investigators have published on *Gambierdiscus toxicus* from Florida (**30**). Tindall and his group (**31**) have grown in mass culture and extracted toxins from three dinoflagellate species which were isolated from areas of the Caribbean in which ciguatera intoxication was prevalent. Thus far, three particular dinoflagellates are implicated in the production of ciguatera toxins: *G. toxicus* in which four toxic fractions have been identified, GT-1, GT-2, GT-3, GT-4: *P. concavum* in which five toxic fractions have been described, PC-1, PC-2, PC-3, PC-4, PC-5: and *P. rathymum*(= *P. mexicanum*) in which one toxic fraction, PR-1, has been described.

Toxin producing dinoflagellate strains

There is ample evidence to indicate that different strains of the same species of dinoflagellates produce different numbers and amounts of toxin (**32, 33**, and **34**). There are also reports of loss of toxicity of dinoflagellates after culture. Under our conditions we have found that the initiation of mass cultures from unialgal or pure cultures has confirmed the strain differences but our cultures (*G. toxicus*) have produced toxins through continual subculturing for a period of 7 years and through over 30 large scale cultures. The same holds true for the other species.

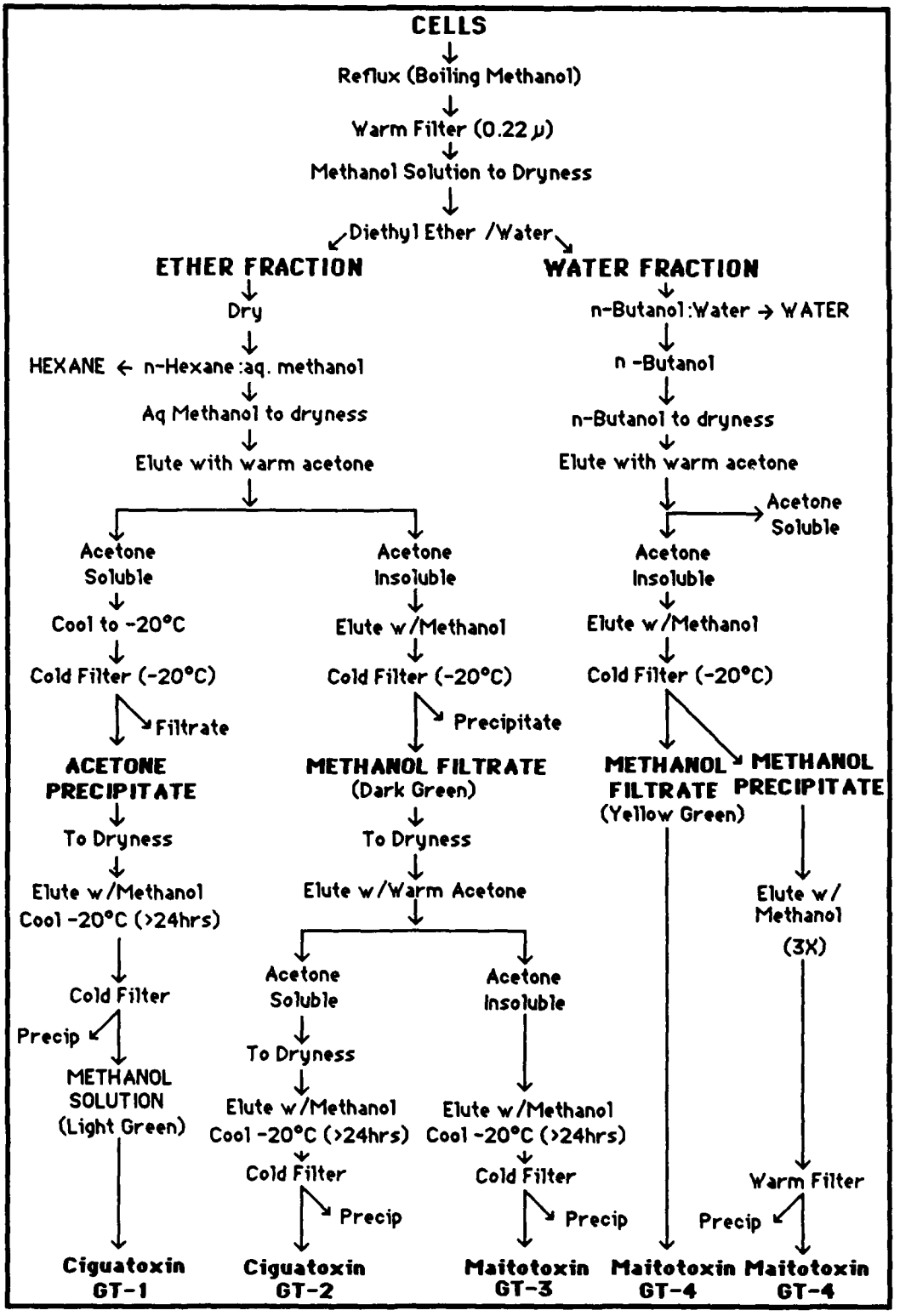


Figure 1. Schematic flow diagram of separation procedures for *Gambierdiscus toxicus*.

Isolation of dinoflagellate toxins

Yasumoto (**16**) extracted toxic components from *G. toxicus* cells utilizing a boiling methanol extraction prior to doing a water-ether partitioning. The ether extracted portion was further treated with acetone to derive a toxic fraction. Since we have found this fraction to precipitate in very cold acetone we term it the ether soluble acetone precipitate (ESAP) fraction. Most cell isolation procedures used an initial partitioning of the cells with a water-ether mixture. The treatment of the water phase of the cell extracts have been similar by all investigators. After an acetone partitioning the filtrate is chromatographed to yield a water soluble toxic component (Figure 1).

The treatments for the ether phase of the cell extracts have differed. Yasumoto used a technique which involved an acetone extraction of the ether-water phase. Bagnis and others (**35**) modified the tecnnique to include a cold acetone treatment that resulted in both ether-soluble acetone precipitates (ESAP) and ether-soluble acetone filtrates (ESAF). Even though he utilized this separation procedure, he then combined the ESAP with the WSAP fraction. Tindall and his group discovered that when the ESAF material was kept in the cold acetone all of the toxic activity eventually precipitated out of the filtrate. Thus, they adopted acetone precipitation as a step in the procedure hoping to further purify the toxic component. The toxic fractions obtained by these procedures differ with the particular dinoflagellate species.

Fish toxins

The studies of toxins from fish extracts are difficult to draw conclusions from for several reasons, the most critical of which is that, if there are multiple toxins in fish, determined by their diet, and any one toxin may have different effects on a variety of assay systems, then it is possible that the extraction of toxin from the same species of fish by investigators from different locales will produce different symptoms.

From the foregoing we conclude, that if we want to determine if a particular fish is toxic, we must have a test(s) or assay system(s) which is specific at a known level for each of the particular toxins which may be involved in the ciguatera syndrome. For this express reason we decided at the outset of our experimentation that the utilization of cell cultures would be the most productive approach.

Indeed, the same philosophy argued above for the diagnosis of toxic fish would apply to the treatment of the disease ciguatera. A different treatment would be called for were a person intoxicated with a sodium channel inhibitor rather than a calcium channel activator. Eventually, it would be ideal to have an assay system which would, in fact, reflect the number, kinds and amounts of toxins. The construction of these specific chemical tests is only possible, however, if one has reasonably pure toxin, which is separated from other toxins and identified.

These constraints have dictated our approach to the entire problem since we started our research in 1978, as follows:

1. Identify sources of the toxins.
2. Produce large amounts of toxic organisms.
3. Improve extraction techniques.
4. Find a sensitive bioassay(s) for screening.
5. Use bioassay to assist in purification.
6. Improve purification techniques.
7. Use purified toxins to:
    a. investigate physiology.
    b. investigate structure.
    c. elaborate chemical assay system.

Our preliminary works (**31, 36, 37** and **38**) have established *that at a minimum at least six different toxins and possibly a seventh* are involved in the ciguatera syndrome:

1. Ciguatoxin
2. Maitotoxin
3. Slow acting toxin (unidentified)
4. Scaritoxin-like toxin
5. Okadiac acid
6. Fast-acting toxin I (unidentified)
7. Fast-acting toxin II (unidentified)

Clearly, the understanding of the entire problem of the toxins produced by dinoflagellates requires a definitive test or set of tests which will allow us to discriminate between the toxins and be able to quantify and follow them. Other desirable features of a test procedure would be 1) provide the same baseline for each of the toxins, 2) consume only a small amount of toxin, 3) require a small

amount of time for the assay, and 4) be able to detect modifiers of sodium channels in membrane as well as calcium channels.

Significance

"Ciguatera-toxins" are involved in a variety of short term symptoms for which people commonly present themselves to a doctor for treatment (e g. diarrhea, headache, etc). In addition intoxication can result in prolonged disability or even death. Another aspect of the toxins and one which has yet to be addressed is the long term effects on animals that is: are they cumulative, are there storage sites, what are the affective sites, and how long before excretion? The reoccurrence of neurological symptoms years after intoxication would seem to indicate a retention of the toxin and/or toxic effects in the nervous system.

The toxins also include a novel calcium channel inhibitor (**2**) and a sodium channel inhibitor (**1**). In addition, there is the prospect of discovering other new and important ion channel inhibitors. Ion channel inhibitors have been essential to our present understanding of ion channel physiology and structure. Clearly, the identification, isolation and purification of individual toxins involving ion channels will expedite (1) an understanding of their structure, (2) allow the investigation of their physiological actions, (3) expedite the formulation of ameliorative and prophylactic treatments, and (4) allow the elaboration of a specific chemical assay.

APPROACH TO THE SECOND YEAR OF THE STUDY

Growth of cells

The dinoflagellate cultures which we used for this project are part of the Southern Illinois University Culture Collection, housed in the Department of Botany. At present this collection houses strains representing dinoflagellate species isolated from "ciguatera community" areas of the British and U. S. Virgin Islands and other recently acquired species. Our stock cultures are routinely grown in 50 ml volumes in 125 ml Erlenmeyer flasks. The medium is ES Medium (**39**) made with natural seawater, with 1.5% soil extract added. These cultures are kept in refrigerator-type culture chambers at 27°C and 500 ft-c. cool white fluorescence illumination (either continuous or on a 16:8 light-dark cycle, depending upon the requirements of the particular species). Stock cultures are transferred every 7-10 days. Two generations of cultures are

retained as back-ups to the new transfers. The subculturing and maintenance of triplicate cultures is labor intensive and requires approximately 20 manhours per week by an experienced person. In addition, all cultures are examined periodically by one of us to check for contamination. Currently we maintain stock cultures of over 153 strains of dinoflagellates. Preparation of the growth medium requires millipore filtration (0.45 or 0.22 μm) and sterilization of the sea water, sterilization of the flasks, compounding of the growth medium, inoculation and siting in the growth chambers. Conservatively, this requires approximately 20 manhours per week for a total of 18 carboys. Because the growth cycle for both the subcultures and the mass cultures takes four weeks, we initiate a mass culture every two weeks. It is critical for the cultures, to achieve the maximum toxin production, that they be harvested very close to the 30 day period.

The development of mass cultures involves transferring cells from stock cultures to a series of two liter fernbach flasks containing enriched seawater medium. After the early stationary phase of growth has been reached (approximately 15-20 days) each of these cultures are used to inoculate 18 liters of the same medium in 20 liter carboys. Mass cultures are grown under the same light and temperature regime as noted above and are aerated continuously in order to prevent CO_2 depletion and to provide moderate agitation. Cells from small cultures are harvested by centrifugation or filtration. Cells from mass cultures are harvested by means of a Pelicon concentrator using 0.45 μm membranes after cultures reach the early stationary phase of growth (30-35 days). If the culture has excessive amounts of slime it is first sieved before the use of the Pelicon.

Chemosystematic studies

Systematics is a branch of taxonomy which deals with assessing variation in characters between and within genera from living material (**40**). A systematic study has been incorporated into this project as a means of identifying clones (cultures initiated from individual cells) which are inherently good producers of toxins. Our culture collection now includes twenty-eight clones of *Gambierdiscus toxicus* from a variety of locations including Bermuda, the Bahamas, Florida, the Caribbean and Hawaii. It is this diverse, living material which forms the basis for the systematic studies.

Our approach to assessing clonal differences has been to first acclimate the *G. toxicus* clones to the same conditions (light, temperature and medium) in one liter cultures. Once acclimated, the final one liter culture is harvested and also used to inoculate an additional one liter culture which will serve as the starter for a 15 liter culture. The toxicities of the crude methanol cell-extract from the acclimated one liter cultures are then compared in terms of the number of mouse units per milligram of dried cells. This is a quantitative measure of clonal toxicity, and does not consider qualitative differences in toxins. We assert that because of the acclimation process the varying potency of these extracts among clones is due to interclonal genetic differences and not to environmental differences.

As a control experiment the extract potencies of several clones from the first and the last one liter culture were compared in order to assess how significant the acclimation process is. In addition, the potency of extracts from clone 135 were compared monthly in order to determine when acclimation was achieved and once achieved, how stable a character it is. An additional control experiment was run with the Martinique (Caribbean) clone (clone 175). Clone 175 has been sub-cloned so that we can assess any toxicity differences which may result from micro-environmental differences in the culture chambers. Only toxicities which exceed the methodological errors were used for "chemosystematic" comparisons ("chemo" referring to the toxins and "systematic" to the clonal comparisons). The material from the 15 liter cultures was used to assess qualitative differences in toxins among the clones.

Previous work (**41**) has determined that it is not possible to completely identify a systematic "variant clone" or "race" of *G. toxicus* by analyzing only one character, e.g. extract potency. Consequently, this project includes an "acclimated reproduction rate comparison" (**42**). In this study the response to light of different intensities by the different clones of *G. toxicus* is being monitored. Four light intensities are being used and include 80, 160, 205 and 250 footcandles of illumination. The reproduction rate (divisions per day) of the clones is plotted against the light intensity and the resulting slope of the line is used as a numerical systematic character. It is critical that the cultures be completely acclimated in this study, as in the toxicity study. The slope of the line is a valid genetic character because the reproduction rate is under enzymatic control and varies with the efficiency of enzymatic transcription

(hence chromosomal differences) under different environmental conditions. These data from these studies will also be useful as a data base in physiological studies which will examine the effect of light on toxin production.

Seventeen clones of the ciguatera-causing dinoflagellate *Gambierdiscus toxicus* were physiologically adapted to the same environment over several months. There were significant variance components detected between non-acclimated and acclimated cells for the cell potencies, yields and reproduction rates of these cultures.

Toxin potencies

Outbred Harlan Sprague Dawly ICR(BR) mice weighing approximately 20 g were used to assess quantitative differences in the potencies of the methanol extracts. The potencies were then determined as LD_{50}'s for each clone using from 3 to 10 mice at each of 4 dosage levels. The mice were observed for 48 h. The LD_{50}'s were determined from the linear interpolation tables of Weil (**43**). Alternatively, in some cases the LD_{50} was determined by linear regression (**44**). The final values are expressed as the number of mouse units (LD_{50} dose for a 20g mouse) per mg of dried cells, per cell of *G. toxicus*. and in terms of number of cells per mouse unit.

The acclimated and non-acclimated cell potencies were compared in the same manner as the growth rate and cell yield statistics. In addition, a one-way Analysis of Variance with Replication (**44**) was used to compare selected clones from broad areas. The f_{max} test (**44**) was used to assess homogeneity before conducting all ANOVA's The potencies used in this ANOVA were plotted vs. latitude of collection along with the values for all other clones. The clones used in generating the ANOVA are also plotted with their 95% comparison intervals, calculated from and a-posteriori T-method comparison among means (**44**). Potency and latitude were also tested for correlation by the Pearson test (**44**).

Three clones (177, 350 and 135) were also assayed for potency at 6 to 11 points during the growth cycle and these were plotted vs. days in culture and compared. The two parameters were then tested for correlation by the Pearson test (**44**). For clone 177, 3 of 11 samples were assayed for potency 4x and compared by one-way ANOVA to test for significance of variation in potency through the acclimation process. The means from this test were also compared by the T-method. The potency and reproduction rate of clone 177

was also monitored after continuous batch culturing was stopped and transfers were made in stationary phase. Potency changes for clone 350 were monitored through acclimation to the vita lite bulbs and then when returned to a cool white light environment at a similar light intensity.

As a control experiment on sub-clone variability, clone 175 was sub-cloned 25x, all isolates survived and 4 were selected randomly to determine the coefficient of variation among fully acclimated sub-clones. As a control experiment on the stability of the acclimated condition, clone 135 is still being monitored for potency data accumulation beyond one year.

Five other relationships were also explored by correlation analysis and include potency vs. cell size and potency vs. reproduction rate for all clones examined. The former test was performed on acclimated potencies only whereas the latter test included all data points. Cell size was determined as the transdiameter and computed from a minimum of 20 cells of each clone collected from a log-phase culture. The potency was also tested for correlation with chlorophyll a, chlorophyll C_2 and peridinin content (pg $cell^{-1}$). Pigments were assayed via the methods of Jeffrey et al. (**45**) and Indelicato and Watson (**46**). They were subsequently extracted and analyzed for composition of proteins (Bio-Rad method), lipids (**47**) and carbohydrates (**48, 49**).

A correlation matrix was constructed among clones based on all of the aforementioned characters as well as ammonium and nitrate uptake rates and the gamma slope (light, growth response; **50**; **41**) at four light intensities. In addition, the average number of cells mL^{-1} and the number of days required for complete acclimation were also treated as phenotypic characters. The correlation matrix was subjected to Factor Analysis (**44**) using the computer program Statview 512 (BrainPower, Inc., Calabasas, CA). Two factors were extracted from the matrix and plotted by the principal components method. The analysis was repeated twice by successively removing those characters contributing the smaller percentages of the total variation.

Extraction of toxins

The initial methods for the extraction of the toxins is very similar to what is currently being utilized by other investigators (especially those attempting to isolate toxin from fish tissues) so that,

to some extent, we can compare the toxic fractions which we obtain with what is in the literature. The cells are no longer extracted by refluxing in boiling aqueous methanol. Instead, extraction is now accomplished by crushing of cell, followed by sonication in methanol at room temperature. The methanol extracts are concentrated and subjected to liquid-liquid partitioning followed by cold acetone precipitation of the toxic components.

Purification

Further purification of the toxin will be either by thin layer chromatography (TLC), silicic acid chromatography or high pressure liquid chromatography (HPLC). Eluting solvents for column chromatography consist of chloroform-methanol (1:1), and chloroform-, methanol-water mixtures (9:1:1). The eluting solvent for HPLC is methanol (100%).

HPLC purification

We have been working out the methodology for the separation of toxins with a minimum of loss on HPLC. We feel that a preparative HPLC separation approach conserves toxin, saves time, and is cheaper in the long run as opposed to regular column chromatography. We presently perform the initial purification on C-18 columns with methanol as the solvent. Separation is achieved using three HPLC associated with a single computer controller system. All three are Waters Company instrumentation and consists of a Delta 3000 Preparative-Semipreparative HPLC and a Model 300 Analytical system all interlinked with SIM modules to an 820 Controller System. While we use C-18 columns in each system, the sizes differ, having 15 μ in the Preparative and Semipreparative and 10 μ or 5 μ in the Analytical.

Mouse bioassay

The mouse bioassay is the officially recognized toxicity assay for ciguatera recommended by the Official Organization of Analytical Chemists and the FDA. In addition, it provides a base line against which we can compare our isolated preparation assays. The carrier for toxic extracts is normal saline containing 0.5 ml of a 1% Tween-60 solution. Toxicity is determined by an intraperitoneal injection of 0.5 ml of a suspension of extract into approximately 20 g mice (Strain CRE:CD:BR:ICR). Toxicity is defined as death of the mouse within 48 hours. LD_{50} values are calculated according to the method of Weil (**43**). Four dosage levels are used with three repetitions at each

level. The LD_{50} is calculated from moving average interpolation tables.

Ileum assay procedures

We have utilized the terminal portion of the guinea pig ileum to assay dinoflagellate toxins. The overall setup for the ileum assay is shown in Figure 2.

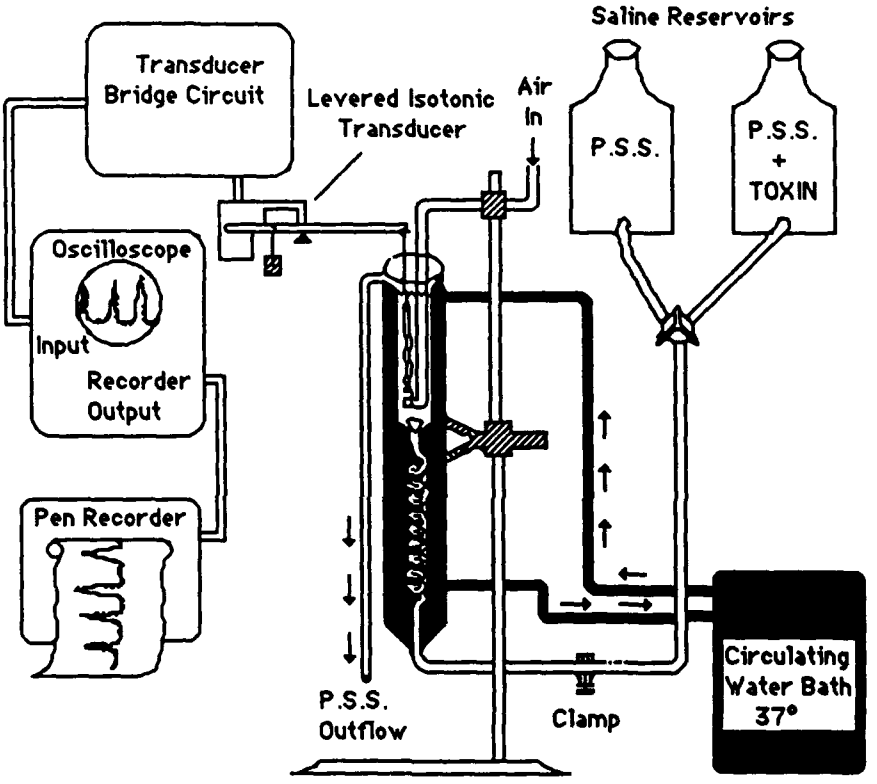


Figure 2. Schematic diagram of the equipment setup for the guinea pig ileum preparation. Individual components are discussed in the textual material.

Guinea Pig Ileum. Female guinea pigs (350-600 g) were sacrificed by a cervical dislocation. A 2-4 cm segment of the terminal ileum was removed and placed in physiological saline solution (PSS) at 37°C. The terminal portion will, however, respond to exogenously applied agonists. Hence its suitability for use in an assay system. The physiological saline solution for the guinea pig ileum consists of the following (mM):

TABLE 1
PHYSIOLOGICAL SALINE COMPONENTS

CHEMICAL	mM	g/L	Stock g/L	amt/L
NaCl	136.9	7.014		weigh out
KCl	2.68	0.372	186.37	2.0 ml
$CaCl_2$	11.84	0.277	55.5	5.0 ml
$MgCl_2$	1.03	0.095	23.8	4.0 ml
$NaHCO_3$	11.9	0.084	8.4	10.0 ml
KH_2PO_4	0.45	0.178	89.1	2.0 ml
glucose	5.55	1.980		weigh out

The excised terminal ileum is allowed to come to temperature in saline for 15 min then a one cm segment is cut and inserted in an Anderson type tissue chamber (**51**) modified as suggested by Bartelstone (Figure 2). The guinea pig ileum is extremely temperature sensitive and changes as small as 0.3°C may affect its contractility and therefore the results. For this reason one must have a circulating water bath or some other means of controlling the temperature. The circulating water bath that we presently use is Fisher Model M8000, Isotemp, Constant Temperature Circulator. The ileum is connected to a locally-made device which is a true, isotonic-tension transducer. The counter weight we use is 2 grams. Amplification of the transducer signal is achieved by the use of a DC oscilloscope with a pen output. For storage of records and recording of data for calculations we chose an inexpensive chart recorder (Fisher Recordall Model 5000). Any good DC level recorder can be used, but a ten inch recorder makes the task of measurement much easier. The ileum has been shown to respond to acetylcholine, histamine, substance P, epinephrine, and several other compounds. Indeed, it has varying sensitivity to each of these compounds. We have found that the toxic fractions will give different inhibitions with different agonists.

Protocol for Reversible Toxins. The first protocol followed is utilized when there is only a reversible toxin in the extract. Initially a control series of acetylcholine or histamine stimulations is performed at different dosages to determine three doses which would give us values between 20 and 80% of maximum contraction of the ileum segment (Figure 3). That particular set of test doses is then utilized throughout the rest of the experiment. All subsequent responses of the ileum to agonist challenge are expressed as a percent of control values.

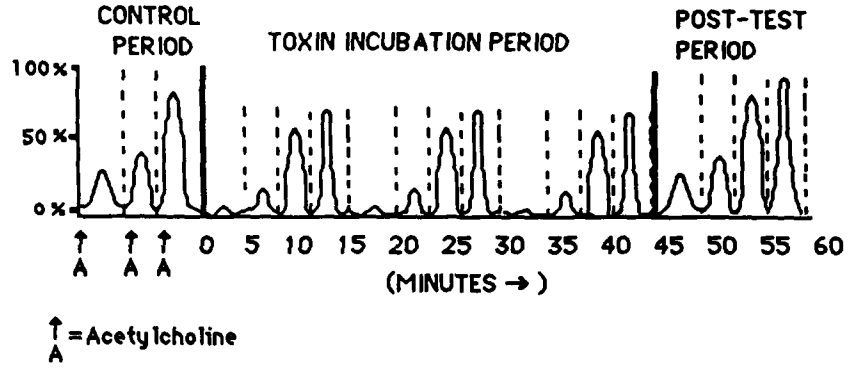


Figure 3. Schematic diagram of the methodology for the assay of a reversible toxin.

The toxin containing physiological saline solution is utilized for subsequent washes. Thus when testing for reversible inhibition, the toxin is always present in the PSS at the same concentration.

Non-reversible Assay. The second protocol is utilized when it is suspected that there only a non-reversible toxin in the extract. First a control series of histamine stimulations is performed at different dosages to determine a dose which would give us approximately 80% of maximum contraction of the ileum segment (Figure 4). That particular test dose is then utilized throughout the rest of the experiment. All subsequent responses to histamine are expressed as a percent of that control value.

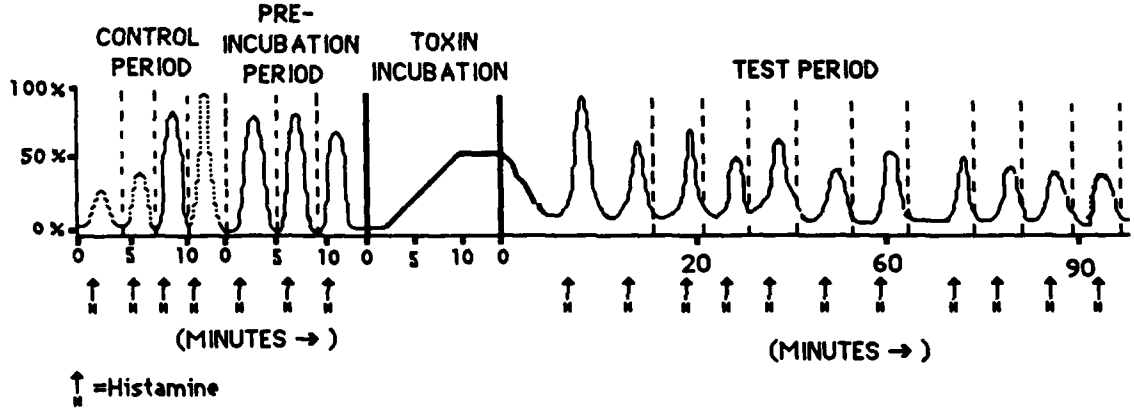


Figure 4. Schematic for the analysis of an irreversible toxin using the guinea pig ileum.

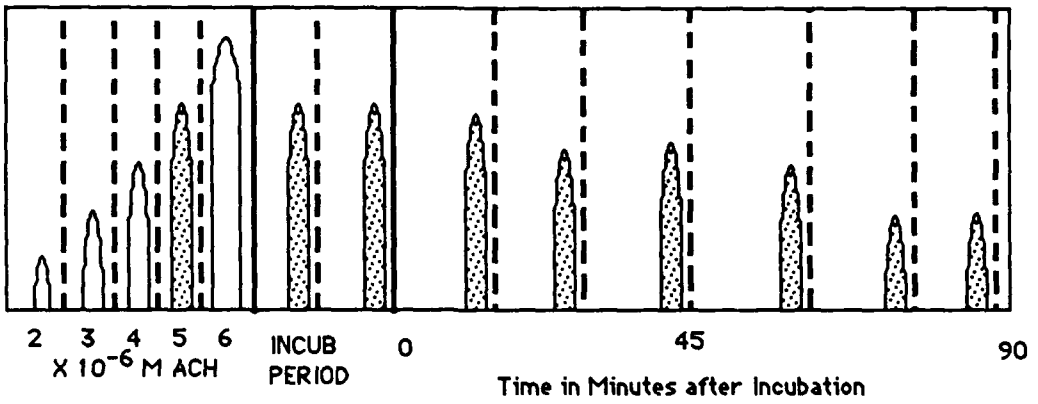


Figure 5. Schematic diagram illustrating methodology for using the ileum assay when both an irreversible toxin and a reversible toxin is present.

Tests for purity of toxins

The easiest manner to test for purity of the toxin is to run in a recycle mode on the analytical HPLC to determine if after a given number of recycles the single peak remains or resolves into more than one peak. We will use several criteria for testing for purity: first the presence of single peak after recycling on analytical HPLC; second, migration on TLC plates in different solvent systems, and structural data from Nuclear Magnetic Resonance.

NMR spectroscopy

At the present time Nuclear Magnetic Resonance (NMR) would provide us with the most informative data and yet is non-destructive to the sample analyzed. For these reasons it is the method of choice. Samples of purified toxins will be sealed in special, thin-walled, small-bore, NMR sample tubes from Wilmad Glass Co. Two state of the art NMR instruments are presently available for our use: specifically a 300 MHz Varian VXR-300 and a 500 MHz Varian VXR-500 multinuclear spectrometer system. Both instruments operate in the pulse Fourier Transform mode and are equipped with a liquid helium VXR superconducting magnet and acquisition hardware. Both have H_2 fixed frequency lock system with 5 mm broad band computer switchable probe. Multiple probes to two chambers accepting, 5 mm (narrow bore) and 10 mm (medium-wide bore) tubes for ^{1}H, ^{13}C, ^{31}P, ^{15}N, ^{19}F and other nuclei. Quadrature detection. Homo- and hetero-nuclear decoupling with spectral limits of 100 to

100,000 Hz. Variable temperature control (-70° to 140°) under computer control for all probes. VXR data station with dual high density disks. The VXR-300 operates at 300 MHz ^{1}H resonance and is presently used primarily for ^{13}C analysis. Automatic performance of standard relaxation experiments, as well as data recording is accomplished by an associated computer. The chemical shifts recorded are then interpreted for structure.

Packaging of toxins for delivery

Purified toxins in 100% methanol will be placed into vials, concentrated to almost dryness under nitrogen gas, sealed and labeled for shipment.

Acquisition of toxic dinoflagellates

Since the inception of our work on Ciguatera in 1978 we have conducted an extensive survey of ciguatera endemic regions of the British and United States Virgin Islands which resulted in the collection of 46 species of dinoflagellates. Sixty-five strains representing 18 of the most conspicuous epiphytic, benthic and planktonic species were isolated and brought into unialgal culture, harvested, and subjected to our standard extraction procedures. We proposed to continue our yearly survey for toxic dinoflagellates.

Overall objectives for the second year

Our second years objectives can be summarized as follows:

(1) Continuation of mass culture of *Gambierdiscus toxicus*.
(2) Biosystematic studies to improve toxin production.
(3) Extraction of crude toxins from mass cultures.
(4) Bioassays each of the isolated extracts.
(5) Purification of toxic fractions.
(6) Investigation of factors affecting the stability of stored toxins.
(7) Quantization of toxins by weight and bioassay.
(8) Examination of toxins by NMR.
(9) Delivery of toxic fractions to U. S. Army Medical Research Institute of Infectious Diseases.
(10) Continue to search for additional toxic dinoflagellates for addition to the SIU culture collection.
(11) Maintain the SIU culture collection.

RESULTS

Results of Systematic Studies

Seventeen clones of *G. toxicus* have been acclimated in one liter volumes and taken through the 15 liter culture phase (Tables 2 & 3). The clones varied in their acclimation periods ranging from 60 days for clone 175 to nearly a year for other isolates. An example of the acclimation process (clone 157) is shown in Figure 6 and Tables 2.

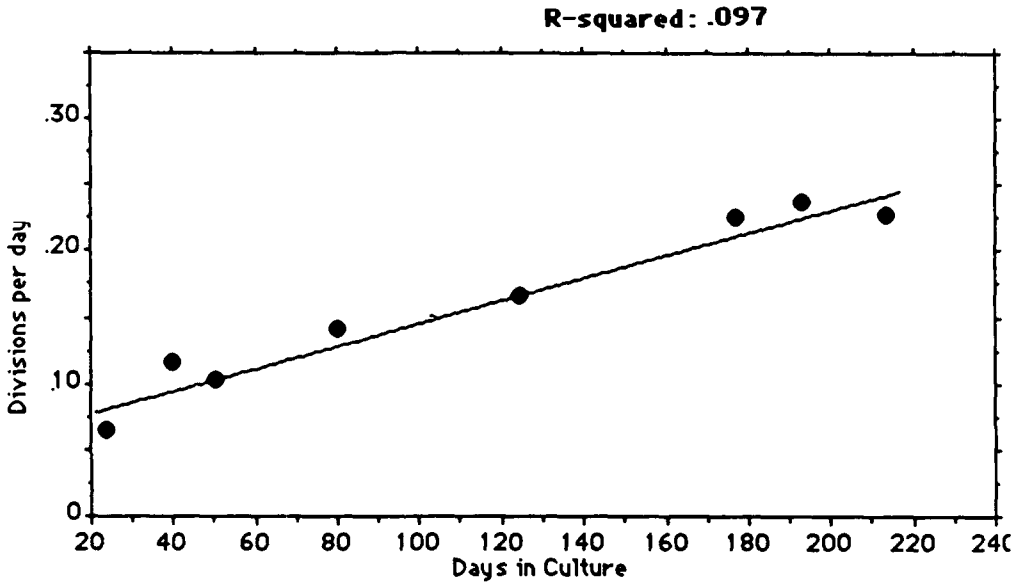


FIGURE 6. Acclimation of clone 157.

The acclimation process is also important in terms of stabilizing growth rates as is evidenced in Table 4, a. two-way ANOVA without replication examining variation in growth rates for 9 clones with similar growth rates through 7 culture periods. This ANOVA reveals that the acclimation process is highly significant and indicates that the physiological status of the cultures changes significantly through time.

Table 2

ACCLIMATION DATA OF *GAMBIERDISCUS TOXICUS* CLONES IN ONE LITER VOLUMES (160 FOOT-CANDLES OF LIGHT)

Clone 175							
	Interval From:	8/5	9/2	9/18			
	To	-9/2	-9/30	-10/19*			
	Div/Day	.20	.24	.27			
	Yield mg/L	(1)	93	269			
	Yield cells/ml	(1)	2166	3085	COMPLETED		
Clone 350							
	Interval From:	8/5	9/2	9/28	10/21	11/9	12/3
	To:	-9/2	-10/9	-11/12	-12/7	-12/22	-2/1*
	Div/Day	.10	.11	.10	.13	.14	.15
	Yield mg/L	(1)	269	103	184	192	(1)
	Yield cells/ml	(1)	809	1333	989	989	(1)
	COMPLETED						
Clone 135							
	Interval From:	8/5	9/3	9/25	10/15	11/5	
	To:	-9/4	-10/9	-11/5	-12/3		
	Div/Day	.12	.11	.17	.17	.16	
	Yield mg/L	107	319	259	171	(1)	
	Yield cells/ml	949	2059	1330	2818	(1)	COMPLETED
Clone 135 continued as time control							
	Interval From:	1/21	2/11	2/25	3/10	3/25	4/11 4/25
	To:	-2/22	-3/7	-3/28	-4/18	-4/25	-4/20 -5.23
	Div/Day	.15	.16	.18	.15	.19	.19 .19
	Yield mg/L	131	164	150	163	105	169
	Yield cells/ml	2022	2439	2628	2249	1870	2249
Clone 177							
	Interval From:	8/24	9/18	10/20	11/12	12/3	1/21 2/4
	To:	-9/21	-11/5	-12/3	-12/22	-1/5	-2/22 -3/7
	Div/Day	.15	.11	.09	.13	.17	.21 .23
	Yield mg/L	118	184	135	227	(1)	130 135
	Yield cells/ml	800	2275	1128	1059	(1)	857
Clone 177 continued from above							
	Interval From:	3/14					
	To:	-4/14					
	Div/Day	.18					
	Yield mg/L	(1)					
	Yield cells/ml	(1)	COMPLETED				
Clone 163							
	Interval From:	8/8	9/2	9/10	10/20	11/9	12/3
	To:	-9/9	-10/7	-9/25	-12/3	-12/22	-1/5
	Div/Day	.17	.12	.12	.18	.14	.14
	Yield mg/L	147	192	207	257	269	(1)
	Yield cells/ml	1040	1166	1655	1912	1014	(1)
	COMPLETED						

Table 2. Continued

ACCLIMATION DATA OF *GAMBIERDISCUS TOXICUS* CLONES IN ONE LITER VOLUMES (160 FOOT-CANDLES OF LIGHT)

Clone 158							
Interval From:	8/5	9/2	9/28	10/19	11/12	12/10	1/14
To:	-9/9	-10/9	-11/5	-12/14	-12/22	-2/4	-2/22
Div/Day	.07	.12	.12	.15	.16	.11	.20
Yield mg/L	134	373	191	234	195	141	
Yield cells/ml	1130	1701	1695	2409	1330	1331	
Clone 158 Continued from above							
Interval From:	2/8	2/25	3/17				
To:	-3/7	-3/28	-4/4				
Div/Day	.21	.20	.20				
Yield mg/L	133	175	(1)				
Yield cells/ml	1331	1485	(1)	COMPLETED			
Clone 157							
Interval From:	8/5	9/2	9/28	10/21	11/12	12/7	
To:	-9/9	-10/19	-11/5	-12/3	-12/22	-1/5	
Div/Day	.08	.08	.10	.14	.16	.16	
Yield mg/L	140	268	176	129	189	(1)	
Yield cells/ml	1260	1970	2240	3003	2791	(1)	
COMPLETED							
Clone 199							
Interval From:	8/8	9/4	9/25	10/23	11/17	12/10	
To:	-9/4	-10/19	-11/5	-12/3	-12/22	-1/5	
Div/Day	.12	.12	.12	.16	.15	.15	
Yield mg/L	99	216	117	113	255	(1)	
Yield cells/ml	949	1400	1730	1896	1503	(1)	
COMPLETED							
Clone 169							
Interval From:	8/5	9/4	9/25	10/20	11/12	12/10	
To:	-9/9	-10/7	-11/12	-12/3	-12/22	-1/5	
Div/Day	.11	.10	.10	.12	.13	.12	
Yield mg/L	162	197	195	142	203	(1)	
Yield cells/ml	1000	844	1320	1165	1000	(1)	
COMPLETED							
Clone 196							
Interval From:	1/25	2/11	2/25	3/10			
To:	-2/22	-3/15	-3/28	-3/25			
Div/Day	.19	.20	.23	.23			
Yield mg/L	178	238	206	(1)			
Yield cells/ml				(1)	COMPLETED		
Clone 165							
Interval From:	2/1	2/11	2/25	3/14	3/24		
To:	-2/22	-3/7	-3/18	-4.8	-4/8		
Div/Day	.15	.19	.24	.22	.21		
Yield mg/L	140	101	222	141	(1)		
Yield cells/ml					(1)		

Table 2. Continued

ACCLIMATION DATA OF *GAMBIERDISCUS TOXICUS* CLONES IN ONE LITER VOLUMES (160 FOOT-CANDLES OF LIGHT)

Clone GT200						
	Interval From:	2/15	3/3	3/17		
	To:	-3/15	-4/8	-4/8		
	Div/Day	.15	.14	.18		
	Yield mg/L	196	114	(1)		
	Yield cells/ml			(1)	COMPLETED	
Clone 172						
	Interval From:	1/21	2/8	2/25	3/10	3/24
	To:	-2/22	-3/7	-3/28	-4/14	-4/15
	Div/Day	.13	.17	.21	.22	.22
	Yield mg/L	157	153	136	178	(1)
	Yield cells/ml					(1)
Clone GT300						
	Interval From:	2/8	3/3	3/17	4/4	
	To:	-3/15	-3/28	-4/14	-4/20	
	Div/Day	.19	.20	.20	.20	
	Yield mg/L	122	151	200	(1)	
	Yield cells/ml				(1)	COMPLETED
Clone 171						
	Interval From:	2/15	3/3	3/17	3/31	
	To:	-3/14	-3/28	-4/25	-4/20	
	Div/Day	.2	.22	.22	.22	
	Yield mg/L	71	122	204	(1)	
	Yield cells/ml				(1)	COMPLETED
Clone 170						
	Interval From:	2/18	3/3	3/21	4/7	
	To:	-3/7	-4/8	-4/25	-4/25	
	Div/Day	.17	.17	.17	.17	
	Yield mg/L	89	184	186	(1)	
	Yield cells/ml				(1)	COMPLETED

Table 3

ACCLIMATION OF *GAMBIERDISCUS TOXICUS* IN 15 LITER VOLUMES

	Acclimated Rate of Reproduction Div/Day	YIELD cells/ml	UPTAKE RATE NH_4 pg/cell/hr	UPTAKE RATE NO_3 pg/cell/hr	TOXICITY Initial MU/mg	TOXICITY Acclim. MU/mg
Clone 175 Axenic Culture, Caribbean ip						
Clone 175-1 Subclone (not Axenic) Caribbean	.26	2,618				112.29
Clone 175-2 Subclone (not Axenic) Caribbean	.25	2,700				110.41
Clone 175-3 Subclone (not Axenic) Caribbean	.27	2,700				128.69
Clone 175-4 Subclone (not Axenic) Caribbean	.25	2,618				127.64
Clone 175 (Standard) Caribbean	.27	2,760	18.8	35.56	55.06	120
Clone 350 Caribbean	.16	1,095	7.3	63.4	6.90	18
Clone 135 Bermuda	.17	1,190	9.3	50.74	3.72	5.39
Clone 177 Hawaii	.21	1,250	10.0	42.1	4.6	30
Clone 199 Florida Keys	.17	1,109	6.9	60.38	4.22	17.5
Clone 300 Florida Keys	.20	1,200	8.0	47		7.3
Clone 200 Florida Keys	.18	723				3.88
Clone 196 Florida Keys	.23	1,108	9.2	41.6		4.0
Clone 163 Bahamas Gingerbread	.14	860	13.5	31.46	4.52	9.85
Clone 171 Bahamas Gingerbread	.22	2,208	5.1	27.4		3.0
Clone 170 Bahamas Gingerbread	.17	800	12.8	32.4		5.1
Clone 157 Bahamas Drift Clones	.25	1,890	19.3	16.43		2.07
Clone 158 Bahamas Drift Clones	.20	848	12.7	22.7		5.60
Clone 165 Bahamas Drift Clones	.21	2,100	6.0	33.9		12.79
Clone 169 Bahamas Drift Clones	.14	660	6.5	39.3	4.09	4.40
Clone 172 Bahamas Great Isaacs	.22	2443	7.8	71.7		
	2.2					

Table 4.

TWO-WAY ANOVA WITHOUT REPLICATION EXAMINING VARIATION IN GROWTH RATES FOR 9 CLONES WITH SIMILAR GROWTH RATES THROUGH 7 CULTURE PERIODS.

Source of Variation	df	SS	MS	Fs
Among clones	7	0.010	0.00143	2.38
Among culture periods	6	0.0390	0.00650	10.83**
Error	42	0.025	0.00060	
	55			

** = $P < 0.01$

An additional ANOVA examining variation in reproduction rates between acclimated and non-acclimated cells (Table 5) also indicates that the variation is significant ($P < 0.01$).

TABLE 5.

PAIRED ANALYSIS OF VARIANCE EXAMINING VARIATION BETWEEN NON-ACCLIMATED AND ACCLIMATED REPRODUCTION RATES.

Source of Variation		Degrees of freedom	Sum of squares	Mean squares	F value[a]
Culture stage	1	0.6241	0.6241	1365.3**	
Clones	14	0.0360	0.0025714	5.6**	
Remainder	14	0.0064	0.0004571		
Total		29			

[a] **Significant at 0.01 level.

There was also significant variation among clones detected in this test. The mean reproduction rate for the non-acclimated cultures was 0.138 and 0.195 for acclimated cultures. Cell yields also improved from 115 mg L^{-1} to 183 mg L^{-1}. The variation between cell yields in the non-acclimated vs. acclimated condition was also significant ($P < 0.01$, Table 6).

TABLE 6.
PAIRED ANALYSIS OF VARIANCE EXAMINING VARIATION BETWEEN NON-ACCLIMATED AND ACCLIMATED CELL YIELDS.

Source of Variation	Degrees of freedom	Sum of squares	Mean squares	F value[a]
Culture stage	1	38964.73	38964.73	19.8194**
Clones	16	22,563.53	1410.22	0.7173**
Remainder	16	31455.77	1965.99	
Total	33			

[a] **Significant at 0.01 level.

Mean cell potencies also improved along with reproduction rates and cell yields through the acclimation process. The number of days in culture and the potency were positively correlated ($P < 0.60$, Figure 7) for clones 177, 135 and 350.

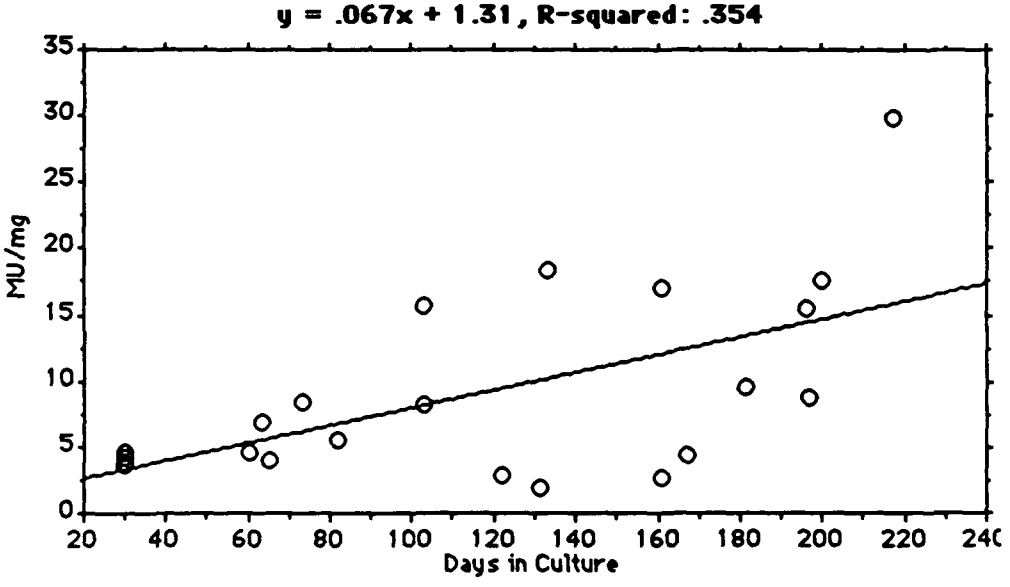


FIGURE 7 Potency (MU mg^{-1}) vs. days in culture for clones 177, 350, 135, 199, 169 and 163.

Table 7 gives data comparing non-acclimated to acclimated potencies for six clones. The initial mean cell potency for clones 175, 350, 177, 199, 169, 163 and 135 was 13.01 MU mg^{-1} compared to the acclimated value for these clones of 25.95 MU mg^{-1}.

A paired ANOVA examining variation between non-acclimated and acclimated cell potencies indicates that there are significant differences between the phases (Table 8).

Table 7.
EXTRACT POTENCY DIFFERENCES IN THE NON ACCLIMATED VS. ACCLIMATED PHYSIOLOGICAL STATE.

CLONE	INITIAL POTENCY (MU/mg)	ACCLIMATED POTENCY (MU/mg)
175	55.06	102.25
350	6.90	18.33
135	3.72	5.55
19	3.92	15.30
163	4.52	9.85
169	4.09	4.40
Mean	13.04	25.95

TABLE 8
PAIRED ANALYSIS OF VARIANCE EXAMINING VARIATION BETWEEN NON-ACCLIMATED AND ACCLIMATED CELL POTENCIES.

Source of Variation	Degrees of freedom	Sum of squares	Mean squares	F value[a]
Culture stage	1	886.11	886.11	7.48**
Clones	6	8230.32	1371.72	11.57**
Remainder	6	711.16	118.53	
Total	13			

[a] **Significant at 0.01 level.

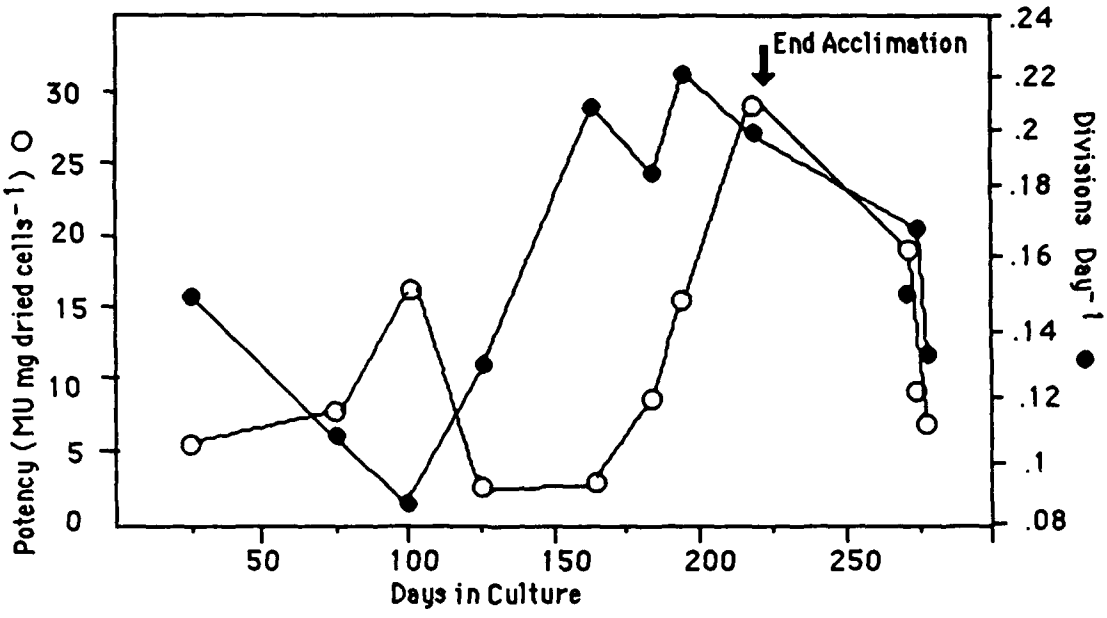


FIGURE 8. Potency (MU mg^{-1}) and reproduction rate (Div. day^{-1}) vs. number of days in culture for clone 177. The arrow denotes the end of the acclimation process. Three potency determinations are shown with their 95% comparison intervals generated by the T-method. Intervals which do not overlap are significantly different.

An examination of the acclimation process in more detail for clone 177 indicates that potency shows a slow improvement with time (Figure 8). The potency appears to be sensitive to environmental changes and the acclimated cell potency could easily be lost as was shown in Figure 8 when successive transferring begin in stationary instead of log phase. The potency appears to follow reproduction rate closely but improvements in the potency lag behind those in growth rate.

TABLE 9.

ONE-WAY ANALYSIS OF VARIANCE WITH REPLICATION EXAMINING VARIATION AMONG POTENCIES ON THREE OCCASIONS DURING ACCLIMATION FOR CLONE 177.

Source of Variation	Degrees of freedom	Sum of squares	Mean squares	F value[a]
Among culture stages	2	1129.612	564.806	31.562****
Within stages	9	161.055	17.895	
Total	11	1290.667		

[a] **Significant at 0.0001 level.

The variation among potencies over time for clone 177 was significantly different ($P < 0.0001$, Table 9) and the means are compared by the T-method (Figure 9). The T-method indicates that the potencies calculated at less than 200 days are significantly different from the one calculated at greater than 200 days. Figure 9 shows the potency changes for clone 350 which indicates that the acclimated potency was lost when the clone was transferred to a new light environment. We did not monitor the potency past 140 days in the acclimated state for this clone. However, we did continue to monitor reproduction rate and this did not improve past 140 days.

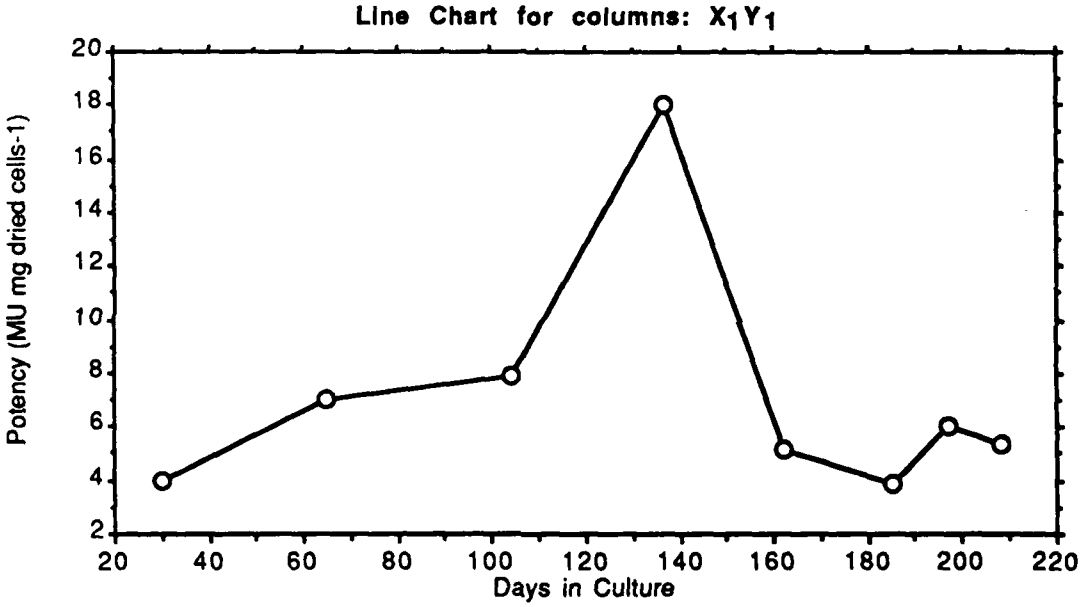


FIGURE 9. Potency (MU mg-1 dried cells) vs. number of days in culture for clone 350. The first four potencies were determined under Vita-lite bulbs and the last four under cool-white bulbs.

A summary of the data compiled on the seventeen clones examined is given in Table 9. A one-way ANOVA with replication examining variation in potencies among six clones indicates that the variation in cell potencies is significant (Table 11). An a-posteriori T-method (Figure 10) indicates that two clones collected from Marathon Key (199 and 200) are significantly different from one another. In addition there are larger differences among clones collected from different areas (Figure 10).

There is a general trend of decreasing potency among the clones with increasing latitude (Figure 10). Latitude and the acclimated potencies are negatively correlated ($r = -0.819$, $P < 0.01$).

TABLE 10.

SUMMARY OF RESULTS FOR THE DIFFERENT CLONES IN THE GENETIC STUDY.

Clone	Cells mL^{-1}	Mg L^{-1}	Div. day^{-1}	MU Mg^{-1}	MU $Cell^{-1}$	Cells MU^{-1}
Caribbean						
175	2659	122	0.26	120	55	183
350	781	116	0.16	18	27	368
Hawaii						
177	1488	134	0.21	30	27	372
Florida Keys						
196	1317	254	0.23	4.1	8.0	1263
199	2158	205	0.16	17.5	16.7	601
200	762	116	0.14	2.6	3.9	2537
300	811	200	0.20	7.3	18.0	555
Bahamas						
162	1300	109	0.13	5.5	4.6	2170
163	938	212	0.15	8.8	19.8	504
170	556	187	0.14	5.1	19.2	522
171	430	79	0.19	3.0	5.5	1804
172	1019	178	0.22	2.2	3.8	2605
Drift Algae						
157	2369	148	0.14	2.9	1.8	5512
158	1485	175	0.20	5.6	6.6	1511
165	1301	176	0.22	12.8	17.3	577
169	860	203	0.11	4.4	10.4	963
Bermuda						
135	2249	109	0.19	11.1	5.4	1861

TABLE 11

ONE-WAY ANALYSIS OF VARIANCE WITH REPLICATION EXAMINING VARIATION AMONG THE ACCLIMATED POTENCIES OF CLONES 175, 177, 199, 200. 157, 163 AND 135.

Source of Variation	Degrees of freedom	Sum of squares	Mean squares	F value[a]
Among clones	6	43.703	7.284	123.182****
Within stages	21	1.242	0.059	
Total	27	44.945		

[a] **Significant at 0.0001 level.

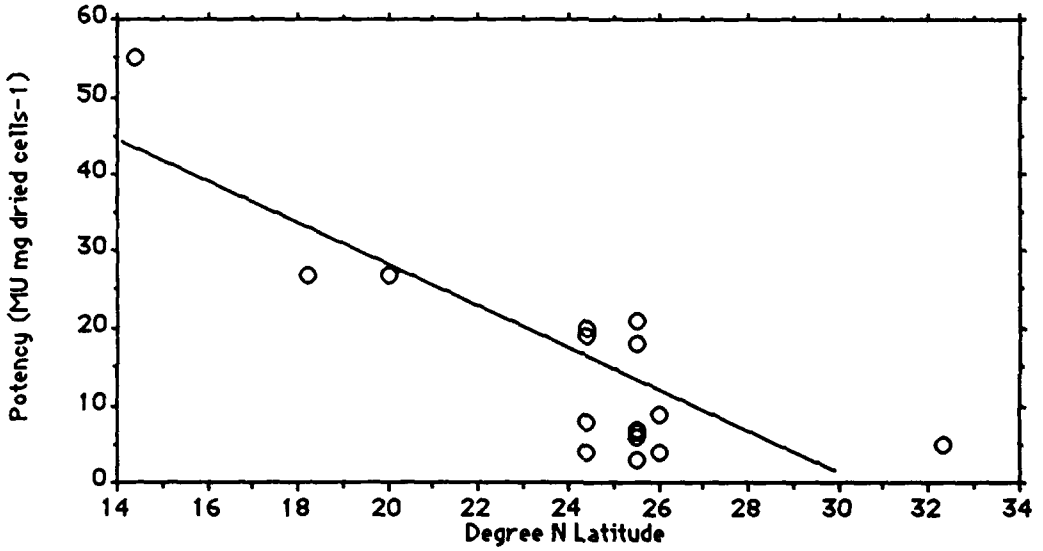


FIGURE 10. Potency (MU cell^{-1}) vs. latitude for all clones examined in the genetic study. Seven clones are shown with their 95% comparison intervals generated by the T-method and based upon the ANOVA in Table 10. Intervals which do not overlap are significantly different.

TABLE 12.

SUB-CLONE VARIABILITY OF CLONE 175.

Sub-clone	Div. day^{-1}	Final cell Yield (cells/ml-1)	MU/mg^{-1}
8	0.27	2,700	128.69
13	0.26	2,618	112.29
16	0.25	2,600	127.64
22	0.25	2,710	110.41
Mean	0.26	2,657	119.78
Stan. dev.	0.01	56	9.74
coeff. of variation	4.09%	2.24%	8.64%

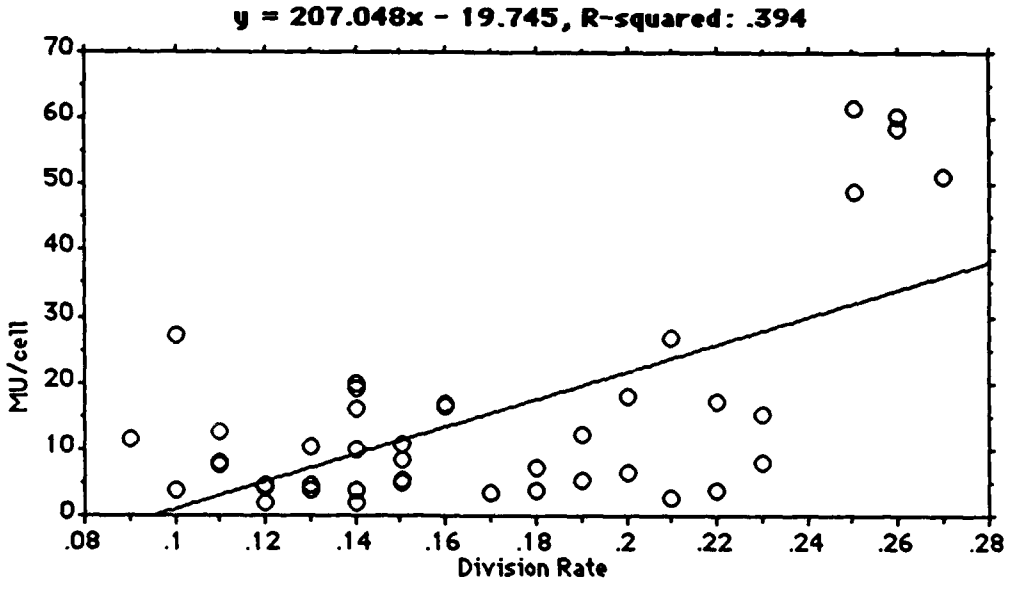


FIGURE 11. Potency (MU cell^{-1}) vs. reproduction rate (Div. day^{-1}) for all clones examined in the genetic study.

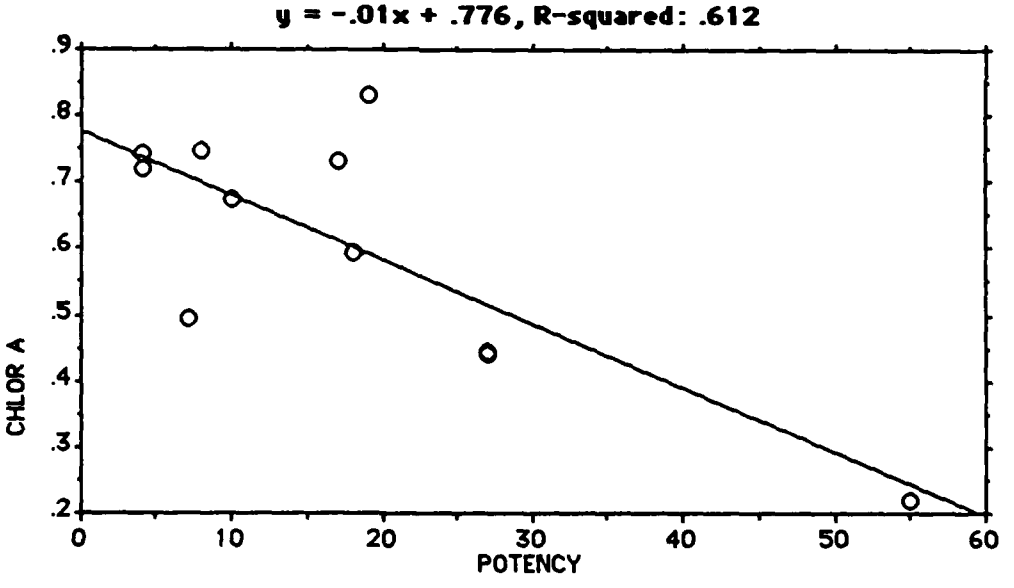


FIGURE 12 Potency (MU cell^{-1}) vs. chlorophyll a concentration (pg cell^{-1}) of twelve clones.

Reproduction rates vs. acclimated cell potencies for all clones examined were positively correlated (Figure 11, $r = 0.628$, $P < 0.01$). However, the correlation between cell size and potency was not

significant. There were negative correlations between acclimated clonal potencies and acclimated pigment content, chlorophyll a (Figure 12, r = -0.782, P < 0.01) and chlorophyll c_2 (r = -0.713, P < 0.01, Figure 13). There was no significant relationship between peridinin content and potency.

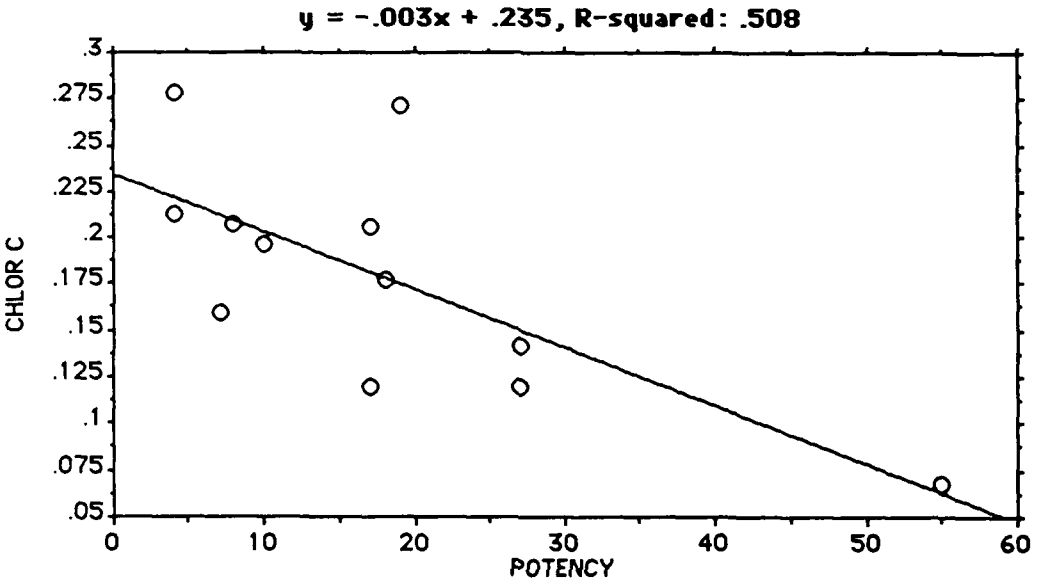


FIGURE 13 Potency (MU cell^{-1}) vs. chlorophyll c_2 concentration (pg cell^{-1}) of twelve clones.

Response of *G. toxicus* clones to different light intensities

Clones have been grown at four light intensities: 40, 80, 160 and 250 footcandles illumination. The toxicity of these clones is currently being compared. In terms of growth rate the clones show significant differences in their light/growth response (Figure 14). These results were generated from tube cultures. In a more detailed examination of the light/growth response for clone 175, there are obvious differences among light intensities (Table 13).

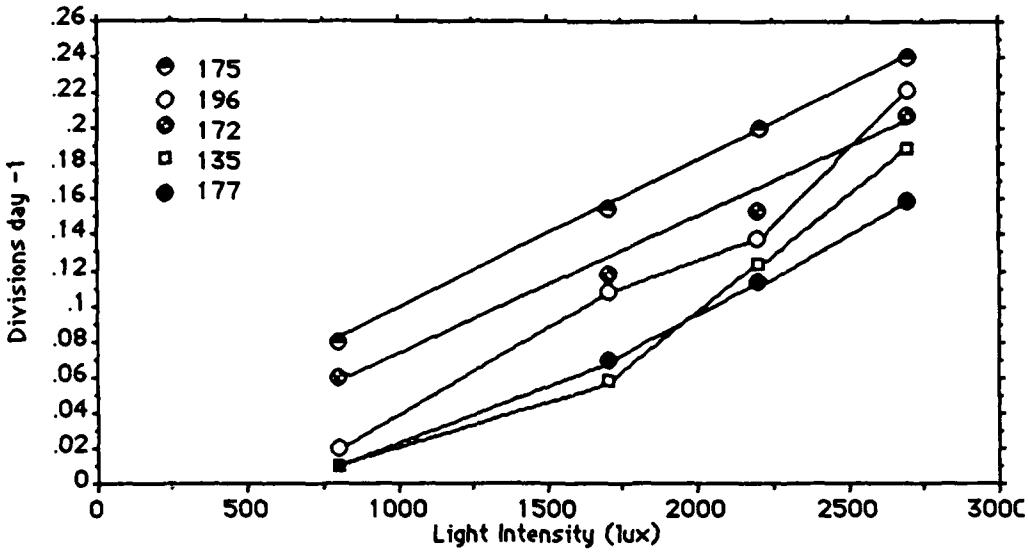


FIGURE 14. Reproduction rates at four light intensities for five of the clones examined in the acclimated reproduction rate study.

TABLE 13.

GROWTH PATTERNS OF CLONE 175 AT 28^{O}C AND AT FIVE DIFFERENT LIGHT INTENSITIES IN APPROXIMATELY ONE LITER CULTURES.

Culture Number	Light Intensity lux	Interval	Reproduction Rate	Cells per ml	Number of cells per mg (x 10^{4})	Total yield in mg
20	3350	8/29-9/8	0.23	1096	1.12	87
26	3350		0.28	1178	1.65	70
37	1999	9/22-10/4	0.18	1013	1.26	76
38	1999	9/19-10/4	0.18	931	1.56	55
24	1081	8/18-9/8	0.14	1301	2.06	58
36	1081	9/6-10/4	0.13	1178	2.13	47
16	648	7/25-8/22	0.10	1301	2.88	33
32	648	8/22-9/30	0.07	808	0.61	112
33	378	8/5-10/4	0.02	520	0.94	49
34	378	8/5-10/4	0.02	602	0.52	107

These cultures were subsequently extracted and analyzed (Table 14) for composition of proteins, lipids, and carbohydrates. The data for the pigment composition, appears in Table 15 The samples for toxicity comparisons are weighed and await the availability of mice for bioassay in our program. One of the major problems that we have encountered in the experiment is that the cultures in the lowest light intensity (378 lux) will not produce cell

numbers above 700 cells/ml in a reasonable time period (< 2 months). In order to avoid cellular degradation by bacteria we are harvesting these cultures at lower densities.

Table 14.
BIOCHEMICAL COMPOSITION OF CLONE 175 AT FIVE DIFFERENT LIGHT INTENSITIES.

Culture Number	Light Intensity	Protein (pg per cell)	Carbohydrates (pg per cell)	Lipid (pg per cell)
	lux			
20	3350	364	--	818
26	3350	667	156	--
38	1999	324	--	1,324
37	1999	400	101	--
36	1081	123	--	470
24	1081	189	463	--
32	648	30	--	269
16	648	307	460	--
34	378	371	--	477
33	378	307	460	--

Thus, it is difficult to compare these cultures directly with the others. Consequently, instead of using cell numbers we have used growth pattern as our criterion for determining the point of harvest. Thus, all cultures have been harvested in what we determine to be mid log phase based on linear growth/time plots. By this method we insure that although cell numbers and external nutrient conditions may vary at the point of harvest, the relative kinetics of growth are the same and differ only in their magnitude. The reader should also keep in mind that the cells at 378 lux are teratogenous and may have leaked cellular components. Therefore, they may not be truly representative of the average cellular condition when grown at low light intensities.

Pigmentation also varies with light intensity. More pigment is produced per cell at higher light intensities (Table 15 & 16). This is to be expected in that more light is available for harvesting and consequently more pigment can be produced. However, the

chlorophyll to carotenoid ratio (Table 16) decreased at both the lowest and highest light intensity.

Table 15.

PIGMENT COMPOSITION AT THE FIVE DIFFERENT LIGHT INTENSITIES AT 28°C. ALL VALUES ARE IN PICOGRAMS PER CELL.

	Light Intensity (lux)				
Pigment	3350	1999	1081	648[1]	378[1]
Chlorophyll a	336	204	105	33	2
Chlorophyll c_2	97	12	15	5	--
Peridinin	395	204	62	22	--
xanthophyll 1	91	26	14	11	--
xanthophyll 2	106	32	17	13	--
β-Carotene	26	8	1	--	--

[1]The dashed lines indicate the quantity was too low to be quantified accurately (< 1 picogram per cell).

Table 16.

TOTAL CHLOROPHYLLS AND CAROTENOIDS PER CELL, RATIOS OF TOTAL CHLOROPHYLLS TO CAROTENOIDS AND TOTAL LIPID TO TOTAL PROTEIN OF THE CULTURES GROWN AT 5 LIGHT INTENSITIES. VALUES GIVEN AS THE TOTALS ARE IN PICOGRAMS PER CELL.

	Light Intensity (lux)				
Parameter	3350	1999	1081	648	378[1]
Total Chlorophylls	433	216	120	38	2
Total Carotenoids	618	270	94	46	--
Chlor./Carot. Ratio	0.70	0.80	1.28	0.83	--
Lipid/Protein Ratio	2.24	4.09	3.82	8.97	1.29

[1]The dashed lines indicate the quantity was too low to be quantified accurately (< 1 picogram per cell).

The plot of the macromolecular data (Figure 15) indicates that the cell weight decreases with increasing light intensity, with the

most cells per mg dry weight occurring at 1999 lux. This is to be expected, with a more favorable environment microalgae tend to partition photosynthates into a larger population (**52**). The protein per cell follows closely with the cell weight plot. This is also to be expected given that more efficient conversion of photosynthate into more offspring requires high enzymatic efficiency. We believe that the increase in protein with increasing light intensity reflects the increase in intracellular enzymes. Conversely, microalgae tend to produce more lipid and carbohydrates per cell than protein in unfavorable environments (**53, 54, 55**). This is thought to be due to the cells preparing storage products for probable dormancy (e.g., encystment) periods (**56**). The increase in lipid and carbohydrate at low light intensities reflects this trend. These data imply that we could expect more lipid soluble toxins (e.g., "ciguatoxin") to occur at lower light intensities. It will be interesting to observe the ileum response and symptoms in mice injected with extracts from the different light intensities. The data so far indicate that we might expect different partitioning of the toxin components with respect to light intensity.

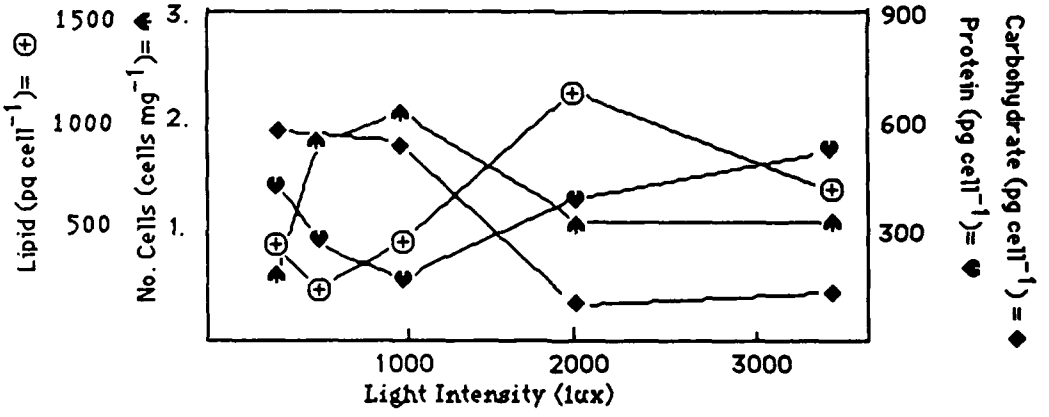


Figure 15. Plot of the biochemical composition (proteins, lipids carbohydrates) and number of cells/mg dry weight of cultures of *Gambierdiscus toxicus* grown at 5 different light intensities. ♠=mean of 2; ⊗= 1 est; ♥=mean of 2.

This implies that at the high light intensity used (3350 lux) saturation is occurring. Saturation is always suspect when more accessory pigments are made per cell relative to chlorophyll (**57**) as the accessory pigments (or other photosynthetic modulators) are

produced to protect chlorophyll against photooxidation. However, general lipid metabolism and pigment metabolism do not necessarily coincide. The lipid to protein ratio at 3350 lux is 2.24 where the pigment per cell is highest. Lipid relative to protein is higher at 648 lux (8.97 ratio). Consequently, we may be able to tell from the toxicity data whether toxin synthesis is linked with pigment production or if it is instead linked with general lipid production.

Figure 16 shows an example thin layer chromatogram of the pigments from clone 175. We achieved excellent separation of these pigments which allowed for their identification via scanning spectrophotometry.

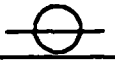
Band No.		Rf	Max Abs λ	Pigment
7		.92	479.0	βcarotene
6		.75	402.6	Chlorophyll a
5		.66	402.6	Chlorophyll a
4		.51	422.3	2 xanthins
3		.30	452.2	peredinin
2		.18	427.6	chlorophyll c_2
		0	472	chlorophyll-protein

Figure 16. TLC pigment separation of acetone extract of clone 175 of *Gambierdiscus toxicus*. Solvent system was acetone:hexane (50:50).

What is encouraging about Figure 16 is that the non-reversible toxin (MTX-like) occurs only in one band (#1). This band only contains 3 major contaminants in addition to the toxin. We are now scaling up to dry-column pigment separation and obtaining results similar to the plates.

LARGE-SCALE CULTURING OF *GAMBIERDISCUS TOXICUS*:

TABLE 17.
HARVESTS OF *G. TOXICUS*

CULTURE DESIG.*	DTE STARTED	VOL. (ltrs)	YIELD GM Wet Wt	Dry Wt	Tot Dry Wt
Material on hand from 1987 culturing					80.51
GT350-2A88D	25Jan	235	117	17.31	97.82
GT350-3A88D	12Feb	31	26.5	3.5	101.32
GT350-5A88D	6Apr	226	81.6	12.2	113.52
GT175-3A88	29Feb	216	80.0	10.0	123.52
GT175-4A88	2Mar	187	28.71	3.58	127.10
GT175-5A/88D	16Apr	230	162.1	30.6	157.70
GT350-7A/88D	8Jun	194	100.4	18.0	175.70
GT350-7B/88D	9Jul	184	112.9	21.1	196.80
GT175-8A/88D	31Jul	199	87.9	16.6	213.40
GT350-8A/88D	5Aug	197	87.99	16.68	230.08
GT350-10A/88D	9/20	190	94.4	17.7	247.78
GT350-11A/88D	10/25	193	81.5	13.2	260.98
GT175-9A/88D	9/6	186	128.5	19.3	280.28
GT175-11A/88D	10/11	168	159.9	26.44	306.72

TABLE 18
Growth Production Data

	Used	Shipped	On Hand
Grams 350	(10.2)		**190.00**
Grams 175	(83.2)		**9.74**
Totals	**93.4**		**213.32**

As can be seen from the growth data above (Table 17 & 18) the production of *Gambierdiscus toxicus* has been good. As a result of our chemosystematic studies this last year we switched from growing clone 350 to clone 175. The significance of this is appreciated when one examines the total estimated mouse units produced as shown in Figure 17.

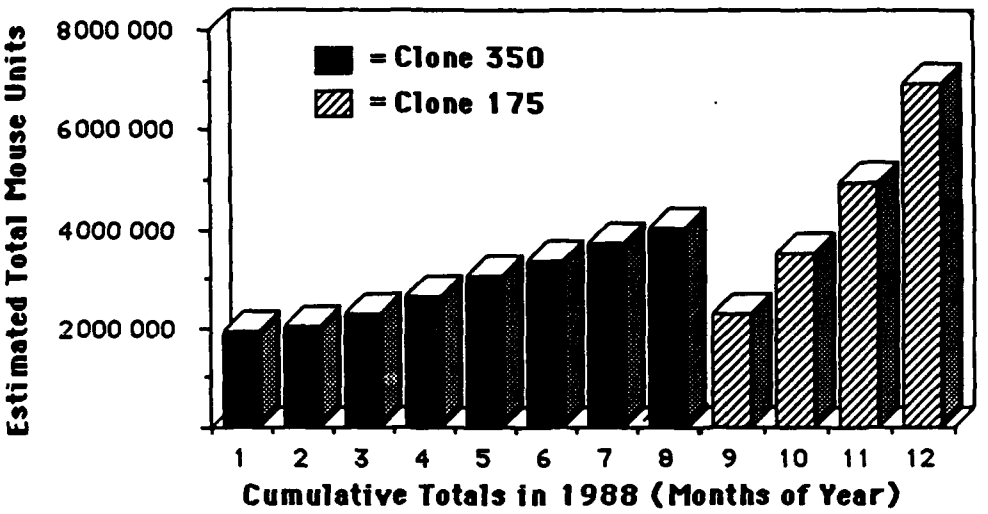


Figure 17. Plot of estimated mouse units contained in cultures of *Gambierdiscus toxicus*, clones 350 and 175 during 1988.

Notice that in just three months production of clone 175 we have exceeded the total mouse units accumulated from clone 350.

TABLE 19
Estimated Production Potential

Clone	350	175
LD_{50s} based on 20 g mouse		
MU/mg DW	18	120
mg DW/L	116	122
Total MU/L	2,088	14,640
MU/15 L Culture	$3.1x10^4$	$2.2x10^5$
LD_{50s} based on Kg Mouse	$6.3x10^2$	$4.4x10^3$
LD_{50s} based on Yasumoto's value of 0.13 ug/Kg		
ug/Carboy	82	572
Monthly production based on 40 carboys per month		
mg/month	3.28	22.88

The switch to clone 175 has given us a ten fold increase in production capacity for GT1-4 toxins. In addition, examination of the results from separation experiments indicate that the material from clone 175 has much less contaminants and therefore provides a much cleaner product for purification.

PURIFICATION OF LARGE SCALE CULTURE PRODUCTS:

Preparative HPLC separation of crude *G. toxicus*

Our preliminary results with preparative separation can best be understood when the results are scaled to actual detector settings and presented as a stereogram as shown in Figure 18.

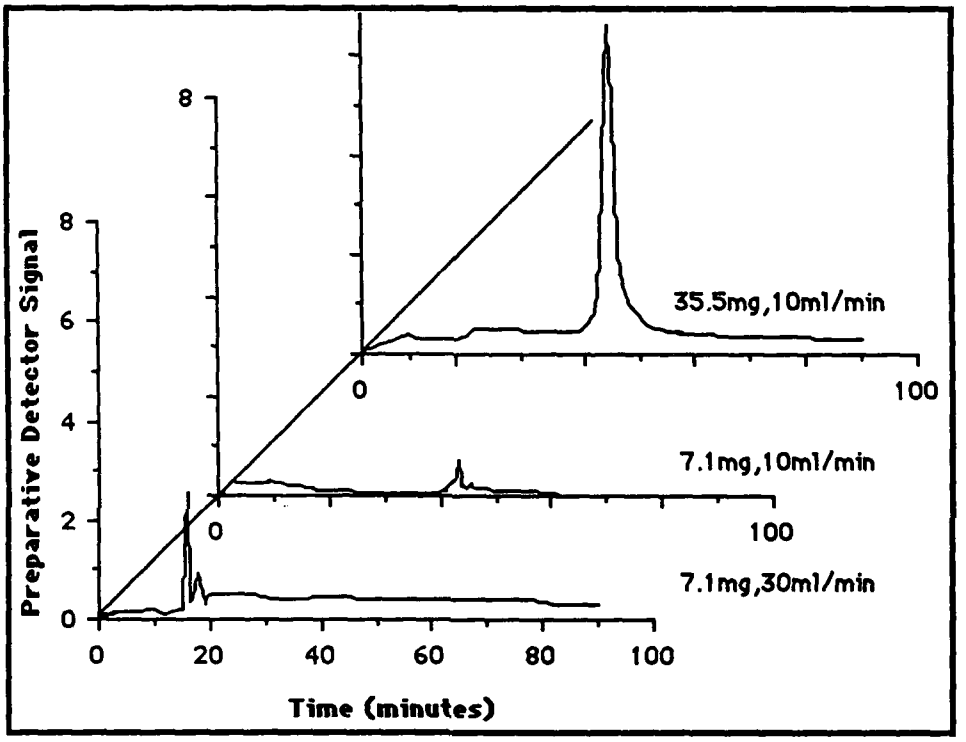


Figure 18. Comparison of preparative HPLC chromatograms of WSAP at different flow rates and quantities. Sample application and flow condition are shown on diagram. Other running conditions were: 15 micron C-18 silica gel, 5 cm X 28 cm column, and 100% methanol as solvent, run time 55+ min.

Essentially when amounts less than 10 mg of WSAP are applied to the column there is some separation of peaks, but when over 10 mg are applied the peaks fuse into a single broad complex of peaks. Running the column at flow rates slower than 20 ml/min results in the elution of the first large 209 nm peak at 40 minutes (with increased base width of the peak), whereas flow rates greater than 20 ml/min make the 209 nm and other peaks elute earlier, but accuracy in the collection of the separate peaks is sacrificed. A flow rate of 20 ml/min was therefore instituted in later experiments.

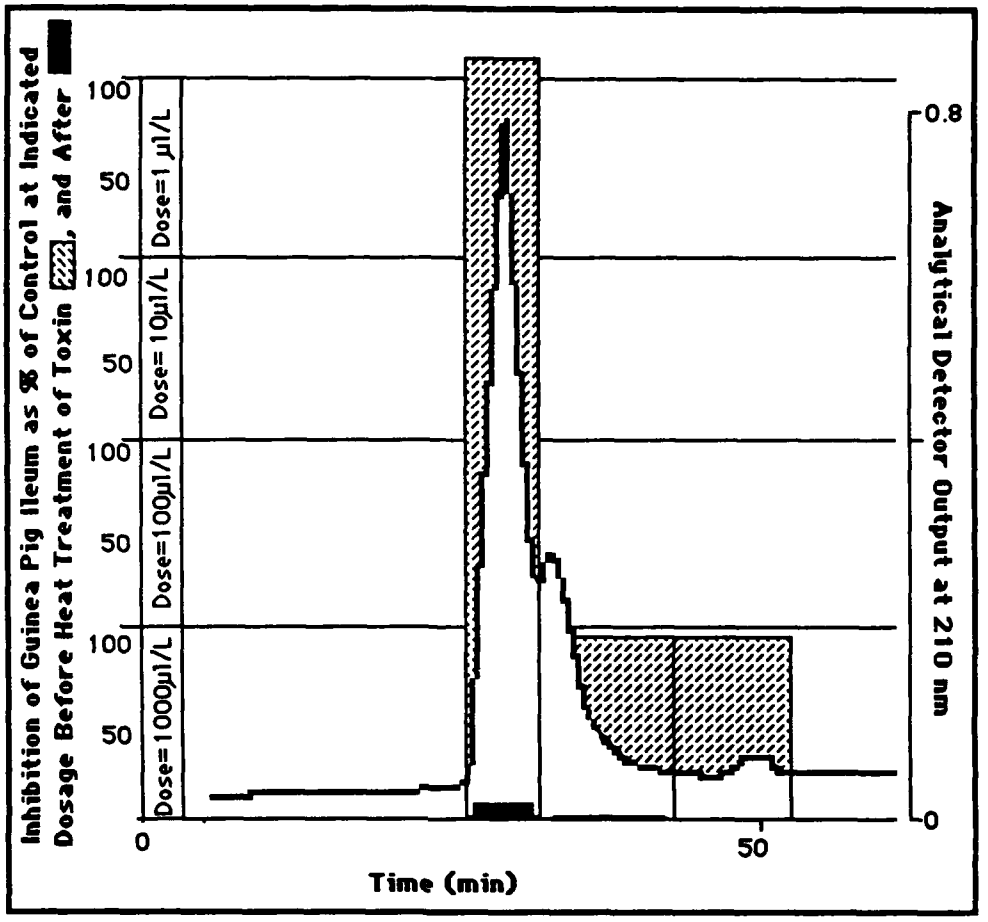


Figure 19. Preparative HPLC chromatogram (P1) of 1 ml of WSAP (7 mg/ml). Running conditions were: 15 micron C-18 silica gel, 5 cm X 28 cm column, flow rate 10 ml/min, and 100% methanol as solvent, run time 70 min. Detector set at 210 nm.

Figure 19 is a summary of our first preparative run (P1). Three fractions were collected from this run of one ml (7 mg/ml) and labeled fraction P1F1, fraction P1F2 and fraction P1F3. When tested on the ileum preparation most of the toxicity appeared in fraction P1F1 which contained the area of the first large 209 nm peak. Some toxicity appeared in fractions P1F2 and P1F3, but it should be noted that these two fractions had levels of toxicity that were two orders of magnitude less than the P1F1 fraction. Once the ileum assays were completed, the fractions were dried down for weighing on the Cahn microbalance. In order to speed the process of weighing them, the fractions were dried on a warm heater. However, after weighing, when the samples were put back into methanol and tested for toxicity, they had lost all their toxicity to the ileum. Our major conclusion from this experimental run is that once the toxin is

purified it becomes unstable to heat or it loses its potency to the ileum preparation

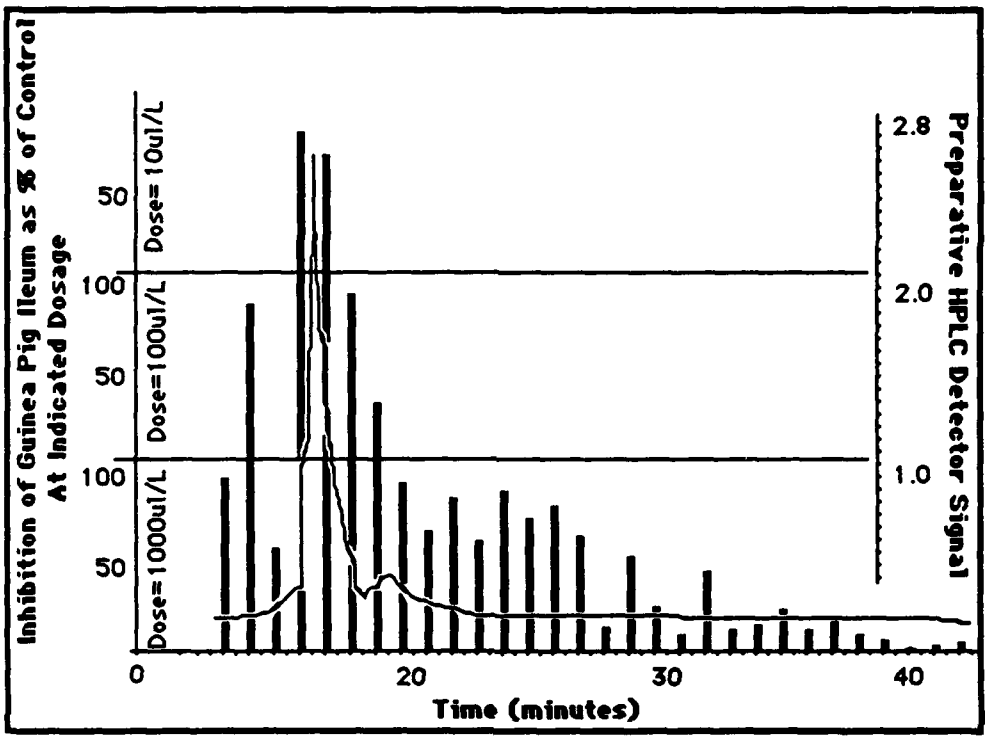


Figure 20. Preparative HPLC chromatogram (P2) of 1 ml of WSAP (7 mg/ml). Running conditions were: 15 micron C-18 silica gel, 5 cm X 28 cm column, flow rate 30 ml/min, and 100% methanol as solvent, run time 55 min. Detector set at 210 nm.

Figure 20 above is a summary of preparative run (P2) which was actually run as a control for the acetonitrile experiments that will be discussed later on in the report. In this experiment, we collected some 50 fractions (P2F1 through P2F50) from a preparative HPLC run of 1 ml of WSAP (7 mg/ml). Each of these 50 fractions were evaporated to dryness and re-solubilized in 1 ml of methanol. As in the previous experiment, the largest amount of toxicity was found to be associated with the first large 209 nm peak. Some levels of toxicity are found after the first large 209 nm peak and we surmise that this is a smearing of the toxin in the HPLC system which comes out after adequate flushing.

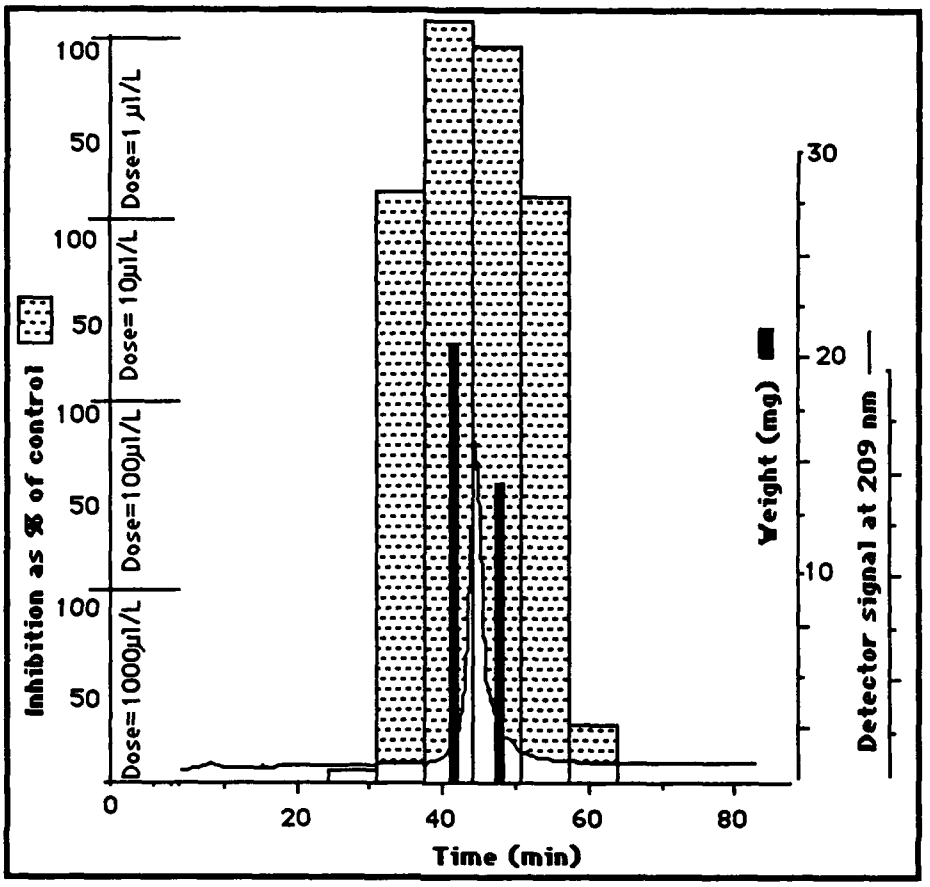


Figure 21. Preparative HPLC chromatogram (P3) of 5 ml of WSAP (a total of 35.7 mg). Running conditions were: 15 micron C-18 silica gel, 5 cm X 28 cm column, flow rate 10 ml/min, and 100% methanol as solvent, run time 65 min. Detector set at 210 nm.

Figure 21 above is a plot of the third preparative run (P3) in which five ml or 35.7 mg of WSAP was applied to the column. In this run six fractions (F1 through F6) were collected around the first large 209 nm peak. These fractions were evaporated down to 1 ml volume and aliquots taken for weighing (100 µl) and guinea pig ileum assay (10 µl). After weighing the 100 µl aliquot was returned to the sample. Fractions P3F3 and P3F4 associated, with the first large 209 nm peak were the most toxic. The fractions on either side of these, P3F2 and P3F5 were an order of magnitude less toxic to the ileum. This experiment also demonstrated that the most of the weight of the sample was recovered in fractions P3F3 and P3F4. Fractions P3F5 and P3F6 were combined into one fraction.

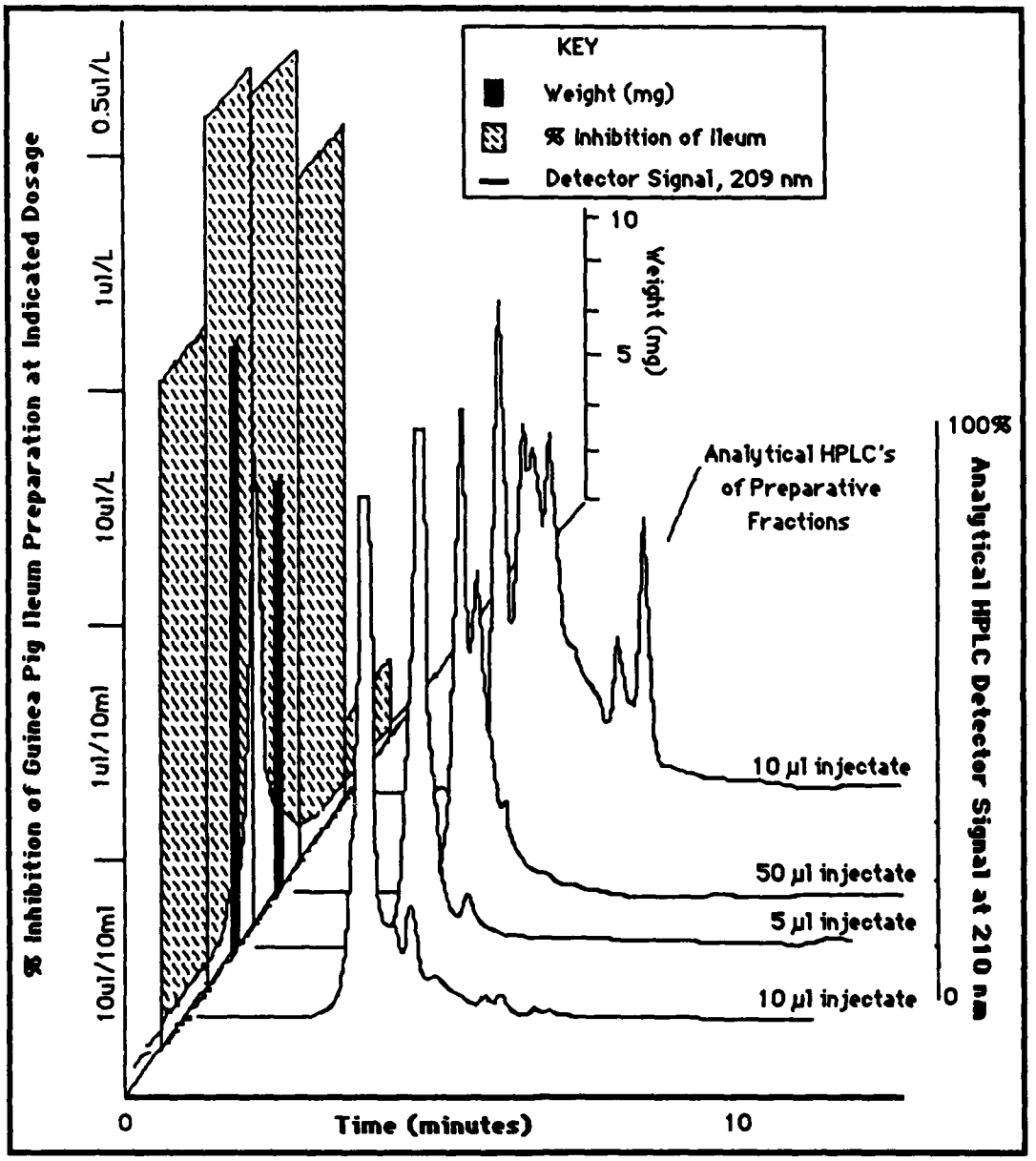


Figure 22. Preparative HPLC chromatogram (P3) of 5 ml of WSAP (35.7 mg total) compared with analytical chromatograms of the fractions collected. Running conditions were: 15 micron C-18 silica gel, 5 cm X 28 cm column, flow rate 10 ml/min, and 100% methanol as solvent, run time 65 min. Detector set at 210 nm. Analytical HPLC aliquot sizes are shown in figure. Other running conditions were 10 micron C-18 RCM column. Flow rate 0.5 ml/min and 100% methanol as solvent.

The fractions from the previous preparative run (P3F1 through P3F4+5) were aliquoted for analytical HPLC. Figure 22 above summarizes these results. First of all, note that different amounts of

injectate were utilized for the analytical runs. Fractions P3F1A and P3F2A have the cleanest first large 209 nm peak and the narrowest base width. For each successive two fractions P3F3A and P3F4+5A, higher amounts were applied to the analytical column (implying a lesser amount of material in the fraction), the chromatogram becomes more complex and the relative height of the first large 209 nm peak decreases.

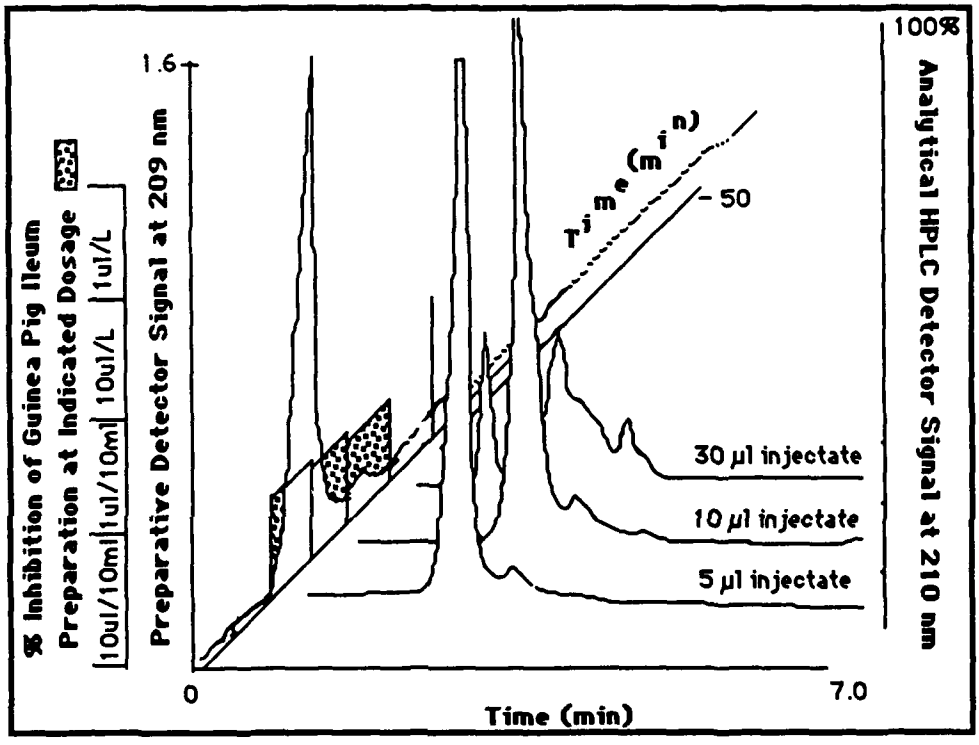


Figure 23. Preparative HPLC chromatogram (P4) of fraction P3F2 dried down to 1 ml. Running conditions were: 15 micron C-18 silica gel, 5 cm X 28 cm column, flow rate 20 ml/min, and 100% methanol as solvent, run time 65 min. Detector set at 210 nm. Analytical HPLC sample aliquots shown in figure. Other running conditions were 10 micron C-18 RCM column. Flow rate 0.2 ml/min and 100% methanol as solvent.

Once the analytical chromatograms were completed for the fractions from preparative run P3, the two fractions around the first large 209 nm peak, P3F2 and P3F3, were re-chromatographed on the preparative HPLC resulting in preparative runs P4 and P5 respectively. Figure 23 above is a summary of the preparative run P4 plotted on the Y-Z plane. Three fractions were collected (P4F1, P4F2 and P4F3) Analytical HPLC chromatograms of these fractions have been completed and the results are plotted on the X-Y plane. As was found in the parent separation (P3), for each successive

fraction P4F1A, P4F2A and P4F3A, higher amounts were applied to the column, the chromatogram becomes more complex and the relative height of the first large 209 nm peak decreases.

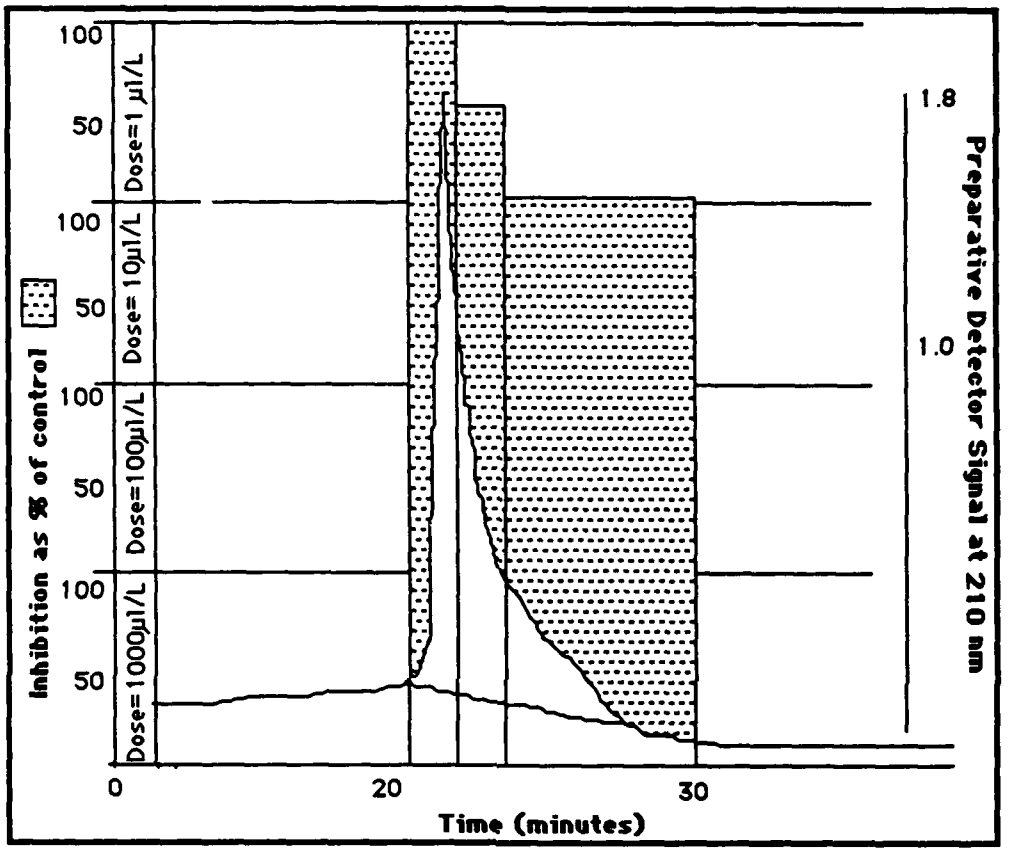


Figure 24. Preparative HPLC chromatogram (P5) of fraction P3F3 dried down to 1 ml (14.03 mg). Running conditions were: 15 micron C-18 silica gel, 5 cm X 28 cm column, flow rate 20 ml/min, and 100% methanol as solvent, run time 55 min. Detector set at 210 nm.

The results of the re-chromatographing of fraction P3F3 in preparative run P5 are presented in Figure 24. Three fractions were collected (P5F1, P5F2 and P5F3) and aliquots of these tested on the ileum preparation. As can be seen, smaller amounts of the first large 209 nm peak are in fraction P3F3 and much larger amounts of the later, low contaminating peak are in fractions P5F2 and P5F3. Nevertheless, since we are dealing with larger samples applied to the column, the amount of toxin is significant and worth collecting.

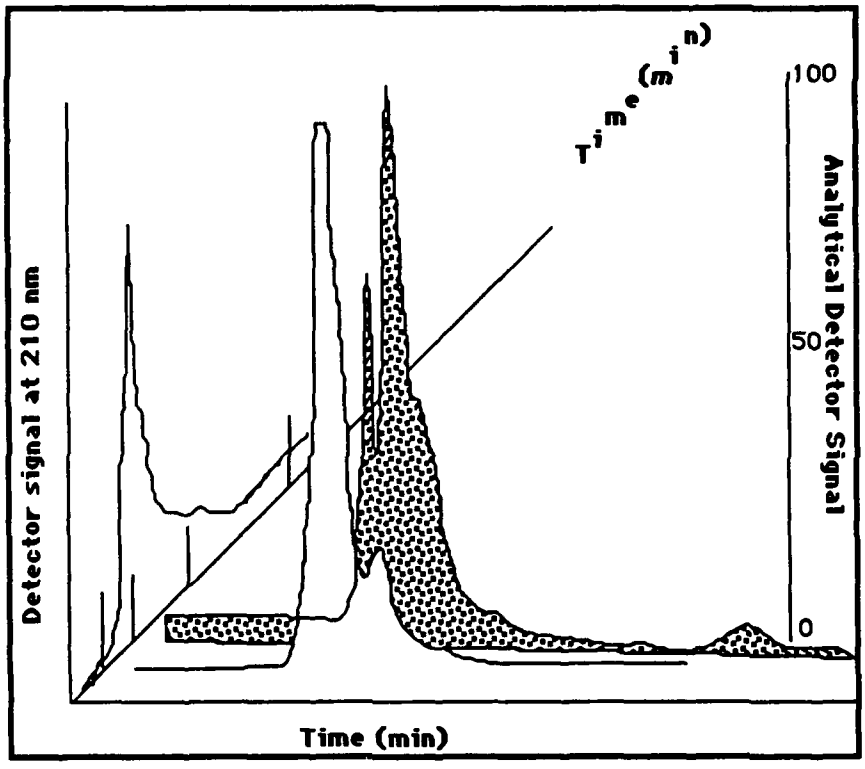


Figure 25. Preparative HPLC chromatogram (P5) of fraction P3F4 on the Y-Z plane and associated analytical chromatograms on the X-Y plane. Running conditions were: 15 micron C-18 silica gel, 5 cm X 28 cm column, flow rate 20 ml/min, and 100% methanol as solvent, run time 55 min. Detector set at 210 nm. Analytical HPLC sample aliquots shown in figure. Other analytical running conditions were 10 micron C-18 RCM column. Flow rate 0.2 ml/min and 100% methanol as solvent.

Analytical HPLC chromatograms were performed on the fractions from the fourth fraction from P3. The results are presented in Figure 25 and they confirm what we surmised from the previous preparative separation.

Preparative separation of crude *G. toxicus*

In an attempt to shorten the purification process and save toxin we entertained the possibility of separating the crude material from *G. toxicus* on the preparative HPLC into two fractions corresponding to the maitotoxin and ciguatoxin fractions directly. Accordingly, we initiated an experiment wherein 60 µl of crude material was run on the semi-preparative HPLC.

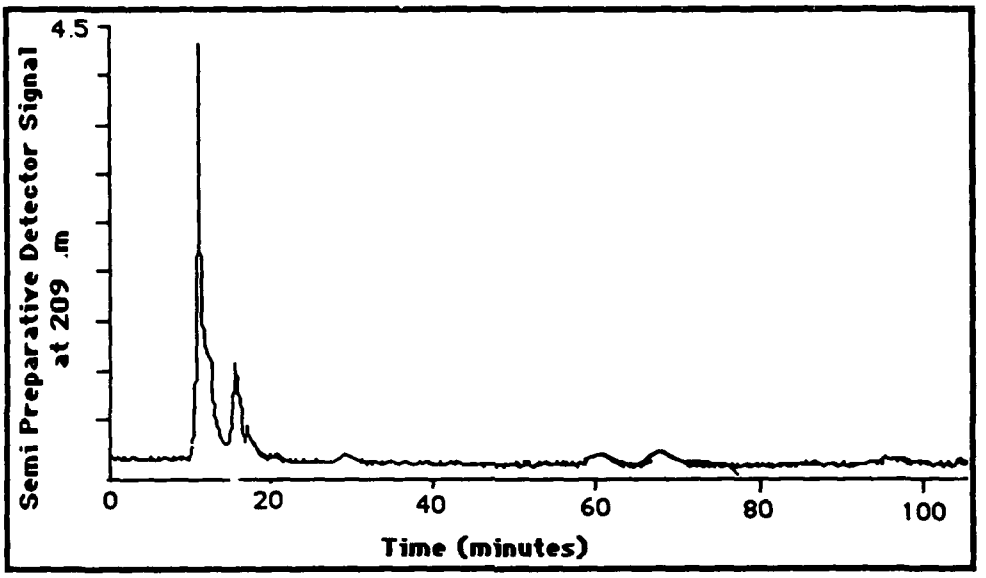


Figure 26. Semi-preparative HPLC chromatogram (SP3) of 60 µl crude extract of *G. toxicus*. Running conditions were: 15 micron C-18 silica gel, 5 cm X 28 cm column, flow rate 30 ml/min, and 100% methanol as solvent, run time 120 min. Detector set at 209 nm.

The effluent from the semi preparative chromatography (SP3) of 60 µl of crude *G. toxicus* extract was collected in nine fractions (SP3F1 and SP3F9). All fraction were collected for a time period of 10 minutes with the exceptions of SP3F3 and SP3F9 which were 20 minutes in length. In this scheme of collection it is expected that GT-3,4 (maitotoxin) would elute in fraction SP3F2 and that GT-1,2 (ciguatoxin) would elute sometime after fraction SP3F5.

The semipreparative fractions were evaporated down to 200 µl volume. Because it was expected that the most of the maitotoxin would be in fractions SP3F2 and SP3F3, 5 µl aliquots of these were utilized for testing on the ileum preparation using the MTX procedure. In the case of the other fractions in which we expected the ciguatoxin activity, the entire fraction was used to assay for ciguatoxin on the ileum preparation.

When processing samples through Sep Pak C18 the color sequence is the same as that seen from HPLC. Experimentation revealed that if the sample was loaded on the sep-pak column and then eluted slowly, the methanol extract of crude was split into three different colored fractions based upon the time of elution.

The procedure for this is as follows: A sep-pak is first wetted with 2 ml of methanol. Then approximately 1.5 ml of crude methanol extract is applied to the sep-pak. This is followed by a 0.25 ml. wash to rinse the syringe. Following this one ml of methanol is flushed through the column and the initial eluent is clear but at the end of the flush is muddy brown in color. A second flush of 0.5 ml. of methanol produces a yellowish-green eluent. Following this a 1.0 ml wash produces a bright green fraction and finally, a 2.0 ml wash produces a more viscous light green fraction. For our purposes we combine the two final washes into a green fraction.

Five ml of GT-350 Combo (=methanol crude) (9.2 mg/ml) was processed by the sep-pak procedure described above and labeled SK-1 br, SK-1 yel, and SK-1 gr. When these fractions were tested on the ileum, the results suggested that the toxic moieties in the three fractions are different. The initial brown fraction produces the typical GT-4 (maitotoxin) effect on the acetylcholine response of the ileum.

The yellow intermediate produces the effects we have seen previously with GT-2, but at this point it is hard to judge. It may simply be a combination of small amounts of GT-1 and GT-4. Because our experience with this fraction has been minimal we decided to simply store all future fractions for later work and to concentrate our efforts on CTX and MTX. It is interesting to note, that this fraction has a very pronounced inotropic effect upon the ileal preparation. Nevertheless, the ileum preparation, with successive washes, recovers from the effects of the toxin and at the end of the run is stimulated.

The green fraction produces the reversible GT-1 (or ciguatoxin effects) that we have reported before. At the present time we are conducting an experiment to assess the relative scale up factor between ileum units and mouse units for ciguatoxin. Our ileal results can be summarized as follows.

TABLE 20
EFFECTS OF TOXIC EXTRACTS ON ILEUM AT LOW DOSES

Fraction from		Assay	Inotropic	Onset of	Recovery
Chrom.	Sep Pak	type	response	inhibition	%
GT-1	Green	CTX	0	gradual	100%
GT-2	Yellow	MTX	+++	immediate	100%+
GT-3	Brown	MTX	++	gradual	Partial
GT-4	Brown	MTX	+	gradual	none

The same fractions were examined on UV-Vis spectrophotometer and the results are shown in Figure 27.

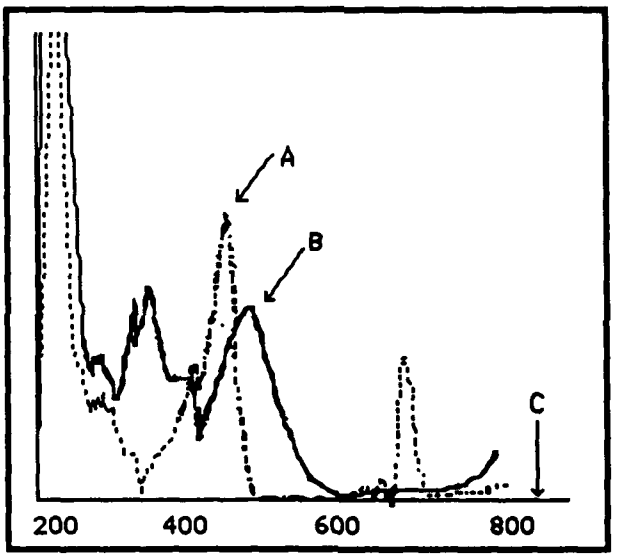


Figure 27. UV-vis spectra of the yellow (A), brown (B) and green (C) fractions (SK-1) from the sep-pak separation of crude methanol extract of *Gambierdiscus toxicus*.

The UV-Vis scans indicate that both the brown and the yellow fraction have maxima at 209-10, 267 and 314. The brown fraction has distinct maxima at 388 and 510, and the yellow has a distinct maxima at 450. The green did not have a distinct maxima, perhaps due to an insufficient concentration.

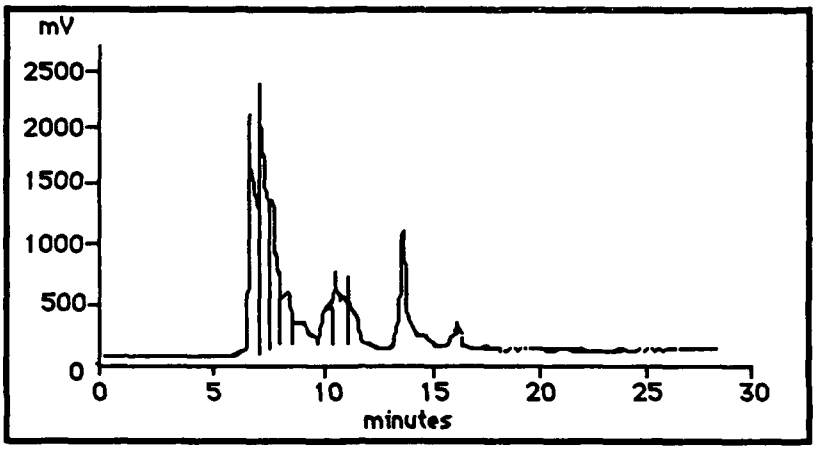


Figure 28. Analytical HPLC chromatograms of the brown fraction (SK-1) from the sep-pak separation of crude methanol extract of *Gambierdiscus toxicus*.

Aliquots of the sep-pak separation were then run on HPLC and the results are shown in Figures 28-31.

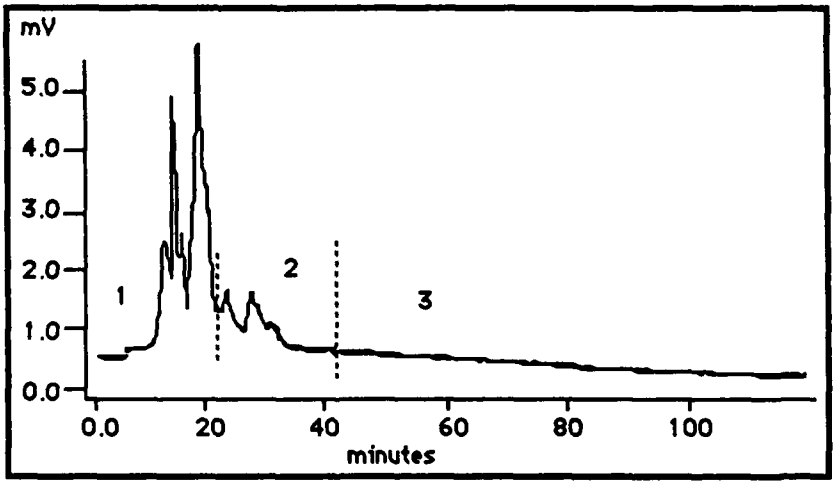


Figure 29. Semipreparative chromatogram of the yellow fraction from (SK-1) the sep-pak separation of crude methanol extract of *Gambierdiscus toxicus*.

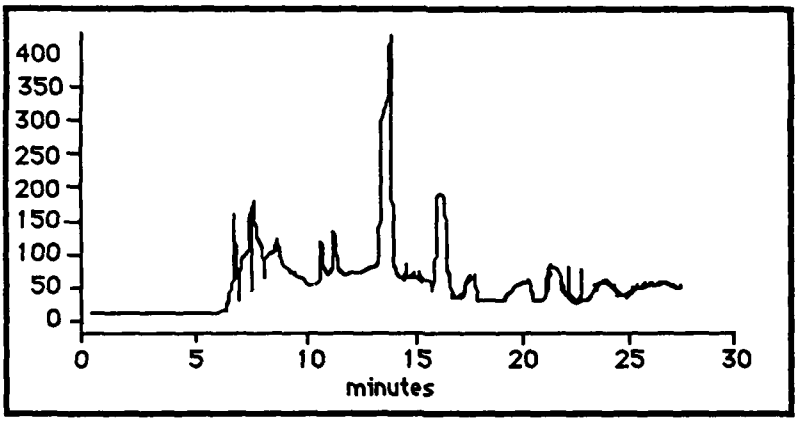


Figure 30. Analytical HPLC chromatograms of the green fraction (SK-1) from the sep-pak separation of crude methanol extract of *Gambierdiscus toxicus*.

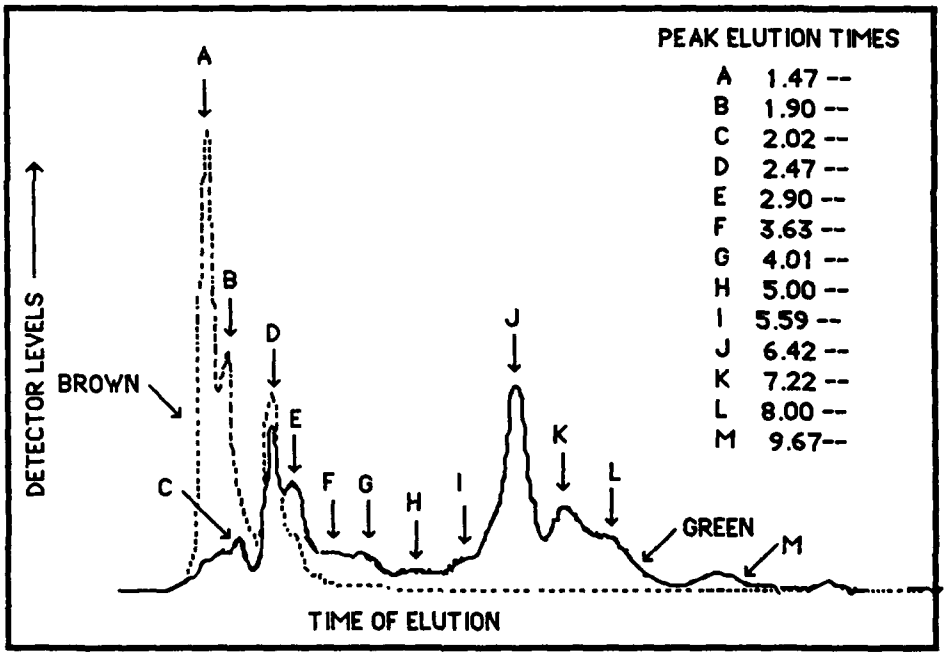


Figure 31. Comparison of analytical HPLC chromatograms of Sep Pak brown and green. Running conditions were: Novapak 5 micron C-18 silica gel, 3.9 mm X 7.5 cm column, and 100% methanol as solvent, run time 20 min.

In the Sep Pak C-18 separation process, the brown portion, When examined at 210 nm using HPLC chromatography, shows four major composite peaks (A, B, D, and E in Figure 31). Only two of these, D and to a small extent E, are common to the green fraction.

The green fraction has the major portion (i. e. 11 major composite peaks) of the 210 scan.

Table 21

QUANTITATION OF CRUDE TOXIC COMPONENTS BY ILEUM ASSAY SEP-PAK SEPARATION OF ONE ML OF CRUDE G. TOXICUS EXTRACT (9.2 MG/ML)

FRACTION OF CRUDE	COLOR OF FRACT	% OF FRACT	TOTAL ACTIVITY *MU-MTX	SPEC ACTIVITY MU/μg	WEIGHT ACTIVITY μg/MU	% PURITY (note 1)	% YIELD
Crude Ext	Dk Br	100.	300	.0326	30.6	.0098	100
Void	Clr	0	0	0	0		
Brown	Brown	83.16	210	.0236	42.3	.0070	70%
Yellow	Yellow	2.71	0.16	.0006	1558	.0001	.05%
Green	Green	7.48	*1.8	.4048	.00247	*	.6%
Straw	Cream	2.89	>>1	nd			
Flush	Cream	3.74	0	nd			

Note 1. To calculate % purity by weight we have tentatively accepted Yasumoto's value for a mouse unit being 0.003 ug/. Therefore the % purity is calculated as:

$$\% \text{ purity} = \frac{\text{weight of pure or .003 } \mu g}{\text{weight of impure } \mu g} * 100$$

Note 2. MTX ileum equivalent unit (MIEU) is 50% inhibition at 90 minutes following a 15 minute incubation with toxin. CTX ileum equivalent unit (CIEU) is 50% inhibition with toxin continually present in the physiological saline for at least thirty minutes and full recovery after a wash of saline contain no toxin.

Note 3. The LD_{50} values used in this table are estimates based upon preliminary mouse data and the actual graph and LD_{50} value will be reported in the next quarterly.

* indicates a CTX assay rather than an MTX assay on the guinea pig ileum

Notice that in the initial Sep-Pak separation, we have achieved a virtually complete separation of GT-1,2 and GT-3,4, we have only doubled the sample volume and achieved a ten fold increase in purity and only lost 30% of the toxicity. Indeed, this may only be an apparent loss, inasmuch as we have no immediate way to assess the contribution of the GT-1,2, and GT-3 toxins in relatively high concentration in the crude to the toxicity seen in the ileum assay for MTX.

Further processing of the brown from SK-1

The brown fraction was run on the semipreparative as SP-9 and tested on the ileum - the results are shown in Figure 32.

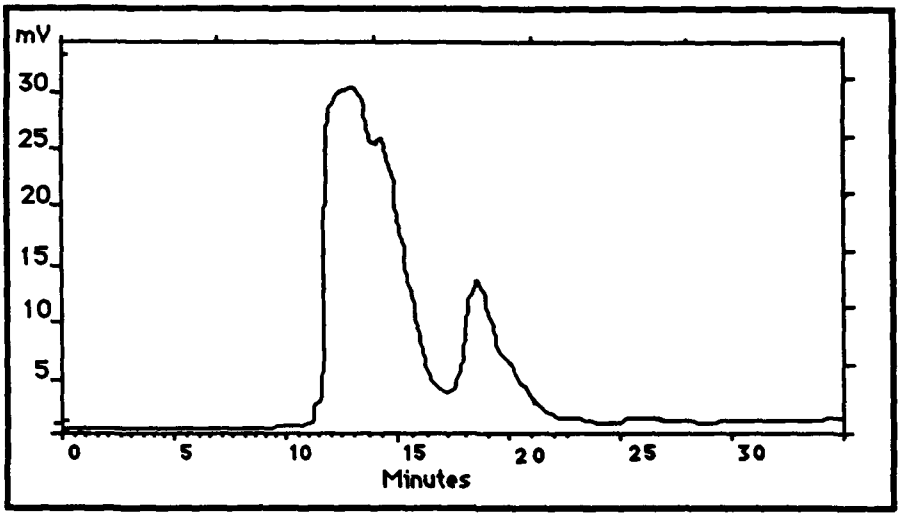


Figure 32. Semipreparative chromatogram (SP-9) of the brown fraction from the sep-pak treatment (SK-1) of crude methanol extract.

Because fraction SP9F4 showed most of the MTX activity it was reprocessed on the semipreparative HPLC as SP10. The area which demonstrated the most MTX activity on the ileum preparation was SP10F4 and it was at this point that we experimented with the formation of crystals.

Introduction of the crystalization step in purification

Both the crystals and the supernatant of SP10F4 were assayed on the ileum preparation.

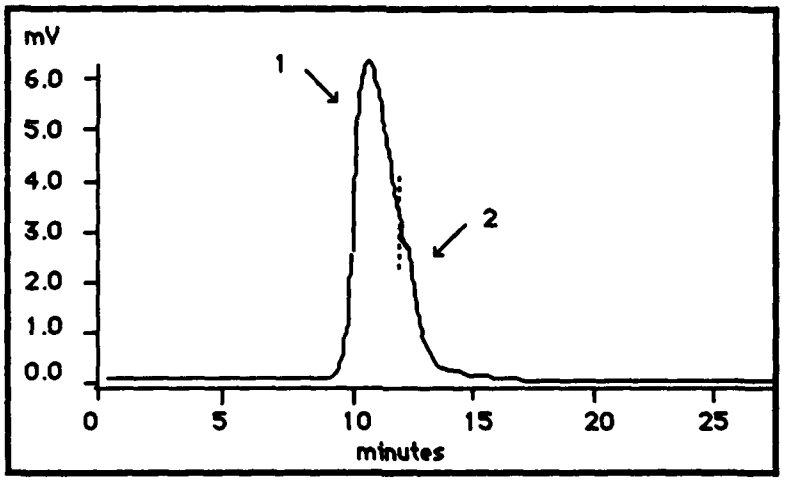


Figure 33. Semipreparative chromatogram of brown supernatant fraction from sep pak treated SK-1, crude methanol extract.

The supernatant from the crystallization step (XTL-1) contained the majority of the MTX activity (approximately 93% by ileal assay).

NMR scans were performed on fraction SP10F4 supernatant. The complete results of this and other NMR data are discussed in the section on NMR. At this point it was decided to incorporate the crystallization as a step in the purification process.

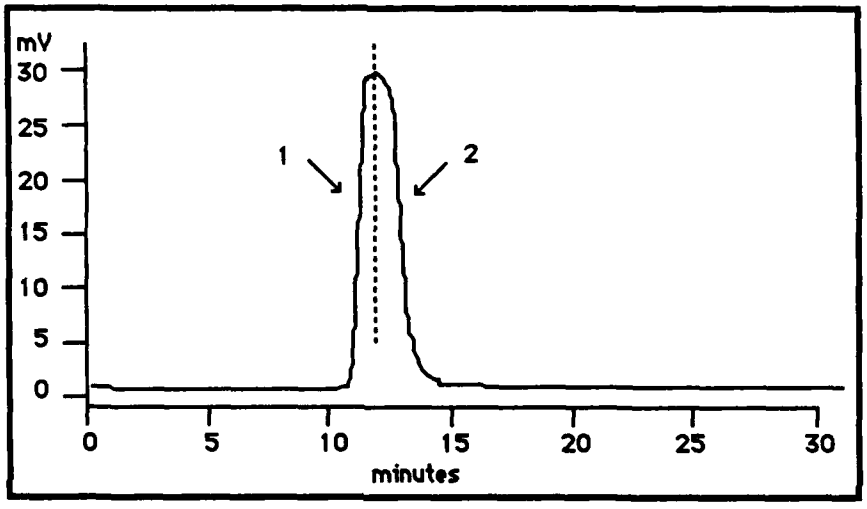


Figure 34. Semipreparative chromatogram of brown xtl fraction from sep pak treated SK-1, crude methanol extract.

Once the brown fraction is separated by means of the sep-pak treatment, the fraction is taken to complete dryness. Then small amounts of methanol are added until the crystals form. They are allowed time to settle and the supernatant decanted. These crystals have been resuspended in methanol and tested on the ileum. The toxicity level of the crystals is very low (approximately 7% of what is in the sample as determined by ileal assay) and we believe it is due to toxin trapped within the crystals when they are formed. The advantages of this step are obvious in removing a large percentage of the contaminants.

Prior to further processing of the brown fraction, it was taken to complete dryness under dry nitrogen gas and then slowly taken up in small amounts of methanol, at which time a crystalline precipitate forms. The supernatant which contains the toxicity was then decanted off.

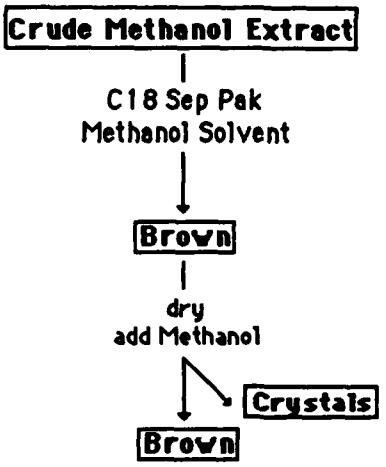


Figure 35 Diagrammatic representation of the removal of crystals from MTX brown.

Separation and acetonitrile extraction of *G. toxicus* WSAP

As indicated in the last report, we did some investigation into the acetonitrile (ACN) extraction of the WSAP extract of *G. toxicus*. Five hundred µl of the water soluble extract was dried down and submitted to acetonitrile extraction. The acetonitrile was decanted off leaving an ACN-insoluble residue. The result was an ACN-insoluble and an ACN-soluble division of the original water soluble extract each of which was dried down and diluted up to 500 µl with dry methanol.

When tested on the ileum both fractions were toxic, but produced subtle differences. Subsequent second and third extractions of the original water soluble extract with ACN were not toxic to the ileum. These results led us to conclude that there are in fact two or more toxins in the water soluble extract and the ACN-soluble fraction is perhaps less polar than the ACN-insoluble portion.

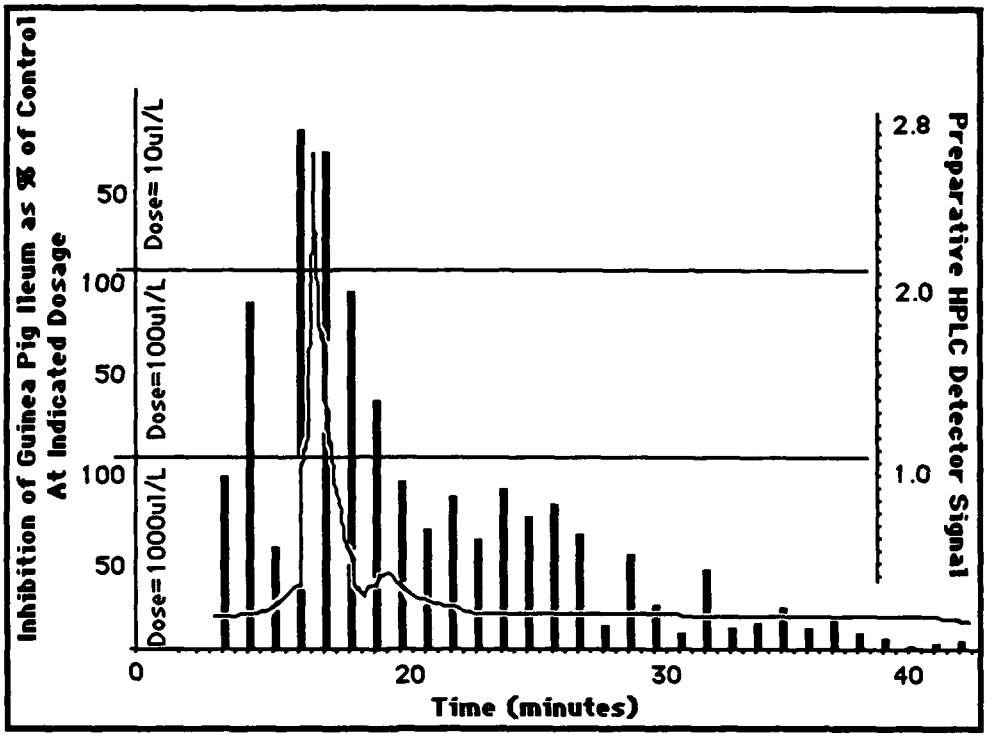


Figure 36. Preparative HPLC chromatogram (P2) of 1 ml of WSAP (7 mg/ml). Running conditions were: 15 micron C-18 silica gel, 5 cm X 28 cm column, flow rate 30 ml/min, and 100% methanol as solvent, run time 55 min. Detector set at 210 nm.

As a preliminary to the ACN experiment, we ran a control chromatogram of the same material we used for the ACN-extraction. The results of this chromatogram was presented in the previous section as Figure 20 and is repeated here as Figure 36 for convenience in comparing with the ACN fractions. Notice in the control, that the most of the toxicity is associated with the initial large 209 nm peak. However there is a small peak of toxicity the precedes the large 209 nm peak and a gradual decrease of toxicity that occurs after the large 209 nm peak.

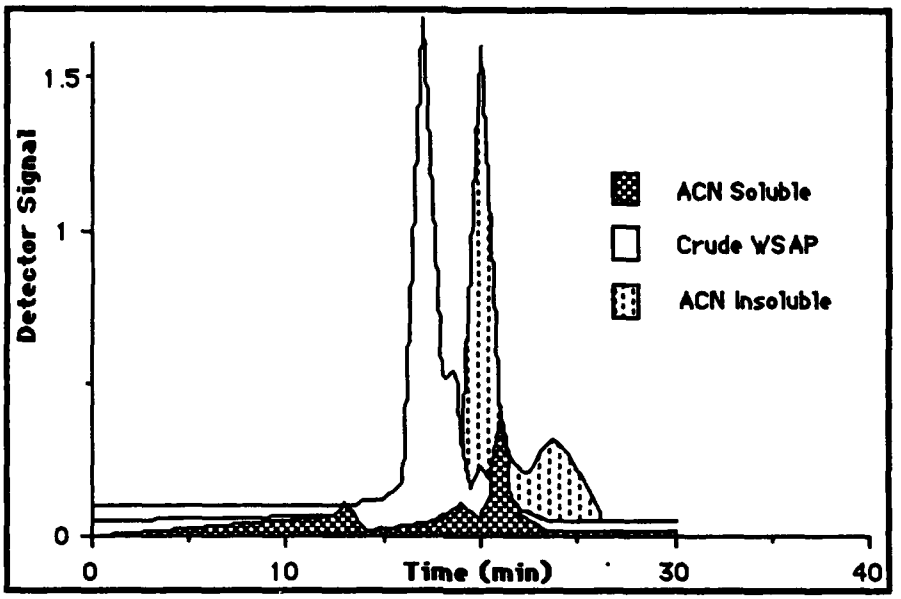


Figure 37. Preparative HPLC chromatograms of WSAP, ACN-soluble and ACN-insoluble. Curves scaled to actual detector levels at 210 nm. Running conditions were: 15 micron C-18 silica gel, 5 cm X 28 cm column, flow rate 30 ml/min, and 100% methanol as solvent, run time 55 min.

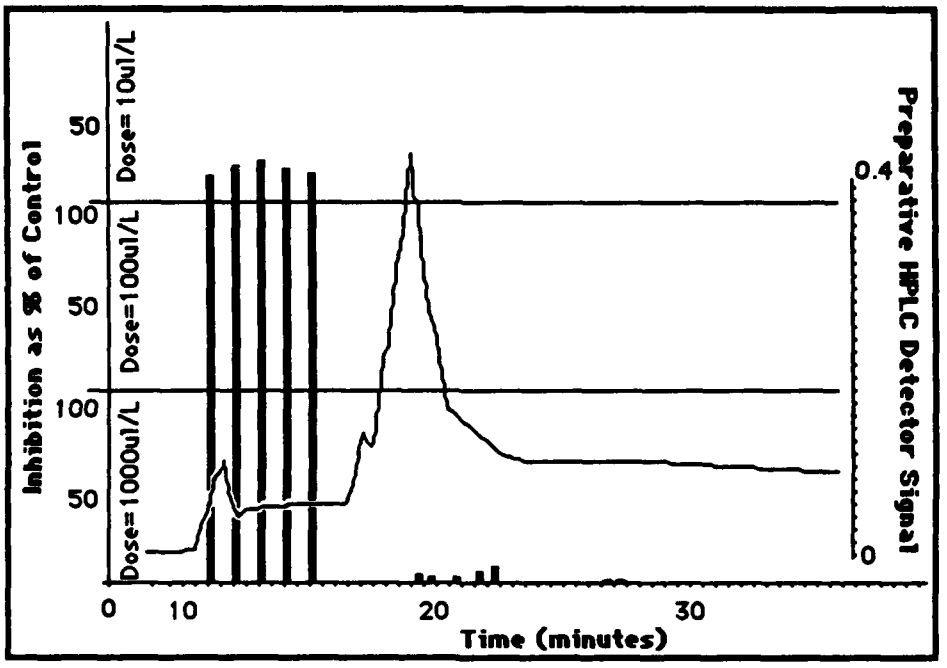


Figure 38. Preparative HPLC chromatogram of ACN-soluble material isolated from WSAP superimposed upon a bar chart of toxicity to guinea pig ileum. Sample application was 1 ml. Running conditions were: 15

micron C-18 silica gel, 5 cm X 28 cm column, flow rate 30 ml/min, and 100% methanol as solvent, run time 55 min. Detector set at 210 nm.

Chromatography of the ACN-soluble and ACN-insoluble fraction is compared with the starting in material in Figure 38. Notice that in terms of the detector output the ACN-soluble is a very small portion of the crude WSAP.

When crude WSAP is extracted with acetonitrile and fraction are collected from preparative chromatography, the results shown in Figure 38 are surprising. All of the toxic activity precedes the normal elution time. In addition, there are two first large 209 nm peaks, albeit at much lower detector levels.

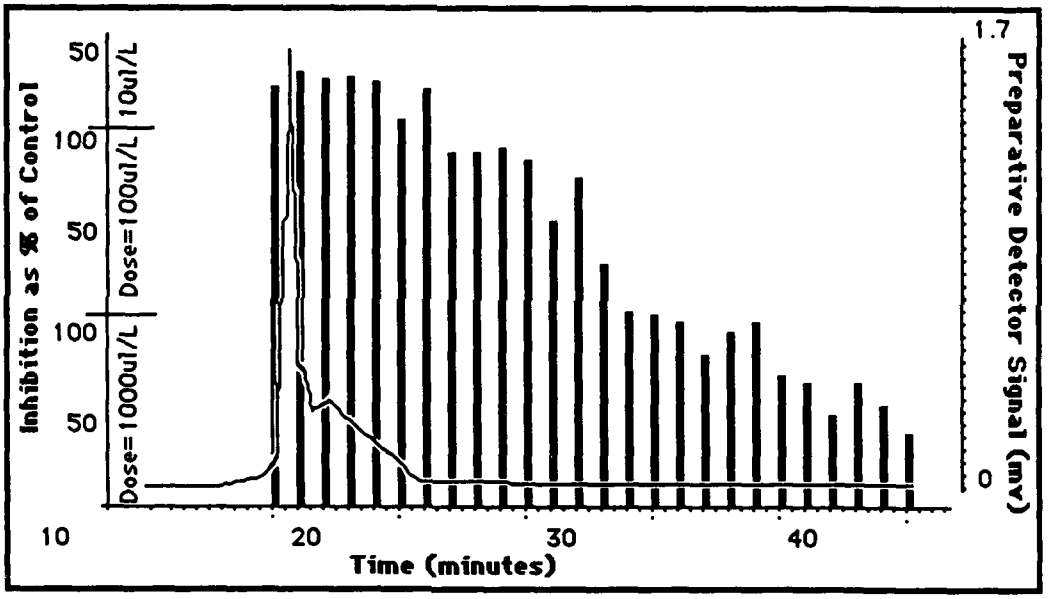


Figure 39. Preparative HPLC chromatogram of ACN-insoluble material isolated from WSAP superimposed upon a bar chart of toxicity to guinea pig ileum. Sample application was 1. Running conditions were: 15 micron C-18 silica gel, 5 cm X 28 cm column, flow rate 30 ml/min, and 100% methanol as solvent, run time 55 min. Detector set at 210 nm.

Preparative chromatography of the ACN insoluble material produces some even more surprising results (Figure 39). After extraction, the toxicity in the ACN-insoluble material is still associated with the first large 209 nm peak at its normal elution time. The gradient of toxicity after the first large 209 nm peak is even higher.

The conclusions from this experiment are that the acetonitrile treatment separates the toxic material into two fractions. Notice in Figure 38 that all toxic activity precedes the 20 minute elution time and that in Figure 39 the toxic activity follows the 20 minute elution time.

When the fractions are examined on the analytical column and the wavelengths scanned, they indicate that the principal brown component extracted by the ethyl acetate is peridinin. Indeed NMR examination of the fractions indicate that the peredinin peaks are present in the crude and almost absent in the ethylacetate insoluble material. After the ethyl acetate separation, we tried to selectively precipitate the toxic fractions with acetonitrile (see Figure 40 below).

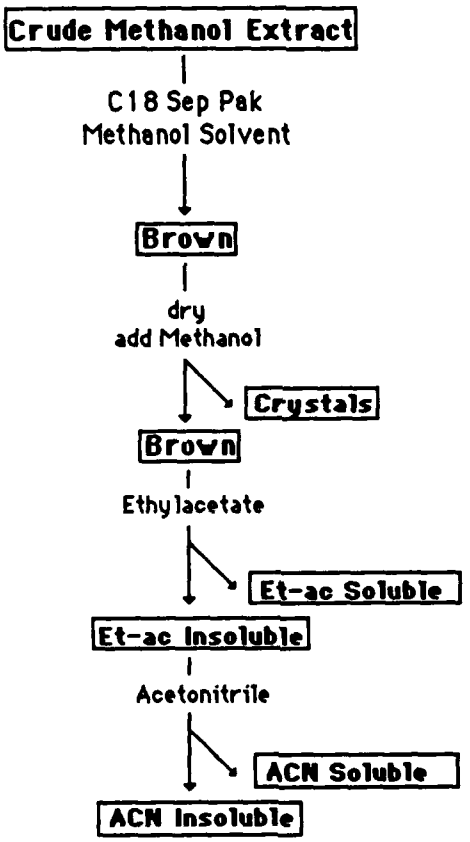


Figure 40. Schematic diagram for the acetonitrile (ACN) step.

When the ACN-soluble and ACN-insoluble material is examined by ileum assay and compared with the starting material it becomes apparent that ACN extraction reduces the total amount of GT-4

activity and converts it to the GT-3 type. Indeed, this is reflected in a decrease rather than an increase in the specific activity.

Because the ACN treatment gave us a decrease in specific activity due to a degrading of toxin, it was decided this was not a viable step. However, the ethylacetate step is a valuable step. Examination of the ethylacetate insoluble material on semipreparative HPLC produced eight peaks which eluted within the first ten minutes. All of these peaks overlap one another and tend to co-migrate. Finally, because the most likely action occurring to degrade the toxin was a methylation, we conducted an ACN precipitation in ethanol rather than methanol. This procedure produced the cleanest HPLC chromatogram of toxin yet. This fraction has yet to be looked at on the NMR and the preparative unit. Nevertheless a major fraction of the toxin is retained and is tentatively associated with the sharp peak at 210 nm and the other wavelengths.

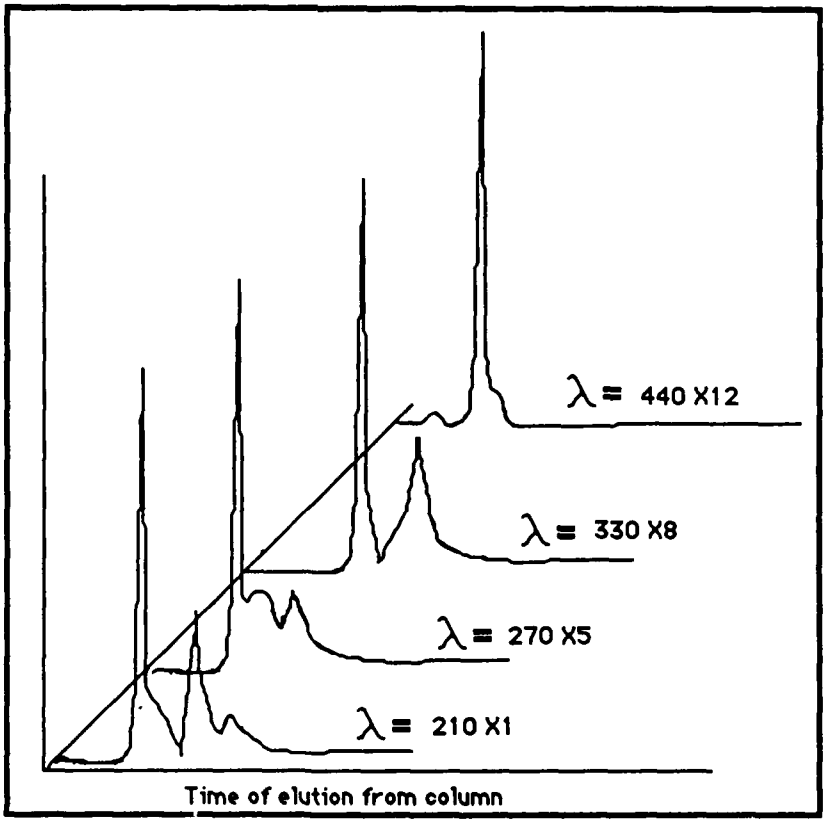


Figure 41. Analytical HPLC aliquot sizes were 5 ul. Other running conditions were 5 micron C-18 RCM column. Flow rate 0.3 ml/min and 100% methanol as solvent.

GT-1 (Ciguatoxin) separation

The green fraction from SK-1 gr was applied to semipreparative HPLC and the resulting chromatogram is shown as Figure 42.

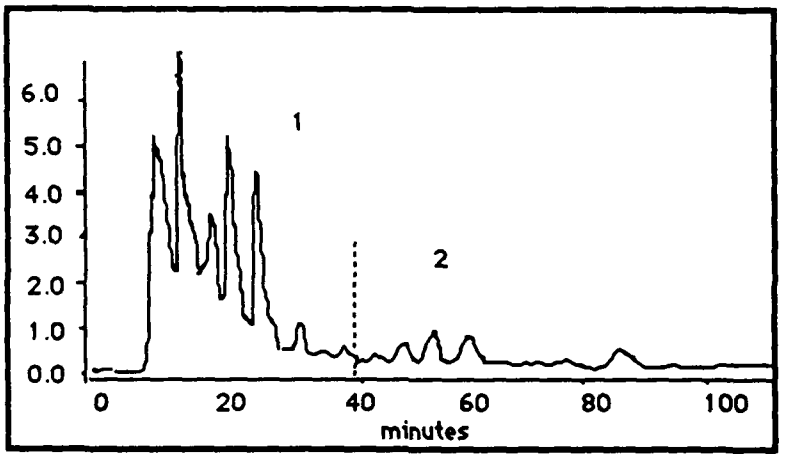


Figure 42. Semipreparative chromatogram (SP-7) of the green fraction from the sep-pak separation of crude methanol extract of *Gambierdiscus toxicus*.

The chromatogram from SP-7 shows some contamination but the significant item was the increase in the more lipid components in relation to the more polar ones. Fractions SP7F4 and SP7F5 were combined and rerun on the semipreparative as SP-8. Table 22 lists the tentative results from these experiments.

Table 22
QUANTITATION OF CTX TOXIC COMPONENTS BY ILEUM ASSAY

FRACTION OF CRUDE	COLOR OF FRACT	PERCENT mg%	TOTAL ACTIVITY MU-MTX	SPEC ACTIVITY MU/ug	WEIGHT ACTIVITY ug/MU	PERCENT PURITY (note 1)	PERCENT YIELD
Crude Ext	Dk Br	100.	300	.0326	30.6	.0098	100
Green	Green	7.48	*1.8	.4048	.00247	.7411	.6%

Note. The LD_{50} values used in this table are estimates based upon preliminary mouse data and the actual graph and LD_{50} value will be reported in the next quarterly.

The data shows that the percent of CTX in the cell extracts is small being less than 0.6% in toxicity. If we assume .009 ug/MU and there is 1.8 MU/9.2mg crude, this means that there is .0162 mg of CTX per gram of dried extract. Then there is 3.24 mg of material in

the 200 grams of dried material that we have thus far accumulated. Preliminary preparative HPLC of the green fraction indicates that toxicity elutes in a broad band within the central portion of the chromatograph.

During the course of the previous preparative run P-4 it was decided that the crystallization step could be performed immediately from the brown sep pak fraction. This made the HPLC run characteristics much better and reduced the chances of fowling the column. Therefore the brown fraction (SK-3 br) was evaporated and crystal formation induced. The supernatant (XTL-1) was decanted off leaving the crystals (XTL-2). Fraction XTL-1 was applied to the preparative HPLC (P-5) and the chromatogram from this run is shown in Figure 43.

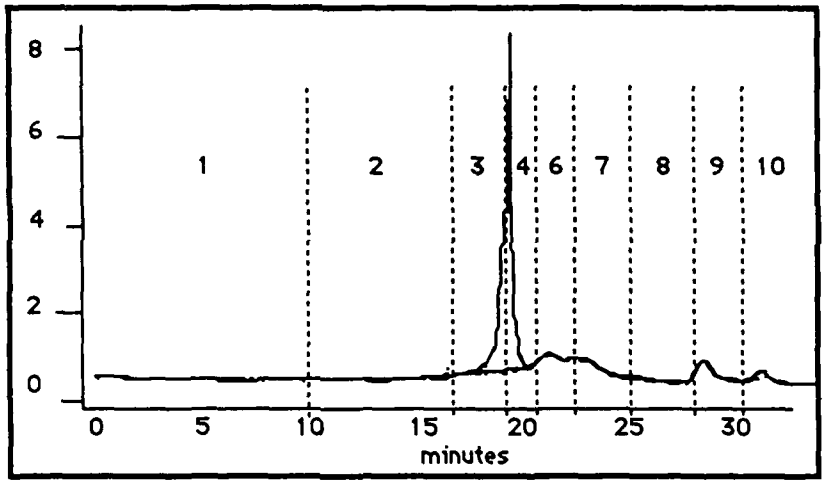


Figure 43. Preparative chromatogram (P-5) of the brown fraction from the sep-pak treatment (SK-3) and subsequent crystallization of ten ml of crude methanol extract (GT-350 Combo).

Preparative run P-5 was collected in nine fractions as was done in the previous one ml run. Fractions P5F4 and P5F5 were assayed by ileum procedure and then combined with P4F4 and P4F5 respectively before running on the NMR.

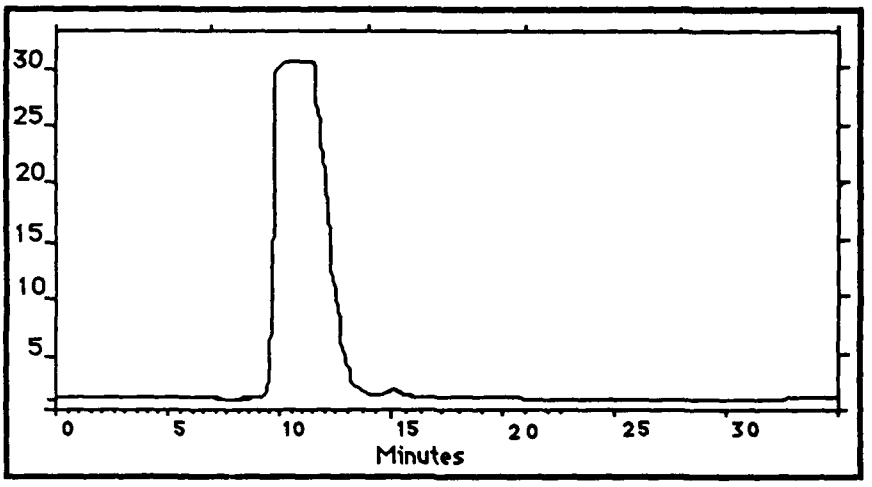


Figure 44. Preparative chromatogram (P-6) of the brown fraction (P5F6) from the preparative run P5.

SK-3br was slowly evaporated until crystals no longer formed and the supernatant decanted. The supernatant was then applied to the preparative HPLC and run as P-6.

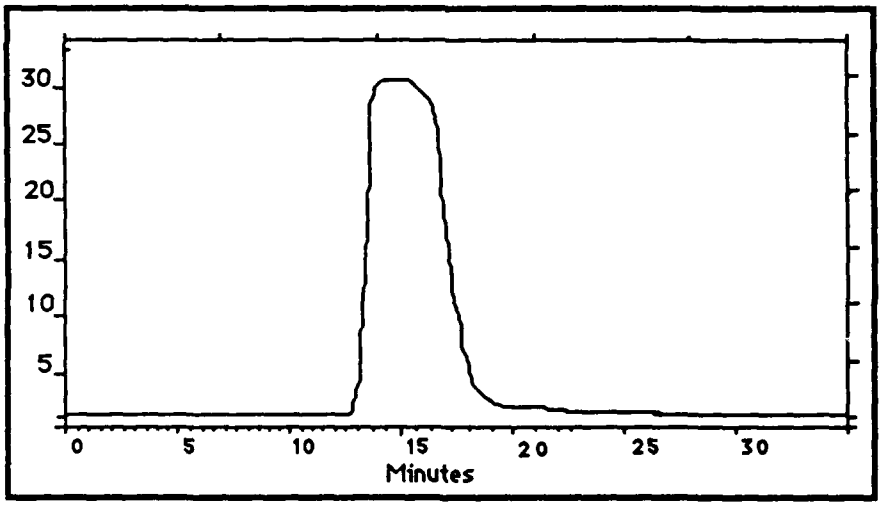


Figure 45. Preparative chromatogram (P-6) of the brown fraction (P5F5) from the preparative run P5.

Nine fractions were collected and MTX activity identified in P6F3. This fraction was then reduced in volume and re-applied to the preparative HPLC as run P-7. Four fractions were collected from this run. In looking over the data from our ileum assays, HPLC chromatograms and NMR scans we came to several conclusions. The ileum records indicated that each time we split the major 209 nm

peak material on the HPLC we split the toxic activity. Even when we split and split again, the toxin was not 100% pure. The conclusions from the NMR data up to this point indicated that several compounds were being scanned. From all of the data then it was surmised that the 209 nm peak seen on the preparative HPLC consisted of seven components (Figure 46).

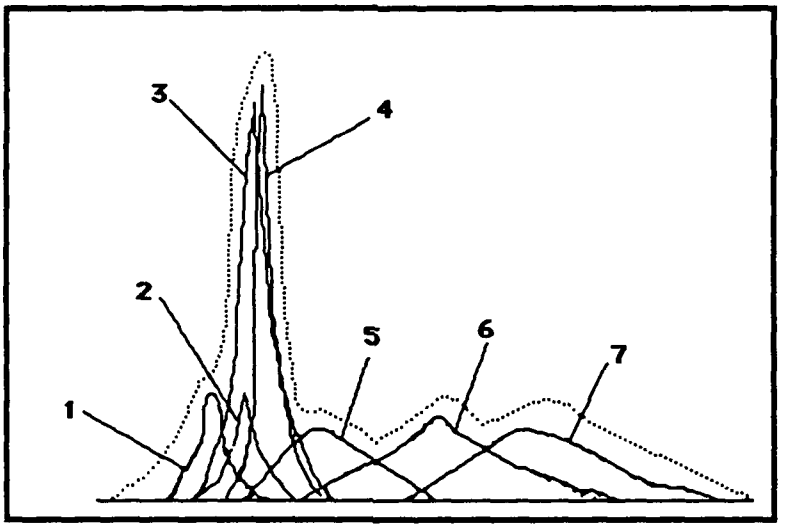


Figure 46. Diagrammatic representation of the components of the major 209 nm peak. Actual peak is dotted line. See text for explanation.

Figure 46 above represents the overall waveform seen in the preparative HPLC of the toxic fraction (dotted line). We have been able to identify a minimum of seven different components that constitute that waveform and they all overlap. Component 2 is green when concentrated, #4 is brown in color, #6 is yellow, and #7 is green.

Some of the solvents which we would like to have utilized in the HPLC either caused continual precipitation within the columns (e.g. acetonitrile) or had an absorption in the range we wanted to monitor (210 nm). We rationalized that we could effect the selective solvent separation prior to HPLC detection and fractionate the similar compounds.

Using the prior rationale, we began a separation process which was a radical departure from the usual. Our philosophy was to attempt solid phase extraction of the *G. toxicus* extracts with selected solvents prior to preparative HPLC chromatography. To accomplish

this we first utilized C-18 Sep Pak's to separate the *G. toxicus* extracts into color based fractions: brown, yellow, and green.

Separation of the pigment toxin complexes

At this point then, the decision was made to reverse the process for purification once the precipitation step was finished. That is, the pigments and associated toxin would be selectively extracted from the maitotoxin fraction while it was in the dried form. Because (as previously reported) only GT-3 was soluble in acetonitrile, it was utilized to force crystallization of the GT-4. Once a pigment complex was isolated it was taken up in methanol and then an equal volume of acetonitrile was added and mixed. The sample was then slowly dried under dry nitrogen gas until precipitation occurred. The supernatant was decanted and the process repeated until no more crystals formed. Each was then dried down and taken up in the original volume of methanol and aliquots taken for ileum assay.

Accordingly, a one ml sample of GT350-Combo was processed by sep pak (SK-4). The green and yellow fractions were set aside. The brown fraction was then processed as follows:

The fractions from the (solid-phase) separation of pigments were tested on the ileum preparation. The amount of toxin required to produce 50% inhibition 90 minutes after a 15 minute inhibition has been defined as an ileum equivalent unit (IEU). We know from experience that approximately 2,000 IEU will equal 1 mouse unit of crude maitotoxin. When compared with the SK-brown starting material, the chloroform extract contained 50% of the initial toxic activity. The DMSO contained 25%, the ACN 10% and the residue approximately 20% of the toxic activity.

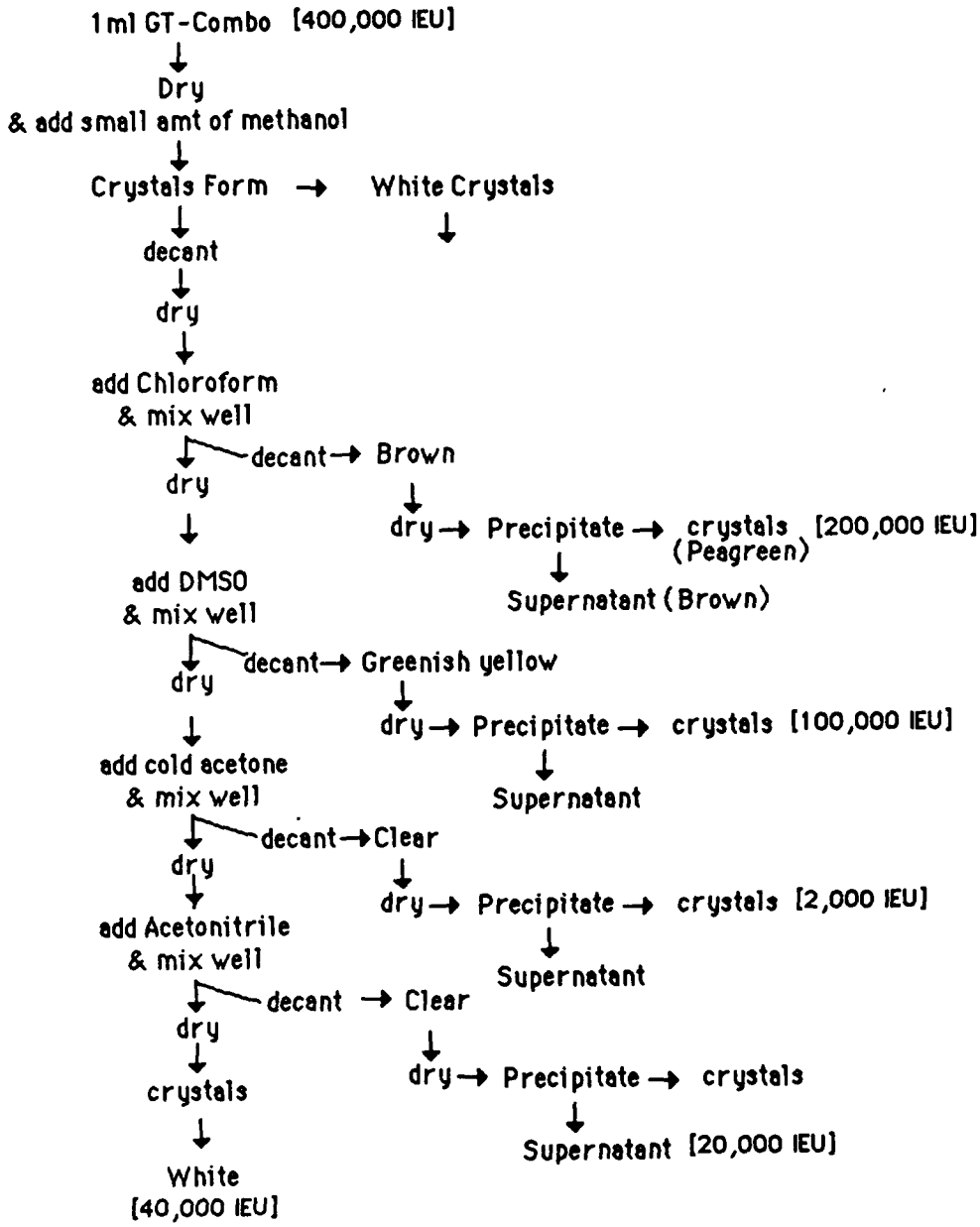


Figure 47. Diagram of the solvent extraction of SK-4

This procedure allows us to recapture much more toxin in the process of purification then we have ever been able to do before. Notice that we can recover up to 90% of the total toxin assayed in the crude. All but one of the toxic fractions recovered are multi-component systems, having pigments associated with them. Thin layer chromatography of the toxic precipitates (as well as NMR) indicates at least three components.

Separation of GT-4 (MTX)

To continue the separation of the maitotoxin fraction we investigated selective solvent extraction of the solid phase material. To this end we found that extraction of the dried material with ethyl acetate produced an ethyl acetate soluble and an ethyl acetate insoluble fraction. The bulk of the toxicity was found in the insoluble fraction.

Table 23
QUANTITATION OF MTX TOXIC COMPONENTS BY ILEUM ASSAY

FRACTION OF CRUDE	COLOR PERCENT OF PURITY FRACT	PERCENT YIELD mg%	TOTAL ACTIVITY MU-MTX	SPEC ACTIVITY MU/ug	WEIGHT ACTIVITY ug/MU	PERCENT ACTIVITY (note 1)
Crude Ext	Dk Br	100.	300	.0326	30.6	.0098	100
Brown	Brown	83.16	210	.0236	42.3	.0070	70%
EtOacIns	Green	40	200	.0530	18.25	.0164	66
ACN ppt	Green	13	45	.0384	26.57	.0112	15

Note. The LD_{50} values used in this table are estimates based upon preliminary mouse data and the actual graph and LD_{50} value will be reported in the next quarterly.

The ethyl acetate extraction of the brown fraction is an especially fortuitous step in that, with it, we (1) partially separate the complexes, (2) split off the majority of the peridinin carotenoid from the toxic fraction, (3) achieve an increase in specific activity with a ten fold increase in purity and (4) retain 66% of our starting material. This is a two and a half fold increase in the cost benefit ratio. HPLC chromatograms of the ethyl acetate fraction is shown in the following figure.

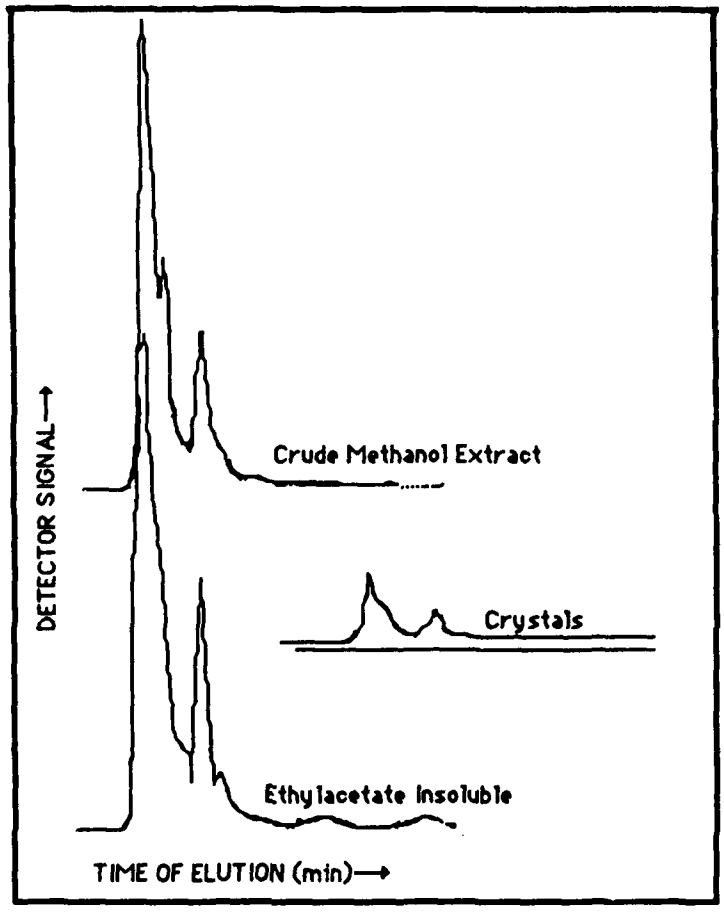


Figure 48. Comparison of analytical HPLC chromatograms of crude brown versus ethylacetate insoluble. Running conditions were: Novapak 5 micron C-18 silica gel, 3.9 mm X 7.5 cm column, and 100% methanol as solvent, run time 55 min.

Separation of crude methanolic extract

As a result of discussions during a contractors meeting in November, 1988, we have initiated the processing of 60 g of dried cells of *Gambierdiscus toxicus* (clone 175). We have determined that 2 g of dried cells is the ideal sample size for extraction in workable volumes of methanol. Five 2 g samples have been extracted (plus one such sample delivered on 11 November 1988 as delivery item number 0001BL). The yields from these extractions ranged from 102,782 to 169,377 mouse units per sample (LD_{50}/20 g mouse = 1 MU). Corresponding mouse unit values of these samples ranged from 4.6 to 3.5 μg.

Further purification of bulk quantities of toxic extracts is being accomplished using three basic approaches. The method which yields the highest recovery of most potent material will become our standard. The first method involves low pressure C_{18} fractionation into three major components (brown {containing polar toxin(s)}, yellow, and green {containing less-abundant less-polar toxin}) followed by preparative HPLC of the brown fraction. This treatment yields 90-100% of the total mouse units (in two fractions) with potency as high as 2.9 µg/MU. Major impurities in these two fractions are limited to chlorophyll C_2, peridinin, and one or a few heavy unknown compounds which absorb at 210 nm. Initital analytical HPLC fractionation of the above fractions yielded products with a potency as high as 1.09 µg/MU (with nearly 100% recovery). The latter value represents an LD_{50} of 55 µg/Kg mouse. Although quantities obtained by analytical HPLC are small, they are quite sufficient for experimentation using nerve-muscle preparations and primary cell cultures.

The second method for initial bulk purification of methanol extracts being tested is standard silicic acid column chromatography. We have been successful in processing 200-300 mg levels of crude toxin with yields having potencies approaching 1 µg/MU. However, recovery of material with this potency is at the 40-50% level. The remaining toxin is not lost but recovery requires laborous rechromatography. We are attempting to increase purity of all toxic fractions from the silicic acid column using preparative TLC. Preliminary results are promising. Initial mouse assays of toxin from one TLC treatment revealed potencies at about 0.5 µg/MU (LD_{50} = 25 mg/Kg). These experiments must be repeated in order to confirm potency and to determine efficiency of the procedure.

The third method currently being employed represents a return to our standard liquid-liquid partitioning procedure As previously reported, this method yields two fractions containing "maitotoxin(s)" [GT3 and GT4] and one fraction containing small quatities of "ciguatoxin(s)" [GT1 and GT2]. We had hoped to avoid using this method because it has consistiently resulted in the loss of over 50% of the toxic activity present in the initial methanol extract. However, after numerous experiments with varying solvent to toxin ratios, we have achieved nearly 90% recovery of the initial toxic activity in terms of mouse units. The maitotoxin fraction (GT3) which partitions to ether in the initial ether:water partitioning has a

potency of 1.5 μg/MU. The bulk of the maitotoxin (GT4) which partitions to water has a potency of about 0.6 μg/MU (48 hour mouse assay is being conducted at this time). Analytical HPLC of this fraction (GT4) shows it to be quite clean compared to the toxic fractions from single preparative HPLC and silicic acid column treatments. Interestingly, a comparison of results from analytical HPLC of GT3 and GT4 revealed the possibility that these toxic components may differ to some degree.

It is hoped that the quantity and purity of GT4 derived by liquid-liquid partitioning are such that preparative HPLC or preparative TLC may be used to efficiently process large quantities with a resulting potency of about 0.1 μg/MU (LD = 5 μg/Kg mouse). During the next reporting period we will continue to extract large quantities of *G. toxicus* (175) and continue experimentation with bulk processing methodology. Also, it is our plan to deliver a sizable sample of partially purified maitotoxin (GT4) with a potency of about 1 μg/MU. Should we achieve our next goal of obtaining measurable quantities of toxin with potencies of 0.1 to 0.2 μg/MU, such samples will be forwarded immediately.

Results of ileal assays

Concomitantly with our separation work, we began attempting to quantitate the ileum assay with each of the three components that we isolated from the crude extract. Toxicity to the whole ileum does not imply the same toxicity to the whole mouse. We simply established reproducible end points for the ileum assays, defined these as an "Ileum Equivalent Unit" and then attempted to correlate these with mouse bioassays. For instance, using the 50% inhibition at 90 minutes after a 15 minute incubation as the end point for one MTX ileum unit (MIU), the ratio for crude *G. toxicus* extract is approximately 2000 ileum units equals 1 mouse unit.

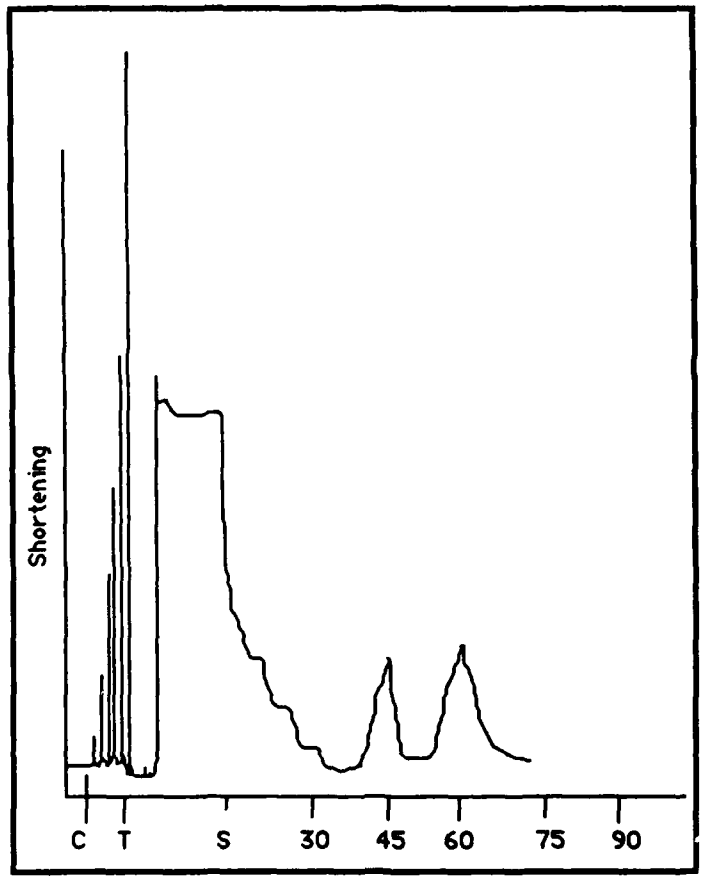


Figure 49. Records of guinea pig ileum assay (MTX assay) of the brown fraction from the sep-pak separation (SK-1 br) of crude methanol extract of *Gambierdiscus toxicus*. C=control stimulations, T=application of toxin, and S=start of experiment. 4 µl of extract was added to 10 bath of PSS and incubated for 15 min.

Simply stated then:

$$\text{MTX Ileum Equivalent Unit} = \frac{\text{MTX Mouse Unit}}{2000}$$

and

$$\text{CTX Ileum Equivalent Unit} = \frac{\text{CTX Mouse Unit}}{10000}$$

These equivalents are invaluable in allowing us to determine which clean up steps are worth incorporating into our purification technique.

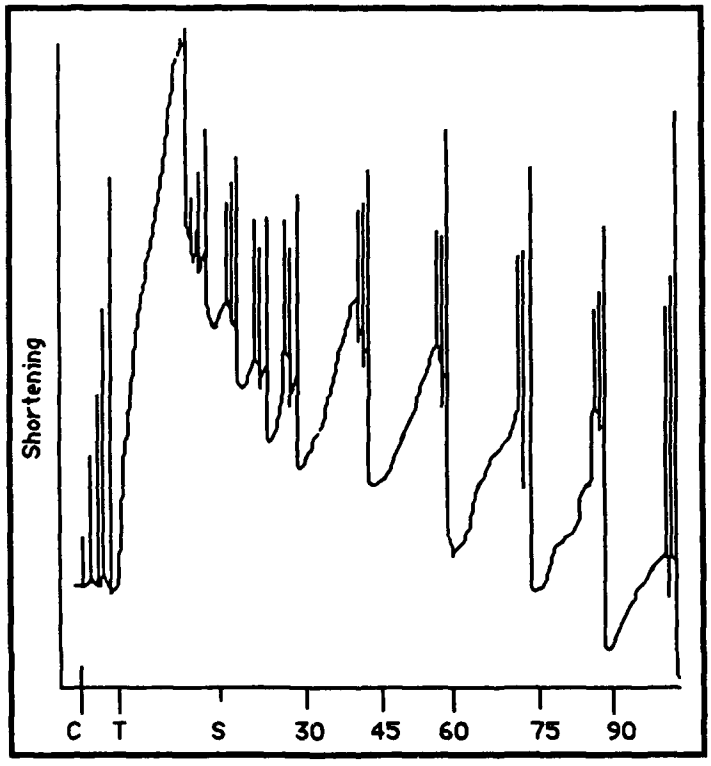


Figure 50. Records of guinea pig ileum assay (MTX assay) of the yellow fraction from the sep-pak separation (SK-1 yel) of crude methanol extract of *Gambierdiscus toxicus*. 1 µl of extract was added to 10 bath of PSS and incubated for 15 min.

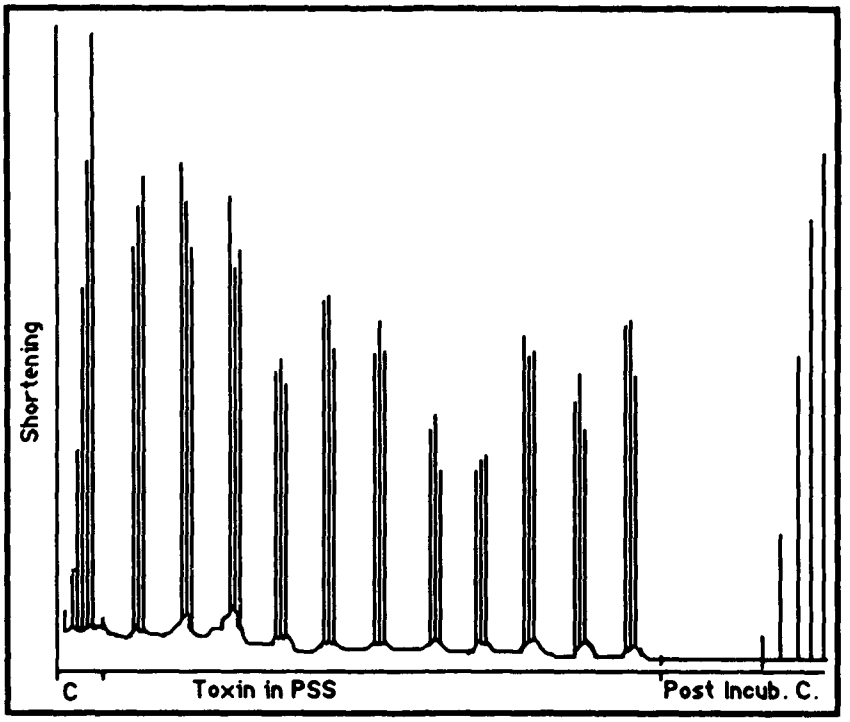


Figure 51. Records of guinea pig ileum assay (CTX assay) of the green fraction from the sep-pak separation (SK-1 gr) of crude methanol extract of *Gambierdiscus toxicus*. 50 µl of extract in 500 ml of PSS.

During this second year over 1,000 ileum assays were conducted. Review of the ileum assays indicates that certain solvent and reagents have a distinctive effect upon the type of result that one sees on the ileum. Some tend to change the response seen from an irreversible to a reversible effect (e.g. ACN soluble, acetone). Acetone also causes an erratic response of the base line of the preparation. Still other solvents cause a decided change in the inotropic response of the ileum.

NMR SPECTRUM OF HPLC PURIFIED MAITOTOXIN

We have obtained the 300 MHz proton spectrum of preparative HPLC purified maitotoxin (Fraction P4F3) in methanol as shown in Figure 52. The amount of sample used was minimal as indicated by the solvent to sample peak ratio, even though we used 99.95% deuteriomethanol.

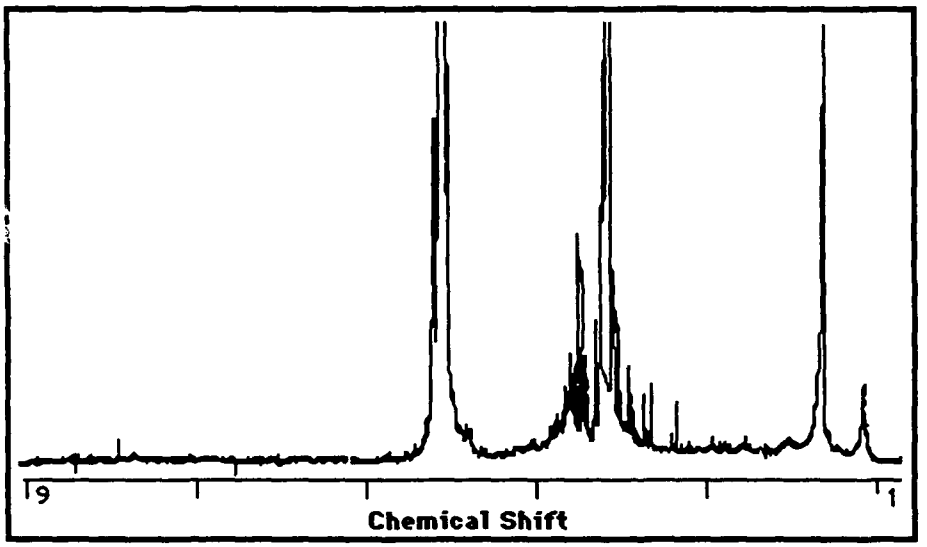


Figure 52. 300 MHz Spectrum of Preparative HPLC Purified Maitotoxin.

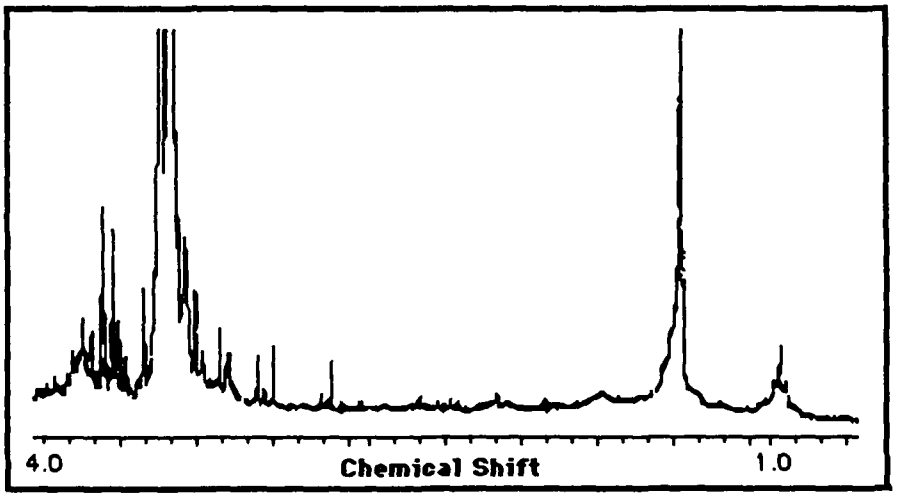


Figure 53. 300 MHz Spectrum of Preparative HPLC Purified Maitotoxin

Furthermore the OH peak at 4.8 PPM may have been enhanced by proton exchange with OH groups on the sample - it being out of proportion with the CH3 peak at 3.30 PPM. Figure 53 is the enlarged right side of Figure 52.

The spectrum indicates that this sample exists as a two component system. One set of large peaks is seen in contrast to a set of smaller peaks. The larger set of peaks may be an aliphatic type molecule closely associated with the toxin molecule, presumably the series of smaller peaks. Figure 54, reveals the contrasting larger and smaller peaks in even greater detail.

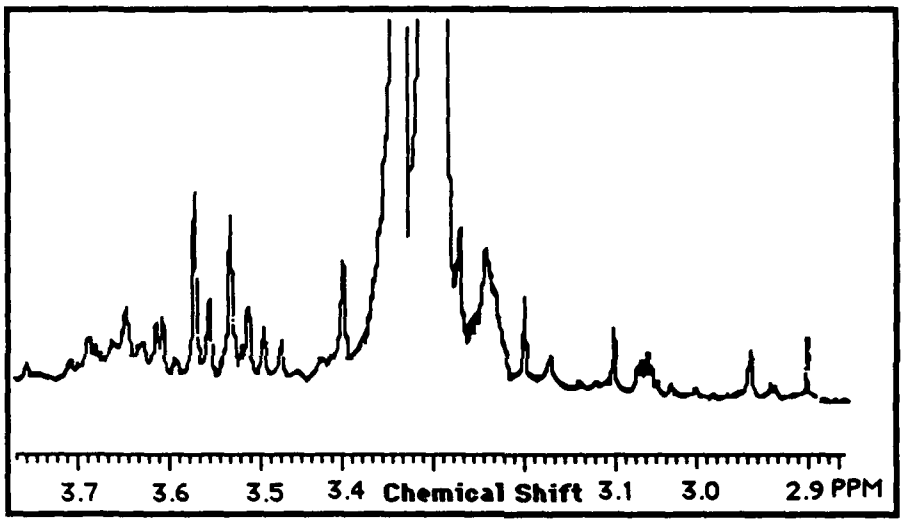


Figure 54. Enlarged Right Portion of Spectrum of Preparative HPLC Purified Maitotoxin.

We believe that the toxin molecule is represented by the smaller peaks because it has been reported that maitotoxin contains highly polar OH, NH_2, and ether linkages. These resonances occur at lower field than 3.7 PPM, the lowest field resonance of the larger peaks in the spectrum. As seen in Figure 54, some resonances are seen at lower field although the signal to noise ratio here is questionable, even though the spectrum was accumulated for 16 hours overnite. Resonances in the 5.5-9.0 PPM range would include NH, olefinic, and conjugated ring protons which maitotoxin may contain.

Numerous attempts to obtain an NMR spectrum of the toxin from fractions extracted from the HPLC column of small amounts (1 or 2 ml) of crude toxin were unfruitful. Basically this revealed a

pattern of large and small peaks but none could unequivocally be assigned to the toxin.

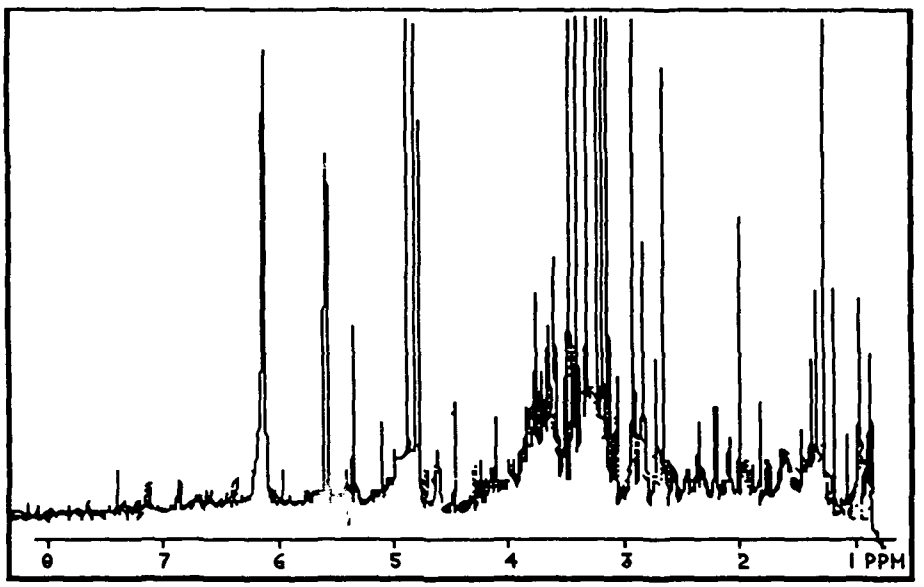


Figure 55. Complete proton spectrum (1-9 PPM) of rust brown fraction (P4F3-4) from color separation on HPLC unit of 10 ml of crude GT-combo. This sample contained 1,000,000 IEU.

However, when larger amounts of crude toxin were used, small peaks in the low field region, 5.7-8.8 PPM emerged which were not previously resolved. The spectrum from such a fraction containing 1,000,000 IEU of toxin and the major brown pigment is shown in Figure 55.

This fraction was obtained from color separation on the HPLC preparative unit from 10 ml of crude GT-combo. Another fraction from 10 ml of crude GT-combo containing 2,000,000 IEU was taken from the upside of the dominant 209 peak. This spectrum is shown in Figure 56. and reveals very distinctly small peaks in the 5.5-8.8 PPM region.

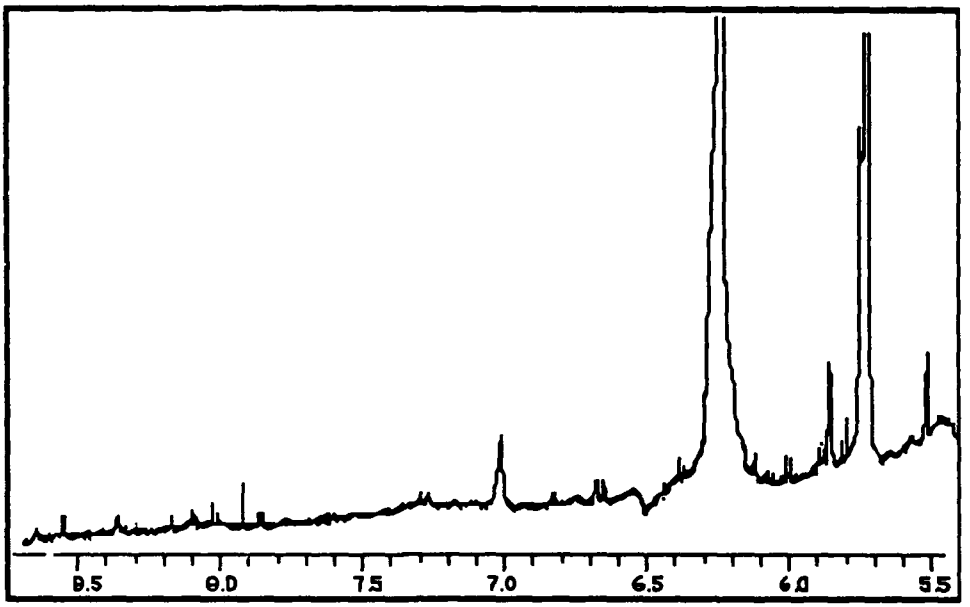


Figure 56. Lowfield region of ^{1}H spectrum (5.5-8.8 PPM) of upside of 209 peak off column P4F5 which was light green in color) separation on HPLC unit of 10 ml of crude GT-combo. This sample contained 2,000,000 IEU.

Another fraction containing 600,000 IEU obtained from color separation of the HPLC column is shown in Figure 57.

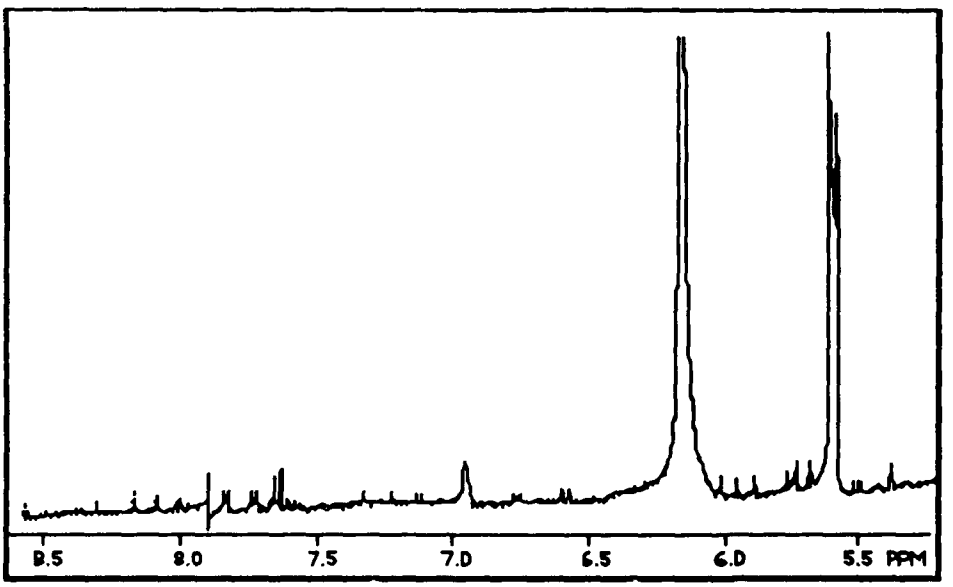


Figure 57. Lowfield region of ^{1}H spectrum (5.5-8.8 PPM) of downside of large 209 peak (P4F6 = brown in color) separation of 10 ml of crude GT-combo. This sample contained 600,000 IEU.

We believe that these small emerging peaks are from the toxin and are observable only when the toxin concentration is several hundred MU. A listing of the peaks common to these spectra and their multiplicity is given as follows:

TABLE 24
PARTIAL PEAK ASSIGNMENTS TO MAITOTOXIN NMR SPECTRA

Δ (PPM)	Multiplicity	Δ (PPM)	Multiplicity	Δ (PPM)	Multiplicity	Δ
8.68	s	8.02	d(9Hz)	6.04	d(7.02Hz)	
8.58	d(6Hz)	7.83	d(4.5Hz)	5.90	d(4.5Hz)	
8.38	s	7.23	s	5.78	d(7.0Hz)	
8.31	s	7.12	d(8Hz)	5.70	d(8.0Hz)	
8.17	s	6.75	d(9Hz)			
8.10	s	6.58	d(13.5Hz)			

Signals in the low field region of the proton NMR spectrum possibly arise from NH and OH groups in conjunction with other polar moieties. This implies that the toxin is very polar and explains many phenomena which we and others have observed. The splitting of the small doublets in the 5-9 Hz region is a strong indication that they do not arise from the protons on the long conjugated carbon chains of the pigment molecules. These couplings from cis and trans double bonds are commonly known in the 11-15 Hz range.

NMR of solvent extracts of Sep-Pak separations

However to further substantiate this we extracted the pigments with various solvents (Figure 58) and examined their NMR spectrum. From the area near the 210 peak on the HPLC column we found three pigments, a brown pigment and 2 green pigments. Choloroform extracts the brown pigment and one of the green pigments. DMSO extracts the other green pigment. The brown pigment can then be extracted from the first green pigment with ACN. The proton spectrum of the brown pigment is shown in Figure 58.

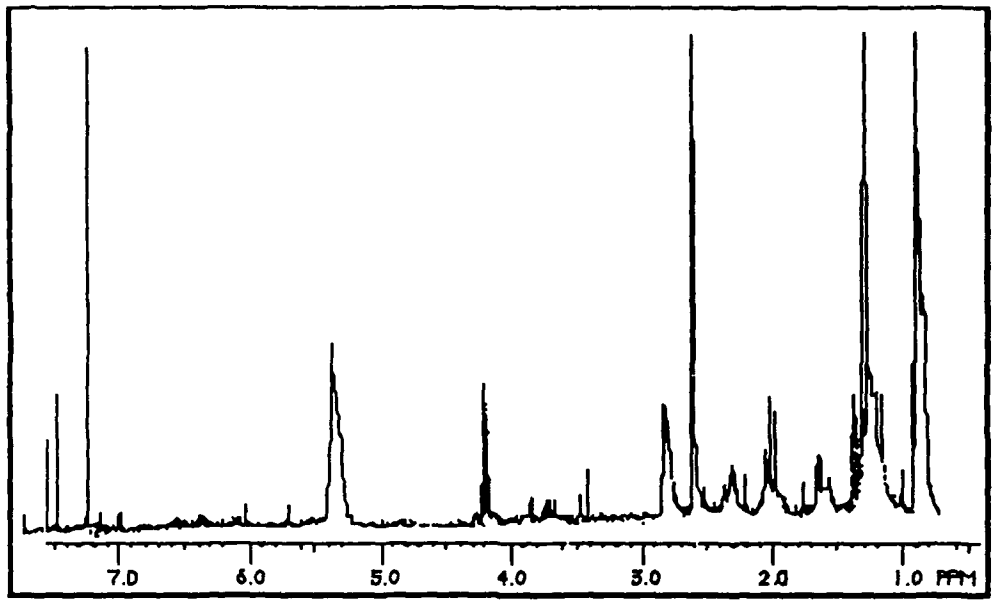


Figure 58. Complete ^{1}H spectrum (1.0-8.0 PPM) of brown ACN soluble pigment after chloroform extraction of 10 ml of crude GT-combo. This sample did not have any activity toward the ileum preparation.

While small degradation peaks are seen in the 5.5-7.5 region, these do not match those listed from the samples containing large amounts of toxin. The spectrum of the second green pigment, the DMSO extract is shown in Figure 59.

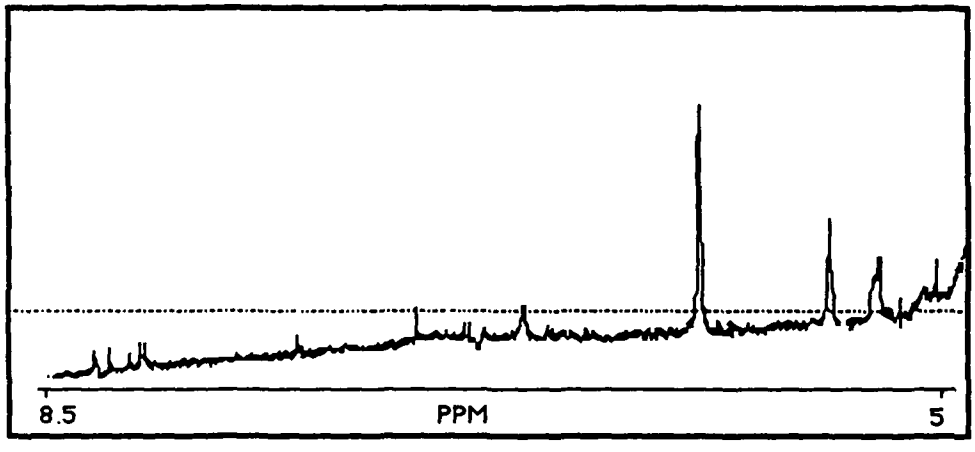


Figure 59. Lowfield ^{1}H spectrum of DMSO extraction 10 ml of crude GT-combo. This sample contained 200,000 IEU.

This sample contains 100 MU of toxin and a few of the signals which we are attributing to the toxin. The NMR spectrum of the first

green pigment with no MTX is shown in Figure 60. and contains one of the toxin signals.

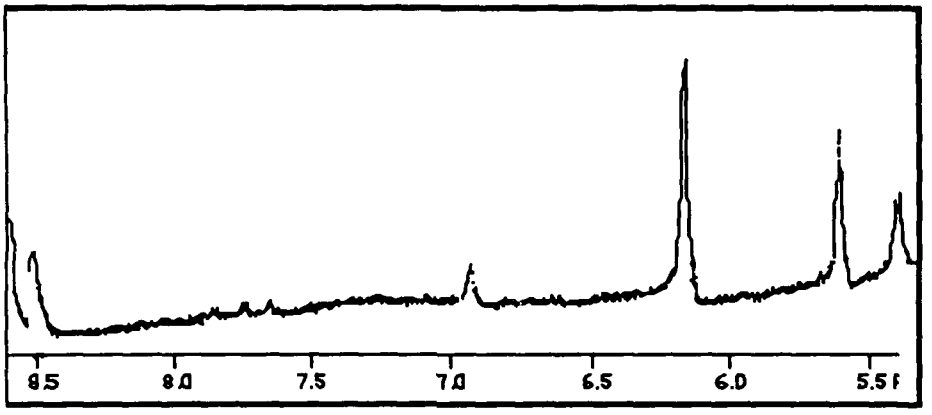


Figure 60 Lowfield region of ^{1}H spectrum (5.3-8.7 PPM) of green pigment from chloroform extract of 10 ml of crude GT-combo. This sample contained was not toxic to the ileum preparation.

We feel that the toxin is very closely associated with the pigments and as has been shown can be extracted with the pigment and further purified.

•**Delivery of toxins:**
The following have been delivered to USAMRIID this past year:

TABLE 25
List of Deliveries to USAMRIID

Lot No	Mass (mg)	Contract Item No	Amt	Date
Lot GT350-COMB	9.2	Item 0001AN	1 ml	15Mar88
Lot GT350-COMB	9.2	Item 0001AO	1 ml	15Mar88
Lot GT350-COMB	9.2	Item 0001AP	1 ml	15Mar88
Lot GT350-COMB	9.2	Item 0001AQ	1 ml	15Mar88
Lot GT350-COMB	9.2	Item 0001AR	1 ml	15Mar88
Lot GT350-COMB	9.2	Item 0001AS	1 ml	15Mar88
Lot GT350-COMB	9.2	Item 0001AT	1 ml	15Mar88
Lot GT350-COMB	9.2	Item 0001AU	1 ml	15Mar88
Lot GT350-COMB	9.2	Item 0001AV	1 ml	15Mar88
Lot GT350-COMB	9.2	Item 0001AW	1 ml	15Mar88
Lot GT350 2B87'u'	.116	Item 0001AA-1	.116 mg	22Apr88
Lot GT350 2B87'u'	.116	Item 0001AA-2	.116 mg	22Apr88
Lot GT350 2B87'u'	.116	Item 0001AA-3	.116 mg	22Apr88
Lot GT175	9.2	Item 0001AX	1 ml	15Aug88
Lot GT175	9.2	Item 0001AY	1 ml	15Aug88
Lot GT175	9.2	Item 0001AZ	1 ml	15Aug88
Lot GT175	9.2	Item 0001BA	1 ml	15Aug88
Lot GT175	9.2	Item 0001BB	1 ml	15Aug88
Lot GT175	1.5	Item 0001BC	1 ml	15Aug88
Lot GT175	1.5	Item 0001BD	1 ml	15Mar88
Lot GT175	1.5	Item 0001BE	1 ml	15Aug88
Lot GT175	1.5	Item 0001BF	1 ml	15Aug88
Lot GT175	1.5	Item 0001BG	1 ml	15Aug88
Lot GT175	23.2	Item 0001BF	1 ml	27Oct88
Lot GT175	23.2	Item 0001BG	1 ml	27Oct88
Lot GT350	23.2	Item 0001BH	4 ml	27Oct88
Lot GT350	23.2	Item 0001BI	4 ml	27Oct88
Lot GT350	23.2	Item 0001BJ	4 ml	27Oct88
Lot GT350	23.2	Item 0001BK	4 ml	27Oct88
Lot GT175	516.6	Item 0001BL	205 ml	11Nov88

•Travel performed during this period:

The Federated American Societies for Experimental Biology meeting in Las Vegas, Nevada, 18 27 Apr 88, was attended by Drs. Tindall, Bomber and Miller. Three posters were presented.

The Association of Marine Laboratories of the Caribbean meeting in Sarasota, Florida, 21-28 May 88 was attended by Drs Tindall and Miller. Three posters were presented.

Drs Bomber, Tindall and Miller atended the International Congress of Toxinology in Stillwater, Oklahoma from 1 to 8 August 1988. Three papers were presented as follows:

Bomber, J., D. R. Tindall and D. M. Miller, Intraspecific variability in acclimated cell toxicities among Atlantic and Pacific clones of the ciguatera-causing dinoflagellate *Gambierdiscus toxicus*.

Tindall, D. R., D. M. Miller and J. Bomber, Culture and toxicity of dinoflagellates from ciguatera endemic regions of the world.

Miller, D. M. and D. R. Tindall, Identification of an acetonitrile soluble toxic fraction from the dinoflagellate, *Gambierdiscus toxicus*.

Drs Miller and Tindall attended the joint U.S.-Japan Conference on Toxic Materials in Foods in Washington, D. C. (1-5 Nov 88), and subsequently visited Fort Detrick for a contractors meeting.

•Visitation:

Dr Aaron Fox of the University of Chicago visited the laboratory 26 Feb 88. Dr. Fox is interested in the pharmacology and function of neuronal calcium channels and hence maitotoxin.

Dr J. Babinchack of the National Marine Fisheries Laboratory, Charleston, South Carolina visited our laboratories on 18 through 21 Apr 88. He presented a seminar for the group entitled ciguatera toxin research. While visiting he observed the ileum assay system, culturing techniques, and mouse bioassays.

Drs Michael F. Capra and John Cameron from the Faculty of Health Sciences, Queensland Insititute of Technology, Brisbane, Queensland Australia visited the laboratory for three days prior to the start of the IST meeting in Stillwater, OK. They were taken through our entire procedures, including the ileum assay technique. They asked for and received samples of crude extract. They intend to see if the effects they see on nerve with extracts from the Australian material (**58**) is the same as ours.

Summary of Results

1. Examined six physiological parameters among seventeen clones of *G. toxicus.*
2. Examined the potency of clones of *G. toxicus* as a function of time during acclimation.
3. Examined the potency of clone 175 of *G. toxicus* at four different light intensities.
4. Examined the biochemical composition of clone 175 at five different light intensities.
5. Examined the relationship between pigment formation and toxin production during growth of clone 175.
6. Grew and harvested 133.27 grams (dried cells) of clone 350 and 92.94 grams (dried cells) of clone 175 for a total of 226.21 grams.
7. Improved toxin production ten fold by switching to clone 175.
8. Tested eight different purification schemes for the isolation of pure toxin.
9. In the course of testing performed: 1008 ileum assays, 100 mouse bioassays, 800 HPLC runs, and 100 NMR runs.
10. Delivered over 1,427 milligrams of crude or semipurified toxin to USAMRIID.

Discussion of the chemosystematic results

There is ample evidence that the acclimation process is essential to comparisons of reproduction rates, cell yields and potencies for *Gambierdiscus toxicus*. These results have serious implications for past studies in this area which based results on material acclimated for short time periods. Even broad, relative comparisons made without a genetic basis in mind must be made in the acclimated condition. For example, had clone 177 been compared at its initial potency (5.4×10^{-4} MU cell^{-1}) it would have been grouped along with clones from the Bahamas, the Florida Keys and Bermuda. In fact, it has a much higher potency of 26.9×10^{-4} MU cell^{-1}, when completely acclimated.

The continuous batch culture method of Brand et al. (**59**) with concommitant monitoring of reproduction rates appears to be an excellent method of measuring the total fitness of clones of *Gambierdiscus*. For example, the cell potencies and cell yields improved along with reproduction rates. The pattern of changes in reproduction rates and potencies was particularly striking when the data for clone 177 was compared over time. Despite the lag time between reproduction rate increases and potency increases, the two characters appear to be closely associated.

We believe that we eliminated environmental and developmental processes from genetic differences by comparing clones only in the acclimated cell condition. The results corroborate previous results closely. For example, Babinchak et al. (**60**) found a value of 250 cells MU^{-1} for acclimated cultures of clone 175. whereas we calculate an acclimated cell potency for 175 of 183 cells MU^{-1} The relative differences among clones are clear and highly significant by statistical analyses. In addition, sub-clone variability (**61**) does not appear to be a problem for *G. toxicus*. Durand (**62, 63**) also did not find sub-clone potency variability in *G. toxicus* to be significant. Consequently, we conclude that the differences in cell potencies are genetic in origin.

Laurie-Ahlberg et al. (**64**) and Salzman (**65**) point out that quantitative characters can be used genetically. Simple analysis of variance have been successfully used with phytoplankton (**59**; **66**). However, the cell potency differences for a clone of *G. toxicus* in the two phases, acclimated vs. non-acclimated, can be greater than the

genetic differences between clones and this stresses the need for a rigorous acclimation process. We were able to investigate environment-genotype interaction by terminating the transferring of clone 177 in log phase and by placing clone 350 back into the cool white lite environment. In both cases the acclimated cell potency was lost. These results suggest that the environment does influence the cell potency in the same way it affects total fitness, i.e. reproduction rates, which also decrease initially when the environment is changed.

Alternatively, there may not be genetic differences in the amounts of toxin produced, rather, there may be quantitative differences in the enzymes necessary for deactivation of the toxins. Previous work (**62**, **63**; **41**) suggests that *G. toxicus* produces toxins for allelopathic use. If this is true, than *G. toxicus* could have the deactivation enzymes typical of other unicellular organisms that produce bioactive ectocrines (**67**). This would prevent *G. toxicus* from being auto-inhibited. However, this too is unlikely as we would expect the clone with the lowest production of deactivation enzymes to have the slowest growth rate and in fact the opposite is true (clone 175, most toxic, fastest growing).

Most MTX extracts produced identical symptomology in the mice used in the bioassay indicating that we have compared differences among clones in the same toxin. However, clone 135 from Bermuda was an exception in that mice inoculated with material from this clone consistently underwent lumbar contractions. Lumbar contractions have hereterofore not been reported for extracts from *G. toxicus*. Consequently, we realize that this matter will need to someday be resolved at the molecular level as it is possible that different forms of maitotoxin will be found much like were derivatives of saxitoxin. Regardless, this does not exclude the use of the cell potency as a chemosystematic tool. The potency differences either reflect the amount or composition of toxins, both of which appear to be heritible traits that can be compared once the environmental and developmental processes have been removed.

The potency apparently has a fundamental role in the fitness of the genotype and this is supported by two related pieces of evidence. First, the potency begins increasing in early log phase, implying a direct relationship to primary metabolism. Secondly, the maximum cell potency also improves over time along with the

reproduction rate. This suggests possible pleiotropic interactions among potency and other systems related to the reproduction rate.

It is interesting to speculate upon what metabolic events are occurring in the clones as they slowly adapt to the new environment. Meeson and Faust (**68**) speculate that as another dinoflagellate, *Prorocentrum minimum*, adapted to new light quality environments, it may have increased its efficiency in converting photosynthetic products into new cells. The apparent increased efficiency in growth rate was correlated with increasing potency in *G. toxicus* and may reflect a similar trend, with the clones differing in the ability to convert and partition the products, some of which may be toxins. The correlation between increasing growth rate and potency and the pattern of toxin production, coupled with the apparent effect of light bulb type on potency, all strongly suggest that the process of photosynthesis and toxin production are linked. The selective forces modifying photosynthetic response may also effect the potency from south to north. Light intensity could be the major selective force leading to the creation of new genotypes, with clone 175 being the most fit for higher light intensities.

We are currently conducting studies with light to address this hypothesis further. Apparently potency and the gross amount of chlorophyll a and c_2 per cell are negatively correlated. Because clone 175 had the lowest level of chlorophylls per cell, but the highest amount of toxin per cell, studies with light and potency could yield exciting information in determining what metabolic role maitotoxin has in *G. toxicus*. All evidence to date supports the contention that maitotoxin has a role in photosynthesis, including our recent work with thin layer chromatography which pinpoints most of the toxic activity from *G. toxicus* into areas closely associated with chlorophyll c_2.

When comparing the potencies with latitude, we found a general decrease in potency with increasing latitude. This is strikingly reminiscent of results found for the potency of *Protogonyaulax* over a latitudinal gradient (**69**), except that in their case potency increased with increasing latitude. We realize that more clones need to be examined to investigate this pattern completely and within site variation first needs to be more thoroughly analyzed. In this study, there was a high level of variation within a collection site (4 to 10x) and as evidenced by the

T-method analysis but this was not as large as that between sites (up to 30x). This within site variation was highest (10x) for clones from drift algae (see also **70**). Watson and Loeblich (**71**) and Hayhome, et al., (**72**) also found that there could be genetic variation within a site, but far greater variation existed with increasing geographic distance between two source populations. Nevertheless, appreciable supporting evidence was found when a clone from a different Ocean, Hawaiian clone 177, fit into the latitudinal cline were it would be expected to fall based on its acclimated potency.

The variation among drift algae clones could be analogous to that found in the Gulf of Maine for *Prorocentrum micans* by Brand (**42**, **73**). In this region the genetic diversity was high, whereas over Georges Bank the variability was low. This was thought to reflect the relative "stability" of the environments which regulated the mode of reproduction with asexual reproduction being characteristic of the nutrient rich Georges Bank area and sexual reproduction characterizing the Gulf of Maine. We suggest that the drift algal habitat is similar to the Gulf of Maine and sexual reproduction will predominate owing to the poor nutrient levels generally associated with surface waters.

It should be pointed out that there are bound to be exceptions in this overall model. Tosteson et al. (**74**) found that a clone of *G. toxicus* from Puerto Rico was not toxic. Considering the influence of drift algal communities on genetic variation within a site and the possible presence of non-acclimated populations, such results are to be expected. The results of the character survey in this study indicate that *G. toxicus* is genetically polymorphic for certain characters. Additional work using clustering procedures (in progress) indicates that clones from the same patch of drift algae can have the same level of genetic difference as clones from the Florida Keys and Hawaii. These latitudinal patterns suggest that some percent of the polymorphism of certain characters results from selection, the selective forces probably being light and/or temperature in accordance with the potency differences over the latitudinal gradient.

It could also be argued that this study suffers in that potency was only examined in one light environment. Brand (**75**) and Lewontin (**76**) argue convincingly that a character should be examined in more than one environment. As we understand it, this

is generally required to allow full expression of the genome regulating the quality or quantity of the character under examination. Because of the large expense and the current dependence on animal bioassay needed to conduct a study such as this, we decided to conclude the study based on the results from one environment. Consequently, we may not be visualizing all possible differences. Nevertheless we believe that we obtained maximum expression of the toxin potency regulating genes in this environment and detected several races of *G. toxicus*, but again, we cannot argue that all possible races were detected.

When all of the character data are examined by principal components analysis what is interesting are in fact not the differences among clones but the similarities. There may of course be underlying differences in the multiple genes affecting the characters assayed, as each mutation would be of relative small effect. We cannot be sure that the characters assayed arise from isozymes which have different electrophoretic mobilities, although overall the clones examined appear to be closely related. However, it was not the intent of this study to detect such differences and this is left for later work. Still, based upon the overall similarities we conclude that this work resembles what was found by Brand et al (77) who concluded that clones of *Thalassiosira pseudonana* isolated from different neritic areas of the world were genetically similar. He concluded that this was due to these populations experiencing similar selective forces by living in a similar environment. This could also be the case for *Gambierdiscus* as all of the clones that we examined were isolated from highly similar environments although they arise from areas thousands of miles apart.

This work represents the first exhaustive study dealing with the character analysis of *Gambierdiscus toxicus*. We approached the question of variation by anticipating a large number of phenotypic differences. In short, we assessed the character variation in this species much like a taxonomist explores phenotypic differences among congeneric species. However, we could find no evidence to justify separation of an ecotype into a new species. In fact, a tremendous amount of similarity was seen among clones collected from diverse areas. However, the classification into ecotypes or perhaps better, races, is justified, based upon the genetic differences found in the acclimated cell potencies.

Discussion of Sep Pak Material

The HPLC records confirm what has been previously stated about the initial separation process, showing multiple peaks for each fraction at 209 nm.

In summary then, the ileum and mouse assay confirms that the sep-pak process effects a clear separation into three toxic fractions (GT-1, GT-2, and GT-3,4). The UV-Vis scans and analytical HPLC records of these fractions demonstrate an excellent relative separation of three toxic components with just the sep pak step. Notice that this one step of sep-pak separation takes us half-way through the old separation process approximately to the level of WSAP and ESAP (Figure 1).

The advantages of Sep Pak separation are multiple: (1) increased yield of toxin due to (a) elimination of water (b) decrease in the number of solvents interacting with the toxins, and (c) one step separation of three of the toxins. (2) savings of methanol and other solvents and, (3) saving of time. The disadvantages are that the fractions are based visually on color and that the pigments would persist in the separate fractions as contaminants. Nevertheless, the advantages far outweigh the disadvantages in this case and the cost benefit ratio is far to the right.

While we can purify small amounts of toxin by continually splitting peaks on the analytical HPLC, scaling up to purify very large amounts presents problems. As the amount of material processed is increased the separation of peaks decreases and their base width increases. In addition, the peaks represent more than one compound. Therefore, before using the preparative HPLC for separation, we began experimenting to see if we could either selectively extract contaminants or further separate peaks.

Our previous work attempting to separate the toxic factors from *G. toxicus* resulted in the following conclusions. On the HPLC using reverse phase columns, the GT-3 and GT-4 toxins as defined by ileum assay, always eluted immediately after the void volume. Toxic components GT-1,2, however, invariably eluted after twenty five minutes or more depending upon the flow rate. In examining, the fractions eluted from the HPLC, we noted that the toxic peaks, as determined by ileum assay, coincide with the pigments. Analysis of

the Sep-Pak fractions by weight indicated that the bulk of the mass was associated with the GT-3,4 or brown component and the GT-1,2 or green component was small. Solid state extraction of the brown fraction with chloroform, DMSO and acetone proved to indiscriminately separate the toxin complexes, such that there was a continual division of the toxicity.

The separation of *G. toxicus* extracts on HPLC, column and TLC, not only confirmed these conclusions but also indicated that the toxin and the pigments are very large complexes. These complexes have been described in *Glenodinium* sp. and *Gonyaulax polyhedra* (**79**). Indeed our investigations have shown that GT-4 is associated with the chlorophyll c_2 containing complex and GT-1 is associated with the chlorophyll a containing complex.

Hence we began to think in terms of isolating the pigment complexes to extract the toxins. In this respect, we were hampered by the fact that the HPLC detector we have does not cover the portion of the spectrum from 600-900 nm. When crude material is applied to silicic acid columns, the majority of the toxicity sticks to the beginning of the column and cannot be eluted. In TLC plates it sticks to the origin, but some - not 100%- can be eluted from the fines. This would be expected of a molecule with a large number of hydroxyl groups. The material eluted in both cases is light green in color when concentrated.

Our previous work with acetonitrile, led us to believe that only a very minor portion of the GT-3,4 toxic components, the GT-3 was soluble in the solvent. This, of course, would have meant that we had found a mechanism for precipitation of the GT-4 fraction (we now find this is not true).

Finally, preliminary work in attempting to clean up the crude with Sep-Pak systems indicated that we could effect a very economical preliminary separation based upon color. Therefore, we began utilizing these data to formulate a procedure for the clean up and recovery of the toxins.

Our analysis of data indicated that GT-4 was contained in material which migrated at the front when methanol was used as the eluent in the HPLC. This is a composite peak, containing several other closely related compound in terms of solubility-solvent characteristics.

Discussion of the NMR data

To date there has only been one published NMR spectrum for maitotoxin and this is the one published by Yasumoto (77). This spectrum is redrawn here as Figure 61

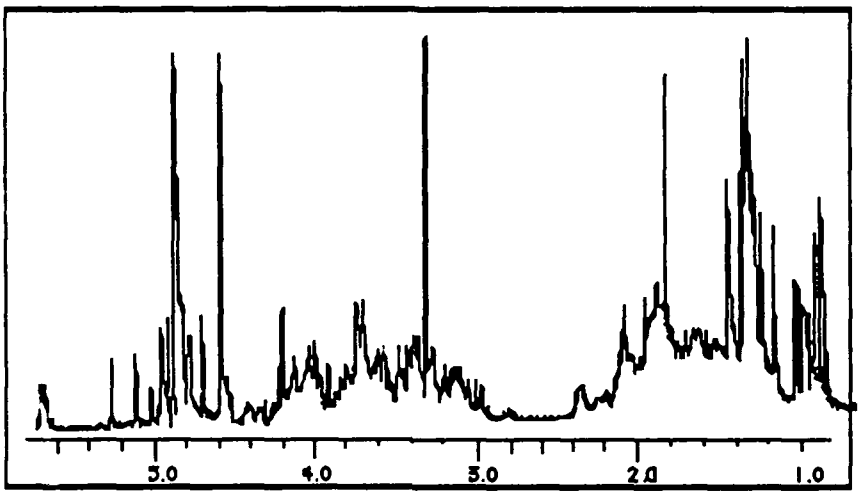


Figure 61. Spectrum for maitotoxin published by Yasumoto (1985). Solvent *system was methanol-*D_2O.

Comparing the spectrum obtained by Yasumoto and the one's we have obtained for crude maitotoxin, they appear to be very similar. However notice, (and this is our conclusion) that the majority of the peaks obtained in the crude scan are due to the associated pigments and the toxin signals are either too small to be seen or are obscured by the contaminants. Notice also, the similarity between Yasumoto's spectra and our spectra on small amounts of maitotoxin (Figure 53 & 54).

While the ^1H NMR spectra published by Yasumoto shows signals only in the 1.0 to 6.0 PPM, we believe that the signals which we observe in the 6.0 to 8.7 PPM arise from OH and NH groups which would exchange in a MeOH-D_2O solvent as was used by Yasumoto for his proton spectra. Our NMR runs have all been made in deuteriated methanol. Our sample shows other small peaks which do coincide with those from the Yasumoto's NMR spectra which we find highly encouraging. As we further process the toxin by solvent extraction to eliminate the other contaminants and to increase the amount of material, we will provide a complete NMR spectrum. Our

observations tends to support the hypothesis that MTX is a large polyether compound with numerous polar sites.

Notice that based on the results so far (i.e. the size of the toxin peaks) the toxin concentration is not more than 100 μg and this was essentially from 11 mls of GT-combo which contained 101.2 mg of material. In order to achieve a separation of 1 mg for an excellent NMR spectrum we would need to process 110 mls of material. This, then would be the next logical step.

Nevertheless, the experiment did provide data relating to the toxic peak. With the larger amount of material we subjected the samples to NMR and found several peaks which correlate with toxic activity. These peaks are described in the following Table 26 and probable structural assignments have been made for them. Both the assignments and their probable structures are tentative at this time.

TABLE 26
TENTATIVE NMR SHIFT ASSIGNMENT AND PROBABLE STRUCTURES

No.	Shift	Descrip	Probable Structural Assignment
(1).	0.89	t(7.0 Hz)	terminal methyl
(2).	0.96	t(7.0 Hz)	terminal methyl
(3).	1.11	s	methyl on tertiary carbon
(4).	1.28	s	chain methylene
(5).	1.46	q(12,5Hz)	methylene on six membered ring
(6).	1.59	q(10, 4Hz)	methylene adjacent to carbon bearing oxygen
(7).	2.08	ddd(10, 8,5 Hz)	methylene adjacent to double bonded carbon
(8).	2.32	dd	methylene protons beta to alpha carbon
(9).	2.45	bs	methylene protons alpha to carbonyl carbon
(10).	2.58	dd	methylene protons adjacent to both carbonyl and oxygen bearing carbon
(11).	2.82	br d	methylene group between two double bonded carbons
(12).	3.48	s	methoxy group
(13).	4.00	dd (8, 3 Hz)	proton on secondary carbon bearing oxygen
(14).	4.13	br s	hydroxyl proton on secondary carbon bearing oxygen
(15).	4.32	d (6 Hz)	proton on secondary carbon bonded to carbon bearing oxygen
(16).	4.48	br d	proton on primary carbon bonded to carbon bearing oxygen
(17).	5.37	dd (14, 6 Hz)	olefinic proton on chain double bonded carbon
(18).	5.40	d(3 Hz)	proton on primary carbon bonded to two oxygens
(19).	5.62	d(14 Hz)	olefinic proton alpha to carbonyl carbon
(20).	5.70	d (8.0 Hz)	olefinic protons on 6 membered ether ring
(21).	5.75	s	OH (observed in DMSO)
(22).	5.78	d (7.0 Hz)	olefinic proton adjacent to double bonded carbon bearing terminal methylene
(23).	5.90	d (4.5 Hz)	NH adjacent to carbonyl
(24).	6.04	d (1.0 Hz)	proton on terminal methylene adjacent to aldehyde group
(25).	6.15	d (1.0 Hz)	proton on terminal methylene adjacent to aldehyde group
(26).	6.58	d (13.5 Hz)	proton on carbon adjacent to both acetyl ester and nitrogen
(27).	6.76	d (9.0 Hz)	olefinic proton on carbon bonded to a carbon with hydroxyl group
(28).	6.95	brs	three protons of a terminal guanidino group
(29)	7.12	d (8.0 Hz)	protons on terminal amino from carbonyl carbon
(30)	7.23	s	NH group bonded to guanidino group
(31).	7.83	d (4.5 Hz)	NH between double bonded carbon and carbonyl
(32).	8.02	d (9.0 Hz)	olefinic proton in resonating double bond adjacent to N and a carbonyl carbon
(33).	8.10	s	same as above; the second of two olefinic carbons
(34).	8.38	s	terminal nitroxyl group
(35).	8.58	d (6.0 Hz)	olefinic hydroxyl group
(36).	8.68	s	aldehyde proton

BIBLIOGRAPHY

1 Lombet, A., J.N. Bidard and M. Lazdunski 1987 Ciguatoxin and brevitoxins share a common receptor site on the neuronal voltage-dependent sodium channel. FEBS Letters **219(2)**:355-359.

2 Santostae, G., R.K. Kuty and G. Krishna 1988 Loss of cell ATP due to increased uptake of calcium as a possible mechanism of maitotoxin-induced cell death in cardiomyocityes. FASEB Journal ABST

3 Palafox, N.A., L.G. Jain, A.Z. Pinano, T.M. Glulick, R.K. Williams and I.J. Schatz 1988 Successful treatment of ciguatera fish poisoning with intravenous mannitol. JAMA **259(18)**:2740-2742.

4 Halstead, B.W. 1964 Fish poisonings -Their pharmacology, diagnosis, and treatment. Clinical Pharmacology and Therapeutics **5(5)**:615-627.

5 Halstead, B.W. 1967 Poisonous and Venomous Marine Animals of the World. U.S. Government Printing Office, Washington D.C. 1070 p.

6 Randall, J.E. 1958 A review of ciguatera, tropical fish poisoning, with a tentative explanation of its cause. Bulletin of Marine Science of the Gulf and Caribbean **8(3)**:236-267.

7 Lawrence, D.N., M.B. Enriguez, R.M. Lumish and A. Maceo. 1980 Ciguatera fish poisoning in Miami. Journal of the American Medical Association **244(3)**:254-258.

8 Bagnis, R.A., T. Kuberski and S. Laugier. 1979 Clinical observations on 3,009 cases of ciguatera (fish poisoning) in the South Pacific. American Journal of Tropical Medicine and Hygiene **28(6)**:1067-1073.

9 Tackett, C.O. 1982 Clinical and Epidemiologic Features of Ciguatera in the Caribbean. U. S. Department of Health and Human Services Pamphlet, PHS, CDC, Atlanta, Georgia, PHS, CDC, Atlanta Ga, 1-8 p.

10 Sylva, D.P. de and J.B. Higman. 1980 A plan to reduce ciguatera in the tropical western Atlantic region. Bulletin of Marine Science **32**:139-153.

11 Baylet, R., C. Beccaria, P.M. Niaussat, F. Boyer and M. Guillaud. 1978 Food poisoning by ciguatoxin in France. Pathological Biology(Paris) **26(2)**:95-97.

12 Bagnis, R.A. 1968 Clinical aspects of ciguatera (fish poisoning) in French Polynesia. Hawaii Medical Journal **28(1)**:25-28.

13 Li, K.M. and A.T.C. Au 1968 Clinical diagnosis Ciguatera poisoning. Far East Medical Journal **4**:313-315.

14 Banner, A.H. 1974 The biological origin and transmission of ciguatoxin. *In* Marine Science, Vol I. Bioactive Compounds from the Sea, H. J. Humm and C. E. Lane (eds.). Marcel Decker, New York, pp. 15-36.

15 Li, K.M. 1968 Action of ciguatera fish poison and multiple toxins in ciguatera fish species. *In*: Poisonous and Venomous Marine Animals, Vol III, B. Halstead, (ed).

16 Hashimoto, Y. and N. Fusetani. 1968 A preliminary report on the toxicity of the amberjack *Seriola aureovittata*. Bulletin of the Japanese Society of Scientific Fisheries **34(7)**:618-626.

17 Yasumoto, T., Y. Hashimoto, R.A. Bagnis, J.E. Randall and A.H. Banner. 1971 Toxicity of the surgeonfishes. Bulletin of the Japanese Society of Scientific Fisheries **37(8)**:724-734.

18 Yasumoto, T. and K. Kanno. 1976 Occurrence of toxins resembling ciguatoxin, scaritoxin, and maitotoxin in a turban shell. Bulletin of the Japanese Society of Scientific Fisheries **42(12)**:1399-1404.

19 Yasumoto, T., I. Nakajima, R.A. Bagnis and R. Adachi. 1977c Finding of a dinoflagellate as a likely culprit of ciguatera. Bulletin of the Japanese Society of Scientific Fisheries **43(8)**:1021-1026.

20 Bagnis, R.A., E. Chungue and S. Thevenin. 1974 Les intoxications par poissons perroquets aux Iles Gambier. Medécine Tropicale **34(4)**:523-527.

21 Poli, M. 1985 Characterization of the *Ptychodiscus brevis* polyether binding component in excitable membranes. *In* Toxic Dinoflagellates, D.M. Anderson, A.W. White and D.G. Baden (eds.) Elsevier North-Holland Press, New York, pp. 357-362.

22 Yasumoto, T., I. Nakajima, E. Chungue and R.A. Bagnis. 1977 Toxins in the gut contents of parrotfish. Bulletin of the Japanese Society of Scientific Fisheries **43(1)**:69-74.

23 Yasumoto, T., R.A. Bagnis, S. Thevenin and M. Garcon. 1977 A survey of comparative toxicity in the food chain of ciguatera. Bulletin of the Japanese Society of Scientific Fisheries **43(8)**:1015-1019.

24 Adachi, R. and Y. Fukuyo. 1979 The thecal structure of a marine toxic dinoflagellate *Gambierdiscus toxicus* gen. et sp. nov collected in a ciguatera endemic area. Bulletin of the Japanese Society of Scientific Fisheries **45(1)**:67-71.

25 Yasumoto, T., I. Nakajima, Y. Oshima and R.A. Bagnis. 1979 A new toxic dinoflagellate found in association with ciguatera. *In* Toxic Dinoflagellate Blooms, D. L. Taylor and H. H. Seliger (eds.). Elsevier North-Holland, New York, pp. 65-70.

26 Yasumoto, T., Y. Oshima, Y. Murakami, I. Nakajima, R.A. Bagnis and Y. Fukuyo 1980 Toxicity of benthic dinoflagellates found in coral reef. Bulletin of the Japanese Society of Scientific Fisheries **46**:327-331.

27 Withers, N.W., R.H. York and A.H. Banner. 1980 Growth and toxicity of the dinoflagellate *Gambierdiscus toxicus* from Hawaiian waters. American Zoologist **20(4)**:849.

28 Withers, N.W. 1981 Toxin production, nutrition, and distribution of *Gambierdiscus toxicus* (Hawaiian Strain). Proceedings, Fourth International Coral Reef Symposium, Manila **2**:449-451.

29 McFarren, E.F., H. Tanabe, F.J. Silva, W.B. Wilson, J.E. Campbell and K.H. Lewis. 1965 The occurrence of a ciguatera-like poison in oyster, clams, and *Gynodinium breve* cultures. Toxicon **3(2)**:111-123.

30 Bergmann, J.S. and M. Alam. 1981 On the toxicity of the ciguatera producing dinoflagellate *Gambierdiscus toxicus* Adachi and Fukuyo isolated from the Florida Keys. Journal of Environmental Science Health A1,**6(5)**:493-500.

31 Tindall, D.R., R.W. Dickey, R.D. Carlson and G. Morey-Gaines. 1984 Ciguatoxigenic dinoflagellates from the Caribbean. *In* Seafood Toxins, E. P. Ragelis (ed.). American Chemical Society Symposium Series 262, Washington, D. C, pp. 225-240.

32 Yentsch, C.M. and Mague, F.C. 1980 Evidence for an apparent annual rhythm in the toxic red tide dinoflagellate *Gonyaulax excavata*. International Journal of Chronobiology **7**:77-84.

33 Schmidt, R.J., A.R. Loeblich and J.W. Hastings 1979 Comparative study of luminescent and non luminescent strains of *Gonyaulax excavata* (Pyrrhophyta). Journal of Phycology **14**:5-9.

34 Oshima, Y., T. Hayakawa, H. Hashimoto, Y. Kotaki and T. Yasumoto 1982 Classification of *Protogonyaulax tamarensis* from Northern Japan into three strains by toxin composition. Bulletin of the Japanese Society of Scientific Fisheries **48**:851-854.

35 Bagnis, R.A., S. Chanteau, E. Chungue, J-M. Hurtel, T. Yasumoto and A. Inoue. 1980 Origins of ciguatera fish poisoning: a new dinoflagellate, *Gambierdiscus toxicus* Adachi and Fukuyo, definitively involved as a causal agent. Toxicon **18(2)**:199-208.

36 Dickey, R.W., D.M. Miller and D.R. Tindall. 1984 Extraction of a water-soluble toxin from a dinoflagellate, *Gambierdiscus toxicus*. *In* Seafood Toxins, E. P. Ragelis (ed.). American Chemical Society Symposium Series 262, Washington, D.C., pp. 257-269.

37 Miller, D.M., R.W. Dickey and D.R. Tindall. 1984 Lipid soluble toxins from a dinoflagellate, *Gambierdiscus toxicus*. *In* Seafood Toxins, R.P. Ragelis (ed.). American Chemical Society Symposium Series 262, Washington, D.C., pp. 241-255.

38 Carlson, R.D., G. Morey-Gaines, D.R. Tindall and R.W. Dickey. 1984 Ecology of toxic dinoflagellates from the Caribbean Sea: Effect of macroalgal extracts on growth in culture. *In* Seafood Toxins, Proceedings, American Chemical Society Conference on Toxins in Seafood, R.P. Ragelis (ed.). pp. 271-287.

39 Provasoli, L. 1968 Media and prospects for the cultivation of marine algae. In Proceedings, Fourth International Seaweed Symposium D. DeVirville and J. Feldman, (Eds) pp 9-17, Pergammon Press, Oxford.

40 Steidinger, K.A. 1983. A re-evaluation of toxic dinoflagellate biology and ecology. *In, Progress in Phycological Research*, Vol. 2, edited by F.E. Round and D.J. Chapman, Elsevier, New York, pp. 147-188.

41 Bomber, J.W., R.R.L. Guillard, and W.G. Nelson. 1987. Roles of temperature, salinity and light in seasonality, growth and toxicity of ciguatera- causing *Gambierdiscus toxicus* Adachi et Fukuyo (Dinophyceae) Journal of Experimental Marine Biology and Ecology **115**:53-65.

42 Brand, L.E., R.R.L. Guillard and L.S. Murphy. 1981. A method for the rapid and precise determination of acclimated reproduction rates. Journal of Planktonic Research **3**:193-201.

43 Weil, C.S. 1952 Tables for convienent calculation of median effective dose (LD-50 or ED-50) and instructions in their use. Biometrics **8**:249.

44 Sokal, R.R. and F.J. Rohlf 1981 Biometry. W.H. Freeman and Co. New York, N.Y.

45 Jeffrey, S.W., M. Sielicki and F.T. Haxo 1975. Chloroplast pigment patterns in dinoflagellates. Journal of Phycology **11**:374-384.

46 Indelecato, S.R. and D.A. Watson 1986 Identification of the photosynthetic pigments of the tropical benthic dinoflagellate, *Gambierdiscus toxicus*. Marine Fisheries Review **48(4)**:44-47.

47 Sperry, W.N. and F.C. Brand. 1955. The determination of total lipids in the blood serum. Journal of Biological Chemistry **213**:69-76.

49 Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Analytical Chemistry, **28**:350-356.

49 Kochert, G. 1978. Quantitation of the macromolecular components of microalgae. *In*, J.A. Hellebust and J.S. Cragie (eds.) *Handbook of Phycological Methods: Physiological and Biochemical Methods*. Cambridge University Press, Cambridge, pp. 189-195.

50 Glover, H.E., M.D. Keller and R.W. Spinrad 1987 The effects of light quality and intensity on photosynthates and growth of marine eukaryotic and prokaryotic phytoplankton clones. Journal of Experimental Marine Biology and Ecology **105**:137-159.

51 Anderson, H.J., D. Barrett and R. Craver. 1949 Archiv Internationale de Pharmacologie et Therapie **Apr 1**:544-547.

52 Fogg, G.E. 1965. *Algal Cultures and Phytoplankton Ecology*. The University of Wisconsin Press, 126 p.

53 Myklestad, S. 1977. Production of carbohydrates by marine phytoplanktonic diatoms. II. Influence of the N/P ratio in the growth medium on the assimilation ratio, growth rate, and production of carbohydrates by *Chaetoceros affinis* var. *willei* (Gran) Hustedt and *Skeletonema costatum* (Grev.) Cleve. Journal of Experimental Marine Biology and Ecology **29**:161-179.

54 Dortch, Q. 1982. Effect of growth conditions on accumulation of internal nitrate, ammonia, amino acids and protein in three marine diatoms. Journal of Experimental Marine Biology and Ecology **61**:243-264.

55 Ben Amotz, A, T.G. Tornabeve and W.H. Thomas 1985 Chemical profiles of selected species of microalgae with emphasis on lipids. Journal of Phycology **21**:72-81.

56 Anderson, D.M., D.M. Kulis and B.J. Binder. 1984. Sexuality and cyst formation in the dinoflagellate *Gonyaulax tamerensis*: Cyst yield in batch cultures. Journal of Phycology **20**:418-425.

57 Faust, M.A., J.C. Sager and B.W. Meeson 1982. Response of *Prorocentrum mariae-Lebouriae* (Dinophyceae) to light of different spectral qualities and irradiances: Growth and pigmentation. Journal of Phycology **18**:349-356.

58 Capra, M.F., J. Cameron, A.E. Flowers, I.F. Coombe, C.G. Blanton and S.T. Hahn 1987. The effects of ciguatoxin on teleosts. In Progress in Venom and Toxin Research. (Proceedings of the 1st Asian-Pacific Congress on Animal Plant and Microbial Toxins, Singapore, June 1987).

59 Brand, L.W. 1981 Genetic variability in reproduction rates in marine phytoplankton populations. Evolution **35**:1117-1127.

60 Babinchak, J.A., D.J. Jollow, M.S. Voegtline, and T.B. Higerd 1986. Toxin production by *Gambierdiscus toxicus* isolated from the Florida Keys. Marine Fisheries Review **48**:53-56.

61 Ogata, T., M. Kodama, and T. Ishimaru 1987. Toxin production in the dinoflagellate *Protogonyaulax tamarensis*. Toxicon **9**:923-928.

62 Durand-Clement, M. 1986. A study of toxin production by *Gambierdiscus toxicus* in culture. Toxicon. **24**:11-12.

63 Durand-Clement, M. 1987. Study of production and toxicity of cultured *Gambierdiscus toxicus*. Biological Bulletin **172**:108-121.

64 Laurie-Ahlberg, C.C. 1985. Genetic variation affecting the expression of enzyme-coding genes in *Drosophila*: an evolutionary perspective. *In:* Isozymes. Current Topics In Biology and Medical Research **12**:33-88.

65 Salzman, A. 1986. Analysis of natural selection on a quantitative physiological trait: Salt tolerance in Western Ragweed (*Ambrosia pfilostachya*). Ph.D. Dissertation. Department of Biology. University of Chicago.

66 Wood, A.M., R. Lande and G.A. Fryxell 1987. Quantitative genetic analysis of morphological variation in an Antarctic diatom grown at two light intensities. Journal of Phycology **23**:42-54.

67 Martin, J.F. and A.L. Demain 1980. Control of antibiotic synthesis. Microbiological Reviews **44**:230-251.

68 Meeson, B.W. and M.A. Faust 1985. Response of *Prorocentrum minimum* (Dinophyceae) to different spectral qualities and irradiances: Growth and photosynthesis. *In* Gray, J.S. and Christiansen, M.E. [Eds.] *Marine Biology of Polar Regions and Effects of Stress on Marine Organisms*. John Wiley and Sons Ltd., London, pp. 445-461.

69 Maranda, L., D.M. Anderson, and Y. Shimizu 1985. Comparison of toxicity between populations of *Gonyaulax tamerensis* of Eastern North American Waters. Estuary, Coast and Shelf Science **21**:401-410.

70 Brand, L.E., R.R.L. Guillard and L.S. Murphy. 1981. A method for the rapid and precise determination of acclimated phytoplantkon reproduction rates. Journal of Planktonic Research **3**:193-201.

71 Watson, D.A. and A.R. Loeblich III 1983. The application of electrophoresis to the systematics of the marine dinoflagellate genus *Heterocapsa*. Biochemical Systematics and Ecology **11**:67-71.

72 Hayhome, B.A. and L.A. Pfiester 1983. Electrophoretic analysis of soluble enzymes in five freshwater dinoflagellate species. American Journal of Botany **8**:1165-1172.

73 Brand, L.E., L.S. Murphy, R.R.L. Guillard and H.-T. Lee. 1981. Genetic variability and differentiation in the temperature niche component of the diatom *Thalassiosira pseudonana*. Marine Biology **62**:103-110.

74 Tosteson, T.R., D.L. Ballantine, C.G. Tosteson, A.T. Bardales, H.D. Durst, and T.B. Higerd 1986. Comparative toxicity of *Gambierdiscus toxicus, Ostreopsis* cf. *lenticularis*, and associated microflora. Marine Fisheries Review **48**:57-59.

75 Brand, L.W. 1981a. Genetic variability in reproduction rates in marine phytoplankton populations. Evolution **35**:1117-1127.

76 Lewontin, R.C. 1985. Population Genetics. Annual Reviews of Genetics **19**:81-102.

78 Yasumoto, T. 1985. Recent progress in the chemistry of dinoflagellate toxins. *In*: *Toxic Dinoflagellates*, (Anderson, White and Baden, eds) Elsevier Science Publishing Co. Inc. pp. 259-270.

GLOSSARY

ACN	Acetonitrile
ACN-insoluble	Acetonitrile insoluble fraction
ACN-soluble	Acetonitrile soluble fraction
ANOVA	Analysis of variance
CTX	Ciguatoxin
DEPT	Distortionless Enhancement Polarization Transfer Plot
DMSO	Dimethyl sulfoxide
ESAF	Ether soluble acetone filtrate
ESAP	Ether soluble acetone precipitate
GT-1	One of two toxins found in CTX group from *G. toxicus*.
GT-2	One of two toxins found in CTX group from *G. toxicus*.
GT-3	One of two toxins found in MTX group from *G. toxicus*.
GT-4	One of two toxins found in MTX group from *G. toxicus*.
HPLC	High Pressure Liquid Chromatography
IEU	Ileum Equivalent Units
MTX	Maitotoxin
MU	Mouse units
NMR	Nuclear Magnetic Resonance
P-	Abbreviation for Preparative
PPM	Parts per million
PSS	Physiological Saline Solution
SK	Acronym for Sep Pak separation of toxins
SP-	Abbreviation for Semipreparative
TEA	Tetraethylammonium
TLC	Thin layer chromatography
WSAF	Water soluble acetone filtrate
WSAP	Water soluble acetone precipitate
XTL	Acronym for crystalization step

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