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ON A CULTURE OF MIXED ALGAE PRODUCING CIGUATOXIN IN HAWAII

Albert H. Banner
University of Hawaii

As is known, Hawaii has had and continues to have sporadic outbreaks of ciguatera, usually mild, and these attacks have continued. In the last decade cases have come from two fishes: Most came from the large carnivore, *Seriola dumerilii* or amberjack (*kahala* in Hawaiian); these cases are so random that one is tempted to suggest that these fish, which are known to migrate, have come to the waters of the main Hawaiian Islands from the leeward Hawaiian chain where many fishes are known to be toxic. However, some cases of ciguatera caused by the pisciverous *Chelinus rhodochrous* (*po'ou* in Hawaiian); this fish may reach the length of 60 cm, but is usually much smaller and it is not migratory. Therefore it must be presumed that the Hawaiian reef ecosystem is producing ciguatoxin, albeit in small amounts.

Therefore, after Dr. Yasumoto announced in February, 1977 the association of the yet unnamed dinoflagellate with ciguatoxin production in the Gambier Islands, and spoke of its benthic habits and association with the alga *Turbinaria* we made an initial exploratory survey of *Turbinaria* on various reefs of the island of Oahu. No dinoflagellates were found and the search was discontinued.

In early January, 1978 I was searching at the Hawaii Institute of Marine Biology for some tanaids, a near-microscopic crustacean living in bottom debris. I looked in an unused water-table where an experiment had been abandoned some months before but the running sea water from the Institute's system had been left on. The source of this water was the shallow water from the reef immediately adjacent to the laboratory. The table is about 0.6 by 1.4 m and the water is 14 cm deep. The bottom of the table was covered with a brown flocculant film which at times would cover small tufts of a red alga a centimeter or more high. Macroscopic life consisted of one xanthid crab, several small tubeworms under small sheets of transite abandoned from the previous experiment, and some amphipods a few millimeters long. Where the currents accumulated the fine debris, the bottom of these deposits were black from anaerobic decomposition.

It should be remembered that Kaneohe Bay has been subject to high levels of plant nutrients from a municipal sewage discharge and has become quite eutrophic. While the sewage outlet was abandoned in mid-December 1977, the nitrate and phosphate levels in the open bay waters have not markedly decreased due to the leaching of the nutrient ions from the organically-rich bottom deposits.

When I examined the bottom crud under the binocular dissecting scope I discovered it to be largely an amorphous grey flocculant material pierced by strands of a blue-green alga - I have later determined that the flocculant material is the "sheath material" produced by the alga. This deposit would

almost engulf the tufts of red alga, leaving only the growing tips bare. Living on - actually "sitting" on - the flocculant material was a biconvex thecate dinoflagellate; examination under ultraviolet microscopy showed it carried chlorophyll a and that it was therefore autotrophic. The behavior of the dinoflagellate was strange: very seldom would it swim into the water column above the deposit, but at times it would slowly move from one place to another and at times it would turn the sulcus down as if it were actually feeding on the deposit. The culture was literally teeming with the flagellate, with at times a hundred or more in the field of a dissecting microscope at 50 diameters magnification.

There were other plants and animals in the wild culture: almost as abundant as the dinoflagellate was a flattened ciliate of about the same size (about 70 µm), also carrying chlorophyll. There were diatoms and a few naked green flagellates. Other animals included a few nematode worms, some harpaticoid copepods but little else.

Upon the discovery of the dinoflagellate-blue-green algal culture I was struck with how parallel this was to Dr. Yasumoto's report of another dinoflagellate (as I then thought) in association with another species of alga. I immediately wrote about it to both Dr. Yasumoto and Dr. F. J. R. Taylor of the University of British Columbia who is the specialist in tropical dinoflatellates to whom Dr. Yasumoto had sent some of his specimens. Dr. Taylor soon replied that he thought from the sketch I had enclosed that the dinoflagellate might be the same as Dr. Yasumoto had found in the Gambiers. I immediately sent him some specimens for confirmation and gave some of the culture to Dr. Hokama for preliminary testing.

Dr. Taylor replied that the dinoflagellate was the same as that found by Dr. Yasumoto, and that it would be described as a new genus, new species. Dr. Hokama applied his ELISA test (enzyme linked immunological sensitivity assay) and found a strong positive for ciguatoxin.

At that point my interest in the culture markedly increased and I hired a technician to help me explore the cultural requirements for ciguatoxin production. I also had the major components identified: as I said, the dinoflagellate will be described as a new genus and species; the blue-green was identified as *Microcoelous lyngbyaceous* - it is the form previously known as *Lyngbya majescula*, a known toxigenic form; the two red algae were identified as *Centroceras clavulatum* and *Ceranium* sp., and a less common fine brown algae as *Giffordia mitchelliae*; I have the common ciliate in the hands of an expert but he has not yet given me the identification.

One of the first things we did was to spot check other algae in the vicinity of the laboratory in Kaneohe Bay. We found the dinoflagellate in many places but not in the abundance found on the water-table.

In our study of the cultures we have had and are having numerous difficulties. For example, while we have been able to separate the blue-green alga and raise it in uni-algal culture, we have not yet been able to grow the isolated dinoflagellate, although the individual cells remain alive for some time. Because of the sessil habits of the dinoflagellate and its spotty distribution, we have been unable to quantify our results except by such subjective methods as "few" or "many." Therefore we are unable to tabulate any firm results.

In general, however, our original wild culture, our sub-cultures in running sea water on tables, and our enriched static cultures, both in flasks and in tanks similar in size to the water-tables, all produce products that give positive tests for ciguatoxin by the radioimmunoassay, in counts per minute per gram of material. Some of these are higher and some are lower, reflecting in part, our crude estimates of dinoflagellate abundance. We have tried enrichment of the sea water with four different media recommended for dinoflagellate culture and all seem to stimulate initially the bloom of the other algal components. It is only when the peak of the bloom of the other algae passes that the dinoflagellates appear to greatly increase in numbers. We have also tried the addition of soil extracts, both in the Erdschreiber medium and with a standard enrichment medium to which soil extract has been added, and found them both to stimulate growth of the dinoflagellates. However, in a series of six test cultures which were harvested in early May, the highest count by the RIA in any of the six experimental cultures was equalled by the control which contained only Kaneohe Bay water with nothing added.

We have not yet been able to obtain a confirmatory test for ciguatoxin by Dr. Rayner's pharmacological test for he needs amounts in the hundreds of grams for extraction, while Dr. Hokama is happy with milligram amounts. However, we have about 100 g of the mixed culture now being extracted under Dr. Scheuer's supervision and we expect that the pharmacological test will be run soon.

If we presume that our dinoflagellate behaves the same as Dr. Yasumoto's dinoflagellate, the cultures present us with an interesting biological problem. Dr. Yasumoto's wild material from the Gambiers and our wild culture is producing ciguatoxin by our tests and if our dinoflagellate like Dr. Yasumoto's produces only maitotoxin in axenic culture, then we have a far more complex situation that is found in the other toxigenic dinoflagellates such as *Gonyaulax*. Four hypotheses suggest themselves:

I. That the dinoflagellate has the capability of producing either maitotoxin or ciguatoxin and the toxin production is switched from one to the other by some regulatory substance given off by another member of the mixed culture.

II. That the dinoflagellate continues to produce maitotoxin but some other component of the mixture, possibly some bacterium, is converting it to ciguatoxin; this would presume that maitotoxin is chemically related to ciguatoxin.

III. That some other member of the mixture is producing a precursor - possibly non-toxic - of ciguatoxin that is modified to ciguatoxin by the dinoflagellate.

IV. That the dinoflagellate has nothing to do with ciguatoxin production but some other member of the mixture is producing the ciguatoxin independently; here the blue-green would be most suspect.

Our plans for the summer include the continuation of our experimental rearing of the mixed culture and the attempts to raise the dinoflagellate in an uni-algal culture. To assist us in the last, we hope to have two dinoflagellate experts join us separately for periods of two to three weeks.

When we have enough favorable data accumulated to warrant a request for research aid, we plan to submit a grant proposal to some agency of the National Institute of Health or the Food and Drug Administration. I now have two preliminary letters of inquiry in the mail. When we submit, we will give two aims: First, to determine the ecological requirements of ciguatoxin production in the laboratory so that the information can be applied to field situations, possibly to prevent or even to reduce epidemics of ciguatera. Second, to develop mass culture techniques so that ciguatoxin can be cheaply and abundantly produced for further studies on the molecular structure of the toxin and for further deliniation of its pharmacological effects. I envision a basic biological staff of one person at the doctoral level experienced in dinoflagellate culture, aided by one or more technicians, and the collateral support of technicians in immunology, chemistry and pharmacology. Our group will again be working as a multidisciplinary team.