Comparative Toxicity of *Gambierdiscus toxicus*, *Ostreopsis* cf. *lenticularis*, and Associated Microflora

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ABSTRACT—Benthic, epiphytic dinoflagellates, Gambierdiscus toxicus, *and an ecological associated dinoflagellate,* Ostreopsis *cf.* lenticularis, *were isolated from macroalgal hosts at a shallow inshore reef habitat off the southwest coast of Puerto Rico. These dinoflagellates were isolated into clonal cultures and are maintained in laboratory culture employing enriched seawater media. Four bacterial strains have been isolated from cultures of* G. toxicus *and* O. lenticularis. *One strain belonged to the family Pseudomonadaceae, two were Vibrionaceae, and the fourth was from the family Nocardiaceae. Three strains could be recovered from culture media in which the dinoflagellates were grown, while* Nocardia *sp. was associated only with the dinoflagellate cells themselves. Methanolic extracts of Puerto Rican* G. toxicus *were not toxic when inoculated (i.p.) in mice, while similar extracts of* O. lenticularis *were toxic.* LD_{50} *values obtained for these extracts ranged from 6.5 to 72.5 mg/kg mice. The highly variable toxicity of* O. lenticularis *extracts appeared to be correlated with the abundance of the* Nocardia *strain recovered from disrupted cells of this dinoflagellate.*

Introduction

The benthic dinoflagellate genera *Gambierdiscus* and *Ostreopsis* are commonly found in tropical marine environments (Besada et al., 1982; Steidinger, 1983). Regions in which they are found are often characterized by frequent outbreaks of ciguatera fish poisoning (Besada et al., 1982). In the coastal waters of southwest Puerto Rico, these dinoflagellates are abundant (Ballantine et al., 1986). Tindall et al. (1984) has speculated that a variety of dinoflagellates contribute to ciguatera fish poisoning. In this paper, we describe the comparative toxicity of methanolic extracts of *G. toxicus* and *O. lenticularis* grown in clonal culture, and the possible influence of their associated microflora.

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Materials and Methods

Microbial Cultures and Extraction Procedures

Gambierdiscus toxicus and *O. lenticularis* were collected from an inshore coral reef located 1 km offshore from La Parguera on the southwest coast of Puerto Rico. Clonal cultures of both dinoflagellates were initiated and subsequently maintained in both F/2 (Guillard and Ryther, 1962) and ES media (Provasoli, 1968). Since February 1983, we have initiated five clonal cultures of *G. toxicus* and seven of *O. lenticularis*. Batch cultures were grown at 26°C in a light regime of 16:8 hours (light:dark) at a flux of 40 microeinsteins/m^2/second. During the study, on the basis of preliminary results obtained using a cross gradient culture apparatus (Edwards, 1970), the light flux for the cultures of *O. lenticularis* was increased to 130 microeinsteins/m^2/second. Cultures of *Gambierdiscus* reached maximum population densities in 3-4 weeks, while *Ostreopsis* required 2 weeks. Volumes of 3-4 liters were harvested weekly with a total yield of 1-3.5 × 10^6 cells of each dinoflagellate. Cells were harvested by filtration (Minitan System, Millipore[1]) and screening (35 μm mesh). Cell aliquots were briefly rinsed with distilled water and sonicated in redistilled methanol. Extracts (final volumes of about 100 ml) were allowed to remain at laboratory temperature (22°C) for 48-96 hours. Extract suspensions were then filtered (Whatman, #1) and the filtrate solvent removed by flash evaporation (Buchi, Rotavapor). The resulting residues were taken to dryness under nitrogen and stored in a vacuum desiccator for later toxicity studies.

The microflora associated with the dinoflagellates were periodically evaluated by streaking aliquots from *Ostreopsis* and *Gambierdiscus* cultures on solid media made from an enriched seawater solution consisting of 2 g peptone, 2 g trypticase, 1 g yeast extract, and 1 mcg vitamin B_{12} per liter of seawater. On several occasions, the microflora associated with aliquots of cell free media and cell concentrates were evaluated as described above. Bacterial isolates were purified, maintained in laboratory culture using the cited medium

[1]Reference to trade names or commercial firms does not imply endorsement by the National Marine Fisheries Service, NOAA.

and Zobell 2216E medium, and later identified to the genus level (Colwell, 1984; Buchanan and Gibbons, 1974; Koneman et al., 1983; Zobell, 1944).

Routinely at the end of each culture growth period, bacteria directly associated with *Ostreopsis* cells were monitored. Aliquots of cells from each culture were disrupted by sonication, and serial dilutions of the resulting particulate suspensions were inoculated onto bacterial media. The number of bacteria associated with the *Ostreopsis* cells was quantitated (spread plate technique) and cellular ratios calculated. Microbes isolated from dinoflagellate cultures were also grown in batch culture, harvested, and extracted with methanol in the same manner as the dinoflagellate cells described above.

Toxicity Assay

Initial screening and LD_{50} determinations were done employing ICR female mice weighing about 20 g each. Animals were maintained on Wayne Laboratory Animal diets (Lab-Glox) and water, ad libitum. Known quantities of dried extracts to be tested were suspended in 0.15 M phosphate buffer solution (PBS) containing 5 percent Tween 80. Inocula of 0.2 ml were administered by intraperitoneal (i.p.) injection. Each extract was tested at from 4 to 8 concentrations, decreasing geometrically from the highest level tested (5-6 mg/20 g mouse). Three or four mice were inoculated at each extract concentration. Extracts that showed no toxicity at the highest level tested were considered nontoxic. Control animals received injections of equal volumes of the Tween PBS media. Mice were observed for periods of 48 hours. LD_{50} values were calculated according to the method of Weil (1952).

Results and Discussion

Methanolic extracts of Puerto Rican *G. toxicus* were not toxic when inoculated i.p. in mice, while similar extracts of *O. lenticularis* were toxic. To our knowledge, this is the first reported toxicity for this dinoflagellate species. Toxicity has been reported for two other species of *Ostreopsis* (Nakajima et al., 1981). The LD_{50} values obtained for the *O. lenticularis* extracts varied considerably, ranging from 6.5 to 72.5 mg/kg mice. These LD_{50} values are comparable to those reported for other tropical benthic dinoflagellates (Tindall et al., 1984). Animals inoculated with toxic extracts of *O. lenticularis* displayed similar symptoms and appearance to mice injected with ciguateric fish toxins (Hoffman et al., 1983).

Four bacterial strains were isolated from *G. toxicus* and *O. lenticularis* cultures. The distribution of these strains is given in Table 1. *Aeromonas* sp. and *Vibrio* sp. were routinely recovered from cell-free media of *G. toxicus* cultures, while *Pseudomonas* sp. was regularly found in the cell-free media of *O. lenticularis*. A distinctive bacteria forming crusty aggregates on the surfaces of both solid (agar) and aqueous media, identified as *Nocardia* sp., was prominently found in preparations of *O. lenticularis* cells. This bacterial strain was only detectable in *G. toxicus* cells of the first clone isolated. *Nocardia* sp. has not been found in repeated sampling of the four subsequently isolated clones of *G. toxicus*. Initial tests indicated that the methanolic extract of *Nocardia* sp. was toxic to mice at dosages between 6 and 9 mg/animal; however, subsequent repeated testing revealed that the methanolic extracts of this bacterial isolate were not toxic to mice. The other bacterial strains associated with the dinoflagellate cultures also failed to show toxicity. The LD_{50} of *O. lenticularis* extracts was inversely related to the *Nocardia*/*Ostreopsis* cell ratio (Table 2). Thus, while *Nocardia* sp. did not remain toxic when repeatedly grown in laboratory culture, its relative abundance in *O. lenticularis* cells may play a role in determining the toxicity of this dinoflagellate. The genus *Nocardia* is characterized by the presence of pathogenic species (Buchanan and Gibbons, 1974). Mitogenic and adjuvant active materials have additionally been extracted from a number of *Nocardia* species (Ciorbaru et al., 1974).

Elevated light intensities stimulated the growth rate of *Ostreopsis* cultures. The steady state number of *Nocardia* cells associated with *Ostreopsis* in the light stimulated *O. lenticularis* cultures significantly decreased (Table 3). In *O. lenticularis* batch cultures, there was considerable variation in growth rate

Table 1.—Dinoflagellates and their associated bacterial strains.[1]

G. toxicus			
Clone I	Clone II-IV	*O. lenticularis*
GT5: *Nocardia* sp. (slight)	None	06: *Nocardia* sp. (heavy)
GT4: *Aeromonas* sp.	GT4: *Aeromonas* sp.	07: *Pseudomonas* sp.
GT16: *Vibrio* sp.	GT16: *Vibrio* sp.	

[1]GT4 and 16 belong to the family Vibrionaceae, 06 is Nocardiaceae, and 07 is Pseudomonadaceae.

Table 2.—*Ostreopsis lenticularis* toxicity and associated bacterial densities.

Preparation[1]	Associated bacteria (per dinoflagellate cell)	[2]LD_{50}
6001 (24 Oct.)	9	[3]0
6015 (20 Nov., 5 Dec.)	74	41
6002 (8 Nov.)	298	6.5

[1]Average number of extracted cells: 1.457 ± 0.068(SE) × 10^6.
[2]Lethal dose 50 percent given in mg/kg mice.
[3]No animals died within the experimental period of 48 hours.

Table 3.—*Ostreopsis lenticularis*: Light flux, growth, and associated bacterial densities.

Light flux[1]	*Nocardia* sp./ *O. lenticularis*	Dinoflagellate doubling time (da)	Dinoflagellate cells harvested ($\times 10^6$)
130	2	4.0	4.82
40	110	6.1	1.27

[1]Microeinsteins/m^2/second.

Table 4.—*Ostreopsis lenticularis*: Growth and associated bacteria densities.

Final diniflagellate concentration (cells/ml)	Bacteria/ dinoflagellate	Doubling time (da)
686	7.3	11.0
1,238	2.6	6.6
645	10.2	11.9

among the culture flasks that constituted a given batch. This effect is illustrated in Table 4. The slower growing cultures were characterized by significantly greater *Nocardia*/*Ostreopsis* cell ratios than that seen in more rapidly growing cultures. It is of interest to note that the cell densities of *Ostreopsis* growing epiphytically on *Dictyota* spp. in the field also showed great variability at a given sampling site (Ballantine et al., 1986). The decrease in *Nocardia*/*Ostreopsis* cell ratios in more rapidly growing dinoflagellate cultures may have been due to the fact that the growth of the associated *Nocardia* populations was not keeping pace with increased dinoflagellate growth in laboratory culture conditions.

A better understanding of the precise relationship between increased *Ostreopsis* growth, *Nocardia*/*Ostreopsis* cell ratios, and *Ostreopsis* toxicity awaits further data and analyses. The data presented here suggest that *O. lenticularis* and its associated microflora may be a primary ciguateric vector in the coastal waters of southwest Puerto Rico.

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