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The effect of elevated temperature on the toxicity of the laboratory cultured dinoflagellate *Ostreopsis lenticularis* (Dinophyceae)

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Abstract: *Ostreopsis lenticularis* Fukuyo 1981, is the major benthic dinoflagellate vector implicated in ciguatera fish poisoning in finfish on the southwest coast of Puerto Rico. Clonal laboratory cultures of *O. lenticularis* (clone 301) exposed to elevated temperatures (30-31˚C) for 33 and 54 days showed significant increases in the quantity of extractable toxin they produced as compared to their toxicities versus cells grown at temperatures of 25-26˚C. *O. lenticularis* samples collected directly from the field following exposure to elevated temperatures for comparable periods of time also showed significant increases in extractable toxin. The increased toxicity of both field sampled and laboratory grown *O. lenticularis* exposed to elevated temperatures may result from the effects of elevated temperatures on their metabolism and/or the bacterial symbionts found associated with these microalgae. The number of bacteria associated with cultured *O. lenticularis* exposed to elevated temperatures was significantly reduced. Increased toxin recovery from *O. lenticularis* exposed to elevated temperatures may have resulted from the direct effect of temperature on toxin production and/or the reduction of *Ostreopsis* associated bacterial flora that consume toxin in the process of their growth. This reduction in the quantity of associated bacterial flora in temperature treated cultures may result in increased toxin recovery from *O. lenticularis* due to a reduction in the consumption of toxin by these symbiont bacteria.

Key words: *Ostreopsis lenticularis*, elevated temperatures, toxin production, associated bacterial flora, ciguatera.

Ciguatera is a food borne illness, which is passed through the marine food web to humans who consume tropical marine finfish tainted by toxin producing benthic dinoflagellates. *Ostreopsis lenticularis* has been demonstrated to be a major vector of ciguatera in Southwest Puerto Rico (Ballantine *et al.* 1988, Tosteson 1995, Faust *et al.* 1996). Ciguatera outbreaks and *O. lenticularis* abundance and toxicity have been reported to have distinct seasonal patterns (Ballantine *et al.* 1988, Morton *et al.* 1992, Tosteson *et al.* 1992, Tosteson 1995). Since both ciguatera and one of its implicated vectors *(O. lenticularis)* show similar seasonal patterns it is important to determine the precise role that seasonal changes in temperature may have on dinoflagellate toxicity and subsequent fish toxicity. The objective of these experiments was to determine what were the effects of elevated temperature exposure on the subsequent toxicity of laboratory cultured *O. lenticularis*.

Numerous researchers have reported that one of the main environmental conditions which appears to determine the seasonal trends and abundance of toxic dinoflagellates is water temperature (Ballantine *et al.* 1988, Morton *et al.* 1992, Hallegraeff *et al.* 1995). Temperature has been reported to affect toxin production in the diatom *Nitzschia pungens* and dinoflagellates *Protogonyaulax tamarensis* and *Gymnodinium catenatum* (Ogata *et al.* 1987, Lewis *et al.* 1993, Oshima *et al.* 1993).

Another essential aspect which must be taken into consideration when studying the factors that influence dinoflagellate toxicity involves the bacterial flora associated with the microalga. Dinoflagellate associated bacteria have been reported to be involved in the development of enhanced toxicity in cultured *Ostreopsis lenticularis* (Gonzalez *et al.* 1995). The presence of bacteria from the genus *Pseudomonas/Alteromonas* are required in the development of enhanced toxicity in clonal

laboratory cultures of *Ostreopsis* cells during the static phase of their culture growth (Gonzalez *et al*. 1995, Tosteson 1995).

Seasonal fluctuations in dinoflagellate blooms and toxicity may be the result of the effect of variable environmental factors on dinoflagellate metabolism, associated bacterial flora or a combination of both of these factors. A basic element of those environmental factors appears to be ambient seawater temperature (Tosteson *et al*. 1998). Delineation of the role of temperature in the regulation of the growth and toxicity of cultured *O. lenticularis* and its associated bacterial flora will aid our understanding of the relationship between these important influences in the initiation of ciguatoxic dinoflagellate blooms in nature.

MATERIALS AND METHODS

Dinoflagellate Cultivation: The toxic benthic dinoflagellate *O. lenticularis* was isolated from the surfaces of macroalgae (*Dictyota* sp.) on the southwest coast of Puerto Rico in February of 1996 and has been maintained in continuous clonal laboratory culture (clone 301) in a natural seawater based Enriched Seawater media (Stein 1973). Experiments were performed in 500 ml flasks using a natural seawater based ES media. All cultures were kept at light intensities of 90 µm Einsteins m^{-2} sec^{-1} with a light/dark cycle of 12:12 hours. Experimental cultures initiated at densities of 100 dinoflagellate cells ml^{-1} were exposed to temperatures of 29.5 to 31°C during their light cycles, for periods ranging of from 0.25 days (6 hours) to 54 days. Control cultures initiated at the same density were simultaneously maintained at temperatures of 25 to 26°C during their culture cycles. Cultures exposed to elevated temperatures for short periods of time (0.25 to 10 days) were subsequently placed at normal control temperatures until they were harvested 21-24 days following their initiation. Experimental cultures maintained for periods exceeding 10 days were transferred at 13 to 16 days after initiation. Transfer cultures were initiated at densities of 100 *O. lenticularis* cells ml^{-1} and maintained in the same elevated temperature regime as the parent cultures. Parent cultures were harvested, extracted and analyzed for their toxicities 21-24 days after their initiation. Thus each sequential transfer of these experimental cultures resulted in an increase of 14 days in time of exposure to elevated temperatures of the subsequently harvested transfer cultures (Fig. 1).

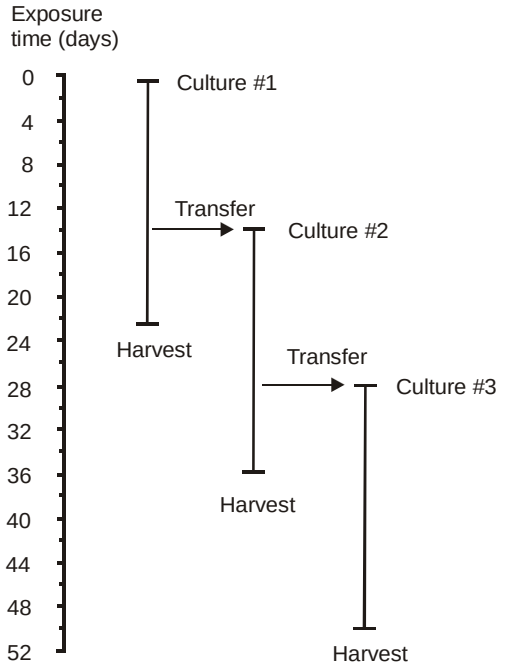

Fig. 1. Sequential culture system: exposure time in experimental *O. lenticularis* cultures.

Dinoflagellate Harvest and Toxin Extraction: Dinoflagellates were harvested after they reached late static phase of culture growth (21 to 24 days of growth) using reported procedures (Ballantine *et al*. 1988). Dinoflagellate concentrations were determined using cell counting chambers (Sedgewick Rafter) before harvesting in order to calculate the total cells harvested (Tosteson *et al*. 1989). Dinoflagellates were harvested onto 0.45 µm Gelman glass fiber filters using vacuum filtration. Harvested *O. lenticularis* cells were subsequently extracted in methanol and evaluated for their toxicity in mice (Tosteson *et al*. 1989).

Toxicity Assays: Dinoflagellate extracts were assayed for their toxicity in white Swiss mice. Known quantities of dried extracts to be tested were suspended in 0.5 ml phosphate buffer solution (PBS) containing 5% Tween 80 and administered by intraperitoneal (IP) injection. Mice were observed for 48 hours and LD_{50} values calculated according to standard methods (Weil 1952). Extracts were evaluated on the basis of their quantity (ng $cell^{-1}$ extracted) and their specific toxicity (mouse units mg^{-1} extract). Extract toxicity was expressed in terms of mouse units/dinoflagellate cell extracted. A mouse unit (MU) is equiva-

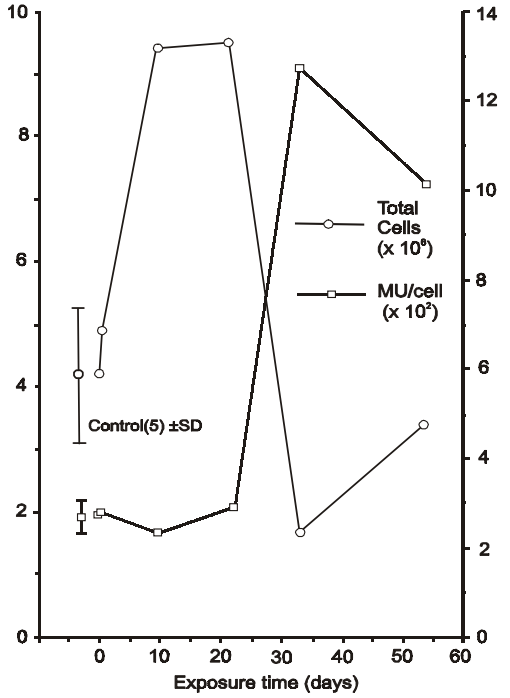

Fig. 2. Effect of temperature (30ºC) on *O. lenticularis* growth and toxicity.

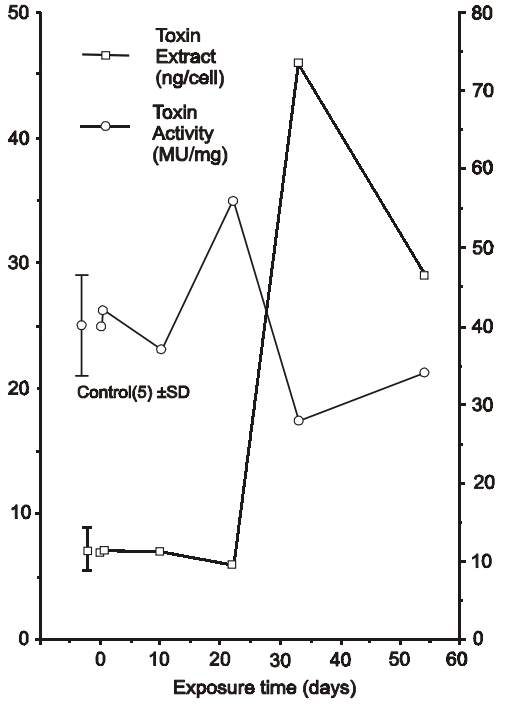

Fig. 3. Effect of temperature (30ºC) on *O. lenticularis* toxin production and activity.

lent to the quantity (mg) of extract that kills 50% of the mice injected with that amount (MU=LD_{50} dose). The total number of MU (TMU) in a given extract = mg extract recovered/MU and the MU/*Ostreopsis* cell = TMU /total number of *Ostreopsis* cells extracted.

Associated Bacterial Flora: At selected harvest times samples of the *Ostreopsis* cells from selected control and experimental cultures were taken for analyses of dinoflagellate associated bacterial flora employing reported procedures (Tosteson *et al.* 1989). The dinoflagellate associated bacterial flora in temperature stressed and control cultures were isolated, quantified and identified. Dinoflagellate cells were disrupted by sonication and streaked onto agar plates in serial dilutions. Inocula of 0.1 ml of the diluted bacterial suspensions were cultured by repeated streaking on nutrient seawater agar for aerobic incubation. Subsequently bacteria associated with the dinoflagellate cells were isolated in pure culture and isolates were identified to the generic level (Krieg and Holt 1984, Tosteson *et al.* 1986).

Classifications were made by using API 20E and other biochemical and antibiotic tests.

RESULTS

The results presented here involve a series of 11 laboratory cultures of *O. lenticularis* (clone 301) which were exposed for variable periods of time to elevated temperatures. The growth and toxicity of 5 of these experimental cultures and their corresponding controls were analyzed. A total of 29 x 10^6 *Ostreopsis* cells were harvested from cultures exposed to elevated temperatures during the light cycles of their cultivation and 21 x 10^6 cells were harvested from their respective control cultures.

Ostreopsis cultures showed significant increases in growth following 10 and 22 days of exposure to elevated temperatures (Fig. 2). After 34 and 54 days of exposure culture growth was similar to that seen in the control cultures unexposed to high temperatures. Coincidentally, *Ostreopsis* toxicity (MU $cell^{-1}$) showed significant increases in cultures exposed to elevated temperatures for 33 and 54 days. The amount of toxin extracted (ng $cell^{-1}$) in cultures exposed to elevated temperatures for 33 and 54 days significantly increased in comparison to control cultures and cultures exposed to elevated temperatures for shorter periods of time (10 to 22 days) (Fig. 3). Toxin specific activity

TABLE 1

Effect of temperature on Ostreopsis lenticularis *toxicity*

	(n)	MU cell^{-1} x 10^6 x ± SD	MeOH cell^{-1} (ng cell^{-1}) ± SE	MU mg^{-1} ± SE
Field data ≥29.5˚C	(4)	209 ± 27	36 ± 9	7 ± 2
Laboratory control 25˚C	(5)	265 ± 43	7 ± 2	40 ± 7
≥29.5˚C 0.25, 10, 22 days	(3)	268 ± 32	7 ± 1	45 ± 10
≥29.5˚C 33, 54 days	(2)	1,133	38	31

(MU mg^{-1}) was not significantly reduced from that found in the cultures exposed for shorter periods or the control cultures.

O. lenticularis sampled directly from the field following prolonged exposure to elevated sea surface temperatures showed extract recoveries (MeOH cell^{-1}) similar to that seen in cultured *Ostreopsis* exposed to elevated temperatures for 33 and 54 days (Table 1). These extract recoveries were significantly greater than those produced by *Ostreopsis* cultured at lower temperatures (control cultures) or those cultures exposed to elevated temperatures for shorter periods of time (0.25, 10 and 22 days). However, the toxicity (MU cell^{-1}) in all field samples is significantly lower than that seen in cultured cells after 33 and 54 days of exposure to high temperatures (Table 1). This reduced toxicity is due to the fact that the specific toxicity (MU mg^{-1} extract) is significantly higher in the cultured *Ostreopsis* compared to the field sampled cells.

TABLE 2

Effect of temperature on Ostreopsis lenticularis *associated bacterial flora*

Exposure time (days)	Control* cultures	Exposed* cultures
16	1.5	1.4
32	1.9	0.8

*Bacteria/Dinoflagellate Ratio (x10^3)

Three distinct bacterial species were isolated and identified. The quantity of these varied with different temperature exposure periods. Preliminary analyses of the effects of elevated temperatures on bacterial flora associated with *Ostreopsis* indicated that after 32 days of exposure to elevated temperatures there was a significant decrease in the bacteria/dinoflagellate cell ratio (Table 2).

The reduction in the quantity of *O. lenticularis* symbiont bacterial flora was correlated with significant decreases in dinoflagellate growth and significant increases in the quantity of extractable toxin.

DISCUSSION

Previous studies involving field sampled *Ostreopsis lenticularis* showed maximum toxicities occurred in October (1985) and November-December (1987, 89, 94) (Tosteson *et al*. 1998). These peak dinoflagellate toxicities were preceded by several months (August-October) of exposure to sustained, elevated sea surface temperatures (SSTs). Results obtained in the laboratory experiments reported here indicate that enhanced *Ostreopsis* toxicity appears to be due to the thermally induced production of more toxic material in cultured cells exposed to elevated temperatures for 33 to 54 days. This increase in *Ostreopsis* toxicity in laboratory cultures resulted from increases in the amount of toxic extract recovered rather than increases in the specific toxicity (MU mg^{-1}) of the extracted material (Fig. 3). Even though pre-exposure to elevated temperatures in field sampled *Ostreopsis* induced the production of

a similar quantity of toxic material to that seen in cultured *Ostreopsis* after similar periods of exposure to elevated temperatures, field samples had a reduced toxicity in comparison to laboratory cultures exposed to elevated temperatures for 33 to 54 days (Table 1). The resulting higher toxicity for laboratory cultures exposed to prolonged periods to elevated temperatures is due to an increased specific toxicity (MU mg^{-1}) when compared to field samples.

The decrease in associated bacterial flora is inversely correlated with the enhanced toxicity of the cultured *Ostreopsis*, suggesting that bacteria may consume the dinoflagellate toxin in *Ostreopsis* cultured at lower temperatures (25-26°C) and in those cultures exposed to elevated temperatures for shorter periods of time (10 and 22 days). Exposure to elevated temperatures for periods of 32 days significantly reduced the number of bacteria associated with cultured *O. lenticularis*, suggesting that reductions in the quantity of associated bacterial flora may result in increased toxin recovery from extracted *O. lenticularis* due to the absence of these symbionts that normally consume the toxin in the process of their growth. This possibility is supported by the fact that the associated bacterial flora of *O. lenticularis* utilize the toxin produced by this dinoflagellate as a carbon source when grown in pure culture (Tosteson *et al*. 1998).

The toxicity of both laboratory cultured and field sampled *Ostreopsis lenticularis* appear to be thermally induced by prolonged exposure to elevated temperatures. Thus fluctuations in both ambient temperature and dinoflagellate-symbiont bacteria interactions affect *Ostreopsis* metabolism, growth (blooms) and toxicity.

RESUMEN

Ostreopsis lenticularis Fukuyo 1981, es un dinoflagelado bentónico y el principal vector implicado en envenenamientos por ciguatera en peces arrecifales en la costa sur-oeste de Puerto Rico. Cultivos clonales de *O. lenticularis* (clón 301) que fueron expuestos en el laboratorio a temperaturas elevadas (30-31°C) por 33 y 54 días demostraron aumentos significativos en la cantidad de toxinas extraíbles que produjeron al comparar su toxicidad con la de cultivos que crecieron a temperaturas de 25-26˚C. Muestras de *O. lenticularis* recogidas directamente del campo después de estar expuestas a temperaturas elevadas demostraron aumentos significativos en la cantidad de toxinas extraíbles. El aumento en toxicidad evidente en las muestras de campo, al igual que en las muestras del laboratorio expuestas a altas temperaturas puede ser el resultado de los efectos de las altas temperaturas actuado sobre el metabolismo o los simbiontes bacterianos asociados a estas microalgas. El número de bacterias asociadas con los cultivos de *O. lenticularis* expuestos a altas temperaturas se vieron significativamente reducidos. El aumento en la cantidad de toxinas recuperadas de *O. lenticularis* expuestos a altas temperaturas pudieron resultar del efecto directo de la temperatura sobre la producción de toxinas o a la reducción en la flora bacteriana asociada a *Ostreopsis*.

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