The use of ozone to degrade *Gymnodinium breve* toxins

Utilisation de l'ozone pour dégrader les toxines de Gymnodinium breve

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Abstract

Gymnodinium breve toxins were exposed to ozone treatment in both extracted form and in intact whole cells. Samples displayed a three log reduction in the total amount for toxin (PbTx-1, -2, -3, -5, -7 and -9) recovered after ten minutes as determined by HPLC analysis. Ozone effectively killed the red tide dinoflagellates when directly contacted ozone and when exposed in a pre-ozonated ASW environment. Both samples, when examined by light microscopy, displayed little difference between the direct and indirect ozone treatments. Reduction in toxin levels directly correlated with reduction of toxicity as observed using by a fish (*Cyprinodon variegatus*) bioassay.

Résumé

Des toxines de *Gymnodinium breve* ont été exposées à un traitement à l'ozone à la fois sous forme extraite et sur des cellules intactes entières. Les échantillons analysés par HPLC révèlent une réduction de 3 facteurs logarithmiques de la quantité totale de toxine (PbTx-1, -2, -3, -5, -7 et -9) récupérée après 10 minutes. L'ozone détruit de façon efficace les dinoflagellés des eaux colorées lorsque ceux-ci sont mis en contact direct avec l'ozone et lorsqu'ils sont exposés à un environnement ASW pré-ozoné. L'analyse par microscopie optique indique que les deux échantillons présentent peu de différence entre les traitements direct et indirect à l'ozone. La diminution des teneurs en toxine est directement corrélée à la diminution de la toxicité observée lors d'un essai biologique sur un poisson (*Cyprinodon variegatus*).

INTRODUCTION

The occurrence of red tide blooms interrupts the culture, production, harvesting and subsequently the marketing of seafood products. Problems include health risks associated with the ingestion of contaminated shellfish, possible contamination of maricultured products, fish kills, morbidity associated with human rhinitis and respiratory and eye irritation.

The causative organism of main interest to the state of Florida is the dinoflagellate *Gymnodinium breve* (formerly *Ptychodiscus brevis*) (Steidinger, 1990). These dinoflagellates produce lipid-soluble neurotoxins (brevetoxins), that are responsible for extensive fish kills in the Gulf of Mexico (Nakanishi, 1985). Shellfish toxicity has also been reported during the occurrence of Florida red

tides and has been cited as the cause of sub-lethal human intoxications known as neurotoxic shellfish poisoning (NSP). Toxin levels normally found in shellfish during *G. breve* blooms are not high enough to be fatal (Baden *et al.*, 1984a).

Ozonated sea water has been shown to be effective in inactivating crude toxins associated with dinoflagellate blooms, as well as in reducing the levels accumulated in shellfish (Thurberg *et al.*, 1975; Blogoslawski *et al.*, 1979). This work reports the action of ozone on the individual toxins associated with *G. breve*.

Methods and materials

Artificial seawater

All experiments were conducted in a minimal artificial sea water mix so as to reduce the interferences generated by the numerous trace compounds found in natural and commercially mixed sea waters. The mix included chloride (30.0 g of NaCl), bromide (0.097 g of NaBr), and carbonate buffers (0.192 g as $NaHCO_3$) for each liter ozone demand free water at a pH of 8.0. A standard ammonia solution was prepared to 1 ppm at a pH of 10.0 using NH_4OH, and added to the appropriate sample using Eppendorf pipettes.

Glassware preparation

Ozone demand free glassware was used in experiments dealing with the quantitation of residual oxidant levels. The glassware was placed in a tank containing 20 liters of artificial sea water that was subjected to ozonation for a period of 1 hour. Glassware remained in the ozonated sea water for 24 hours, at which time it was removed, rinsed in ozone demand free water and placed in a 108°C drying oven for no less than 12 hours. The glassware was then removed and quickly covered with aluminium foil until needed.

Production and delivery of oxidant

Ozone gas was generated by a Welsbach T-408 Ozone Generator supplied with bottled medical grade oxygen (99.5 percent 0_2 with a dew point of – 120°C) and set to an energy output value of 50 V. All lines and connectors were made of Teflon to reduce any reactivity. The generator was switched on for no less than 30 minutes prior to experimentation to allow for warm-up. The gas flow was controlled by a Matheson flow meter set to deliver 0.5 liters per minute. Flow was set using a soap bubble calibration. Ozone was bubbled into artificial sea water via crystalline alumina gas diffusing stones. The ozone generator was allowed to flush with 0_2 for 30 minutes following shut down to purge the generator and lines.

The total ozone production for the generator with a flow rate of 0.5 liters per minute at a voltage of 50 V was determined by bubbling output gas directly into 5 percent potassium iodide. To establish that no off-gasing was occurring, filter paper soaked in a 10 percent KI solution was placed over the reaction vessel. In the presence of ozone gas, the filter paper rapidly turned a dark yellow to

brown color (this effect was not noticed when output tests were conducted). The 5 percent KI solution was then titrated amperomatrically using phenylarsine oxide (PAO) to determine output versus time.

Measurement of oxidant

In the buffered potassium iodide test, 5 ml sample of ozonated sea water was added to a buffered potassium iodide (KI) solution at room temperature and allowed to react for 30 minutes in the absence of light. The absordance of triiodide produced was measured on a Beckman DU-40 spectrophotometer at a wavelength of 352 nm using a 1 cm path length cuvette. A standard curve relating triiodide absordance at 352 nm to oxidant concentration was generated using an amperometric titration.

Gymnodinium breve cultures

A stock culture of *G. breve* was supplied by Dr R.H. Pierce of Mote Marine Laboratory, Sarasota. This culture was grown and maintained in NH-15 media at 25°C on a 12 hours light cycle (Pierce, personal communication). Experiments involving the determination of *G. breve* inactivation utilised a Fisher-Brand cell counting slide in conjuction with light microscopy.

Toxin extraction

Brevetoxins were extracted from the *G. breve* culture in a two step process according to the procedure of Proffitt *et al.* (1989) (Figure 1). Glass columns were assembled using a stopcock fitted with a needle value at the bottom. A small plug of glass wool was set on top of the stopcock to retain the ODS C-18 material. One gram was C-18 was added the column and a second plug of glass wool added to the top of the column bed. The column bed was then washed with 3 full column volumes of methanol. Next, 2 full column volumes of HPLC grade water were gravity feed through the C-18 bed, leaving 2 ml of on top of the column. At this stage the column was ready for use.

One liter portions of *G. breve* were measured out using a 1 liter graduated cylinder. Cultures were vacuum-filtered through a number 1 Whatman (GF/B) glass fiber filter paper using a Buchner funnel. The filter was then rinsed thoroughly with HPLC grade water. The resulting filtrate was then vacuumed through the OCS 5-18 packed column at a flow rate approximately 1 liter per hour. The column bed was rinsed with 2 column volumes HPCL grade water to remove any salts associated with the culture medium. The column was then allowed to run dry for 1 minute. The toxins were eluted from the ODS C-18 bed with four column volumes of methanol. This eluant was collected in round bottom flasks and rotary-evaporated at 40°C, to a final volume of 5 ml. Toxins were placed in volumetric test tubes and stored in a cool, dark storage area until needed.

An analysis of the toxin extract by high performance liquid chromatography (HPLC) was performed using a Varian Model 5000 LC pump in conjunction with a Varian Model UV-50 detector set at 215 nm and a Burbick and Jackson 25 cm, OD-5, C-18 reverse phase column. The instrumentation was allowed to

warm-up for a period of one hour. Isocratic separation was performed, utilising a methanol/water (85%/15%) mobile phase at a flow rate of 1.0 ml per minute. Toxin standards were injected at varies concentrations to ascertain detector response. Twenty five µl injections of experimental samples were made in triplicate and their response values averaged. Data was collected using the Perkin-Elmer Nelson 900 Series interface which used the Model 2600 Nelson Chromatography software (version 5.1) to integrate peak area.

Toxin reduction experiments

To determine the effect of ozone on brevetoxins, two separate experiments were conducted. The first involved the purified toxins extracted from stock cultures and reintroduced into ASW. The second experiment tested ozone directly on whole cells of *G. breve*. Toxin standards for PbTx-1, -2, -3, -7 and -9 were obtained from Calbiochem Corp. (San Diego, CA). Each standard, 100 µg of powdered toxin, was resuspended in methanol. High performance liquid chromatography UV detector response curves were generated for each toxin standard. Detector response for PbTx-5 was estimated from a comparison of known responses for the other toxin. A typical chromatogram for *G. breve* extract can

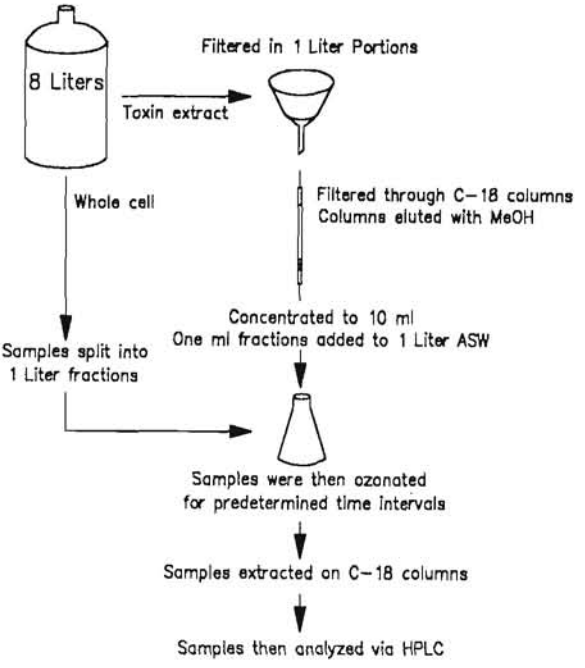

Figure 1: Schematic representation of the solid/liquid phase extraction procedure (Proffitt *et al.*, 1989). The pathway to the upper and right portions shows the purified toxin experiment and the lower and left portion represents the whole cell experiments

be seen in figure 2. It should be noted that PbTx-9 and PbTx-6 elute closely and are hard to resolve (Pierce, R.H., personal communication). Therefore, calculated values for PbTx-9 may reflect the combined total toxin concentration of both PbTx-9 and PbTx-6.

In the first experiment, 8 liters of 6.64 x 10^7 cell per liter was extracted using the solid/liquid phase extraction technique described above. The toxins were collected and concentrated to a final volume of 9 ml. One ml was placed into each of eight 1500 ml glass beakers containing 1 liter ASW and the remaining 1 ml was used as a control. The group of eight beakers was used for two replicates. Each pair was subjected to ozone for the following time periods: 0, 1, 5 and 10 minutes under constant agitation via a Teflon stirring star. After the challenge period, samples were allowed to mix for 60 seconds, at which time 5 ml of 1N $Na_2S_20_3$ were added to quench any further reactions. The 1 liter samples were re-extracted using the solid/liquid phase extraction process (figure 5) and analysed by HPLC.

In the whole cell experiment, 8 liters of 5.7 x 10^7 cells per liter of *G. breve* culture were measured out into eight 1500 ml glass beakers, each with a Teflon stirring star. The group of eight beakers was split into two replicates. Each pair was subjected to ozone for the following time periods: 0, 1, 5 and 10 minutes under constant agitation using a Teflon stirring star at ambient temperature. The 1 liter samples were extracted using the solid/liquid technique and analysed by HPLC.

Fish bioassay

To establish a reduction in toxicity, a fish bioassay using *Cyprinodon variegatus* was performed. Two hundred 10 week old juvenile *C. variegatus* were obtained, having an average length of 14-20 mm. The fish were allowed to acclimate in flow through tanks which were supplied with triple filter sea water from Sarasota Bay, Florida.

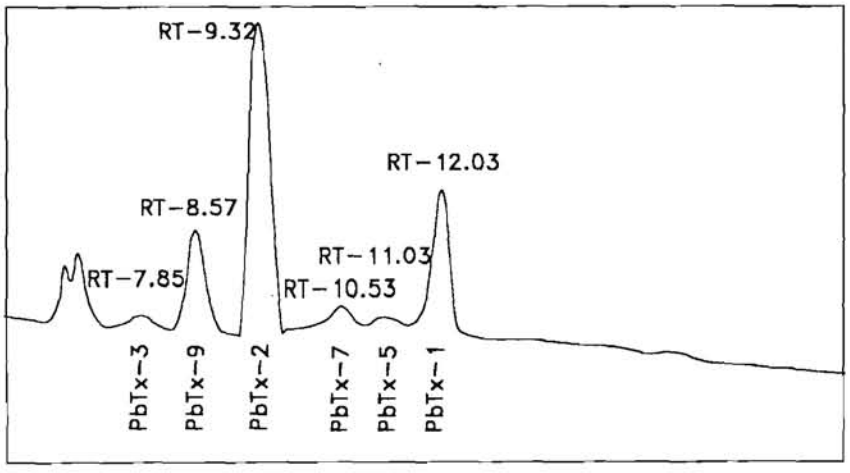

Figure 2: HPLC chromatograph of *G. breve* extract

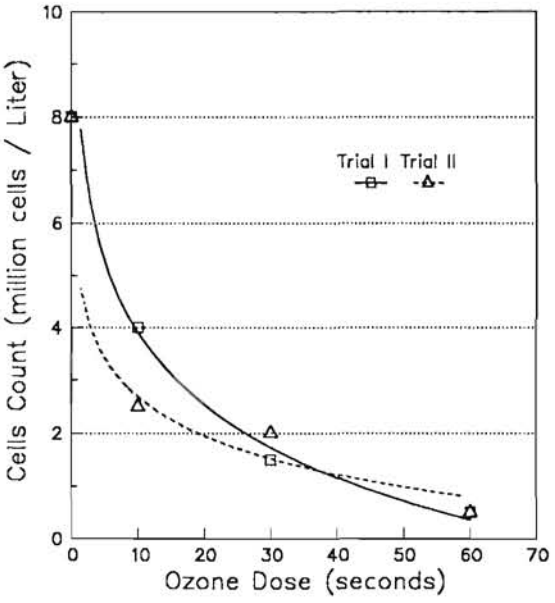

Figure 3: Effect of ozonated ASW on *G. breve* culture. Two hundred ml samples were exposed to ozone for 0, 10, 30 and 60 seconds at standard output. The 200 ml of ozonated ASW was then added to 200 ml of *G. breve* culture and intact cells were counted. Each point represents two replicates

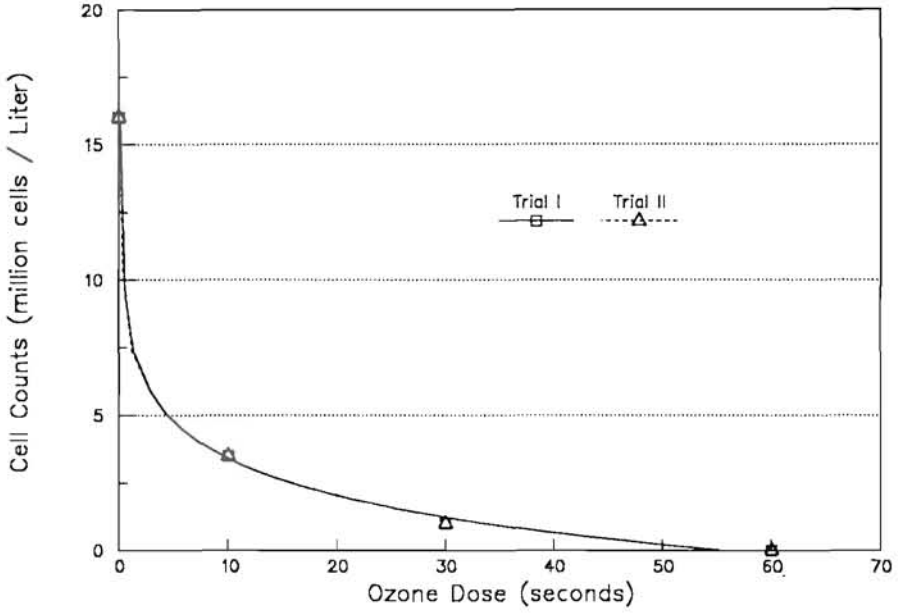

Figure 4: Effect of direct ozonation on *G. breve* culture. Two hundred ml of culture were exposed to ozone for 0,10, 30 and 60 seconds at standard output conditions. Intact cells were then counted. Each point represents two replicates

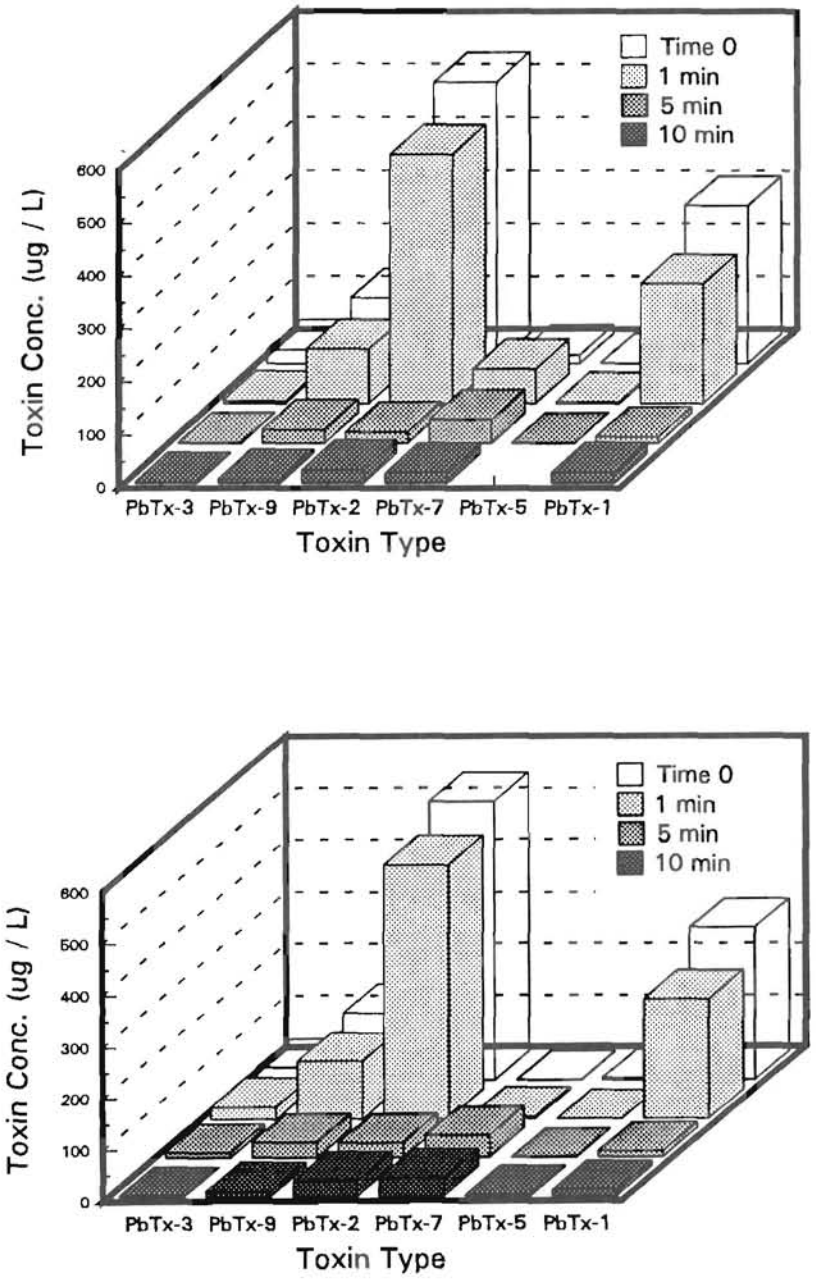

Figure 5: Effect of ozonation on purified *G. breve* toxins reintroducted into ASW. One liter ASW samples were spiked with toxin extract and exposed to ozone treatment for 0, 1.0, 5.0 and 10 minutes with constant stirring. Each graph represents one trial analyzing for six major toxins by HPLC. Toxin calculations are based on the average of triplicate injections. A marked reduction can be seen as ozone exposure increase.

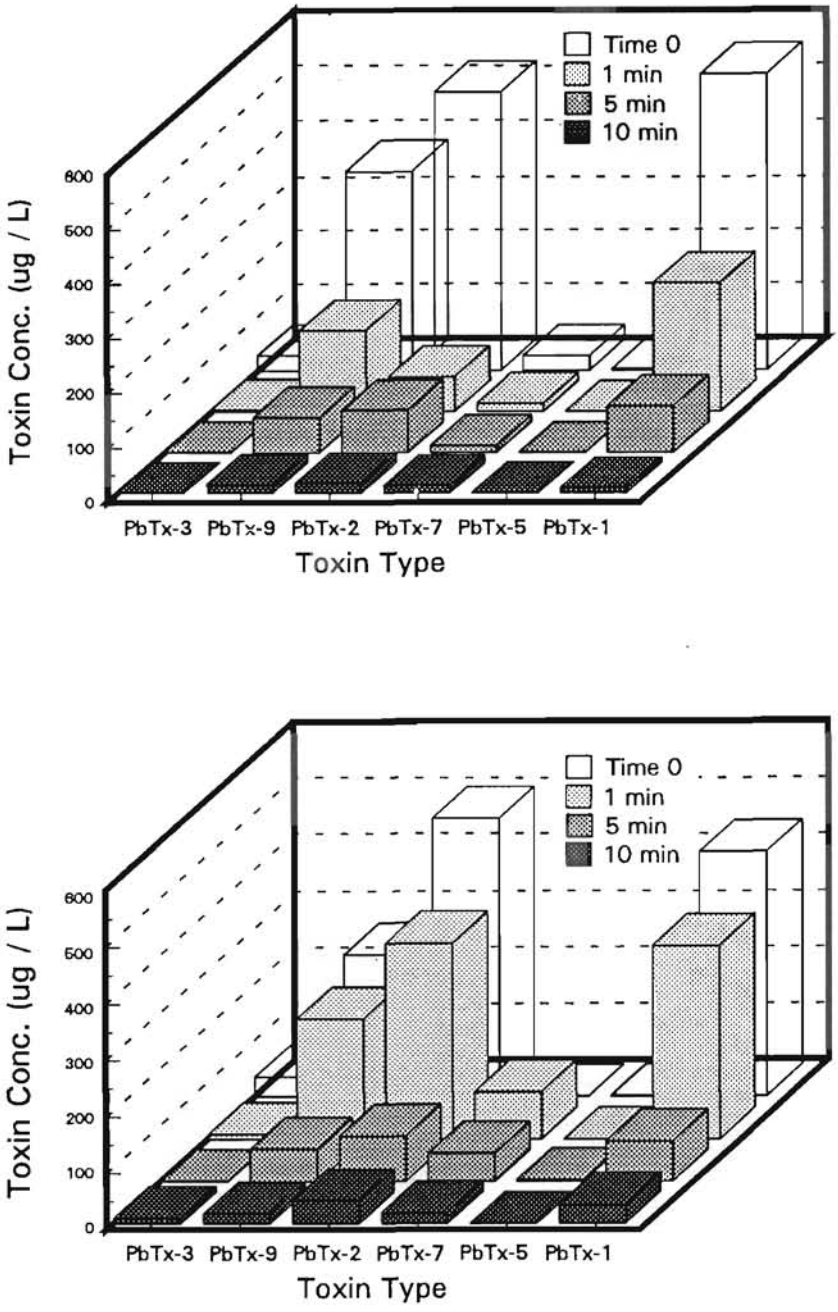

Figure 6: Effect of ozonation on whole cell cultures of *G. breve*. One liter samples of *G. breve* culture were exposed to ozone treatment for 0, 1.0, 5.0 and 10 minutes with constant stirring. Each graph represents one trial analyzing for six major toxins by HPLC. Toxin calculations are based on the average of triplicate injections. A marked reduction can be seen as ozone exposure increases.

Thirty liters of triple filtered seawater were oxygenated for 30 minutes using medical grade 0_2. The oxygenated water was then measured in 1 liter portions into twenty five 1500 ml beakers. Each beaker was equipped with an air stone to provide aeration (30 minutes of ambient air every 6 hours). The use of continuous aeration was not applied to minimise aerosolising the toxins.

Five *C. variegatus* specimens were placed in each 1500 ml beaker. Control beakers were dosed with toxin extract concentrations equal to LD100, LD50, and nonlethal values determined by Mote Marine Laboratory tests. This represented 500 µl, 300 µl, and 50 µl of the 5 ml toxin extract (final concentration) of each one liter sample tested. Samples designated for experimental fractions received a 300 µl dose. Replicate samples tested received a 500 µl and a 300 µl dose. Since toxins were dispersed in methanol, a separate control was run using methanol only to rule out any alcohol interference. Fish mortality was monitored for 24 hours.

Results

The effect of ozone treated ASW on *G. breve* cells is seen in figure 3. Each trial consists of two replicates. In both cases the number of surviving cells decreased approximately 80 percent after 60 seconds of ozone exposure. After 60 seconds of ozonation (approx. 25 mg of ozone) one cell per counting grid could be found. Figure 4 shows the effect of ozone when applied directly to *G. breve* cultures. Each trial consists of two replicates. Both treatments yielded identical curves, however the number of cells remaining was reduced at a greater rate than seen in figure 3. After 60 seconds of ozonation (approximately 25 mg ozone), non intact cells were found.

Figure 5 shows the effect of ozone treatment (time 0, 1, 5 and 10 min) on purified toxins from 1 liter of extracted *G. breve* cells re-introduced into 1 liter of sterile culture medium. Each graph represents a separate test, and each point on both graphs are calculated from the average peak area for each toxin from three HPLC chromatograms. In both studies, seen in figure 5, a dose-dependent reduction in toxin levels was observed for all toxins with the exception of PbTx-7. Figure 6 shows the effect of ozonation (time 0, 1, 5 and 10 mn) on 1 liter of whole cell cultures of *G. breve*. As with the purified toxins, a dose-dependant reduction in the total toxin present is seen for all toxins except for PbTx-7 at the 1 minute time period.

In order to determine if the reduction in peak area seen in the HPLC chromatograms truly reflected a reduction in toxicity, a fish bioassay was performed. Figure 7 shows the results of this test and indicates a clear correlation between peak area as recorded by HPLC and fish mortality. The sample doses were determined using LD50 toxicity levels established for *G. breve* toxins for *Cyprinodon variegatus* generated from a study performed at Mote Marine Laboratory (unpublished data). The toxin levels needed corresponded to a 300 µl dose of the final toxin extract for each litter of *G. breve* culture. Some experimental samples received an LC100 dose or 500 µl of toxin extract, equivalent to 90-100 µg of total toxin, and a nonlethal dose or 50 µl of toxin extract, equivalent to 5-10 µg of total toxin.

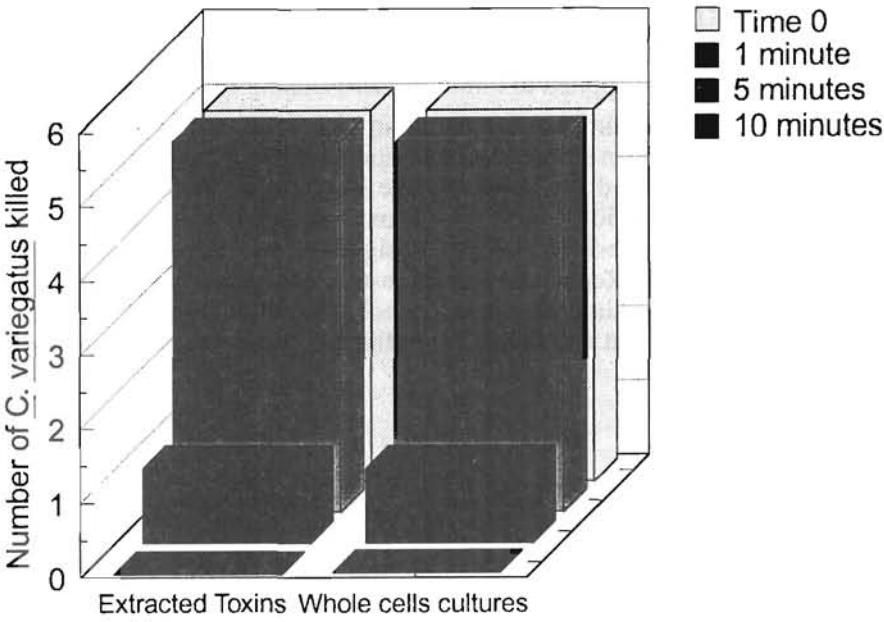

Figure 7: Fish bioassay for ozone treated *G. breve* toxins using *Cyprinodon variegatus*. Fish were exposed to extracted toxins reintroduced into ASW and ozonated and whole cell cultures of *G. breve*, which were ozonated directly. Graph represent the number of fish that died when exposed to a sample volume equal to an LD100 dose at time zero

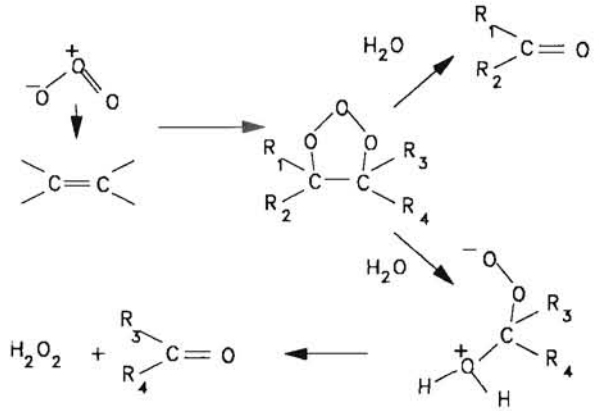

Figure 8: Possible reaction pathway for ozone and carbon-carbon double bonds (Bablon *et al.*, 1991)

DISCUSSION

Previous studies have focused on the use of ozone for the purpose of degrading toxins such as paralytic shellfish poison (PSP) (Blogoslawski *et al.*, 1979; Dawson *et al.*, 1976) and toxins associated with the marine dinoflagellate native to the Gulf of Mexico, *G. breve* (Blogoslawski *et al.*, 1975b; 1973). This study focuses on *G. breve* toxins PbTx-1, -2, -3, -5, -7 and -9.

The first phase of our study focused on the ability of ozone to kill the dinoflagellate. Results of both *G. breve* culture introduced into pre-ozonated ASW environment and the effects of ozone directly introduced in *G. breve* culture were determined. The samples, when examined by light microscopy, displayed little difference between the direct and indirect ozone treatments. Disruption of the cell wall and subsequent release of cellular materials appeared to have occurred in both experiments. As expected, direct ozonation of *G. breve* cultures exhibited faster kill with t1/2=2.5 seconds as compared to t1/2=10 seconds for the oxidised ASW. This was probably due to the direct action of ozone which reacts rapidly with organic molecules, such as the cell membrane, rather than HOBr formation (Hoigné and Bader, 1983b).

Once established that ozone would kill the dinoflagellate by disrupting the cellular membrane, the next phase of this research was to determine the fate of the toxins within these cells. Blogoslawski *et al.* (1973) ozonated crude toxin extracts of *G. breve*. Both mouse and fish bioassay confirmed a marked decrease in the overall toxicity of the crude extract. In a later study, Blogoslawski *et al.* (1975) ozonated whole cultures. Using a mouse bioassay it was shown that the overall toxicity in this test was also reduced. The research performed in this study examined both scenarios.

In the first experiment, *G. breve* toxins were extracted from laboratory cultures, then reintroduced into sea water (figure 5). These samples were then exposed to ozone for predetermined time periods, and reextracted. The second experiment used whole cell culture which were exposed to ozonation for predetermined time periods and then extracted (figure 6). HPLC analysis was used to track the fate of six major toxins (PbTx -3, -9, -2, -7, -5 and -1) associated with *G. breve*. In both figures 5 and 6 a three log cycle reduction was noted in the amount of toxins recovered. The two major toxins present, PbTx -2 (group 1) ant PbTx -1 (group 2) both were effectively removed after five minutes. One unexpected outcome of this experiment was the slight increase of PbTx -7 during the first minute of ozone exposure. This increase was probably caused by the reduction of PbTx -1, the aldhyde form of group 2 type toxin, to PbTx -7, the alcohol form of the same skeleton. This could be caused by reaction with hypobromous acid. An increase in any toxin level is unwanted, but its important to note that PbTx -1 is being converted to a less active form, PbTx -7 (Baden *et al.*, 1988). The increase seen at the one minute time period for PbTx -7 was temporary. As ozone exposure was increased to five minutes, the total amount of all toxins was reduced. The total toxin concentration was reduced 99.9 percent after ten minutes (135 ppm of ozonation). The production of break-down products, as seen during HPLC analysis as newly formed, quickly eluting peaks, were probably the cause of the decrease in the overall rate of

toxin degradation. The competition for available oxidant increased as the toxins break-down products increased. Surprisingly, the whole cell samples did not appear to be affected by the presence of cellular material. In some instances, the reduction of several toxins (PbTx -9 and -2) was actually enhanced over that of the purified toxin assay. One possible explanation could be the release of auto-catalytic enzymes when the cellular constituents were released.

Figure 8 shows a possible pathway ozone might take when attacking *G. breve* toxins. Reactions similar to those seen in figure 8 could possible result in four new keto moieties for both toxins type 1 and 2. This disruption in the ring structure might result in a change in the physical shape of the toxin molecule. This change, in turn, could be responsible for the reduction of the toxicity seen in the fish bioassay (figure 7).

To establish if the reduction in peak area represented a true reduction in toxicity, a fish bioassay (Mote Marine Laboratory, personal communication) was conducted. The ichthyotoxicity of the toxins is related to their ability to alter membrane properties of excitable cells (Poli *et al.*, 1986). The three most potent toxins are PbTx -1, PbTx -2 and PbTx -3 respectively (Stuart and Baden, 1988). Therefore, a reduction in toxicity should be noted which corresponds with the reduction of toxin seen in figures 5 and 6.

Sheepshead minnows, *Cyprinodon variegatus* were exposed to a 300 µl dose of the final toxin extract or approximately 50-60 µg of total toxin (equivalent to previously record LC50 values). The same sample size (300 µl) was used for each ozonation time period. Some experimental samples received an LC100 dose or 500 µl of toxin extract, equivalent to 90-100 µg of total toxin and a non-lethal dose or 50 µl of toxin extract, equivalent to 5-10 µg of total toxin. Each test, both the purified toxin assay and the whole cell assay, were performed in duplicate. In all cases, as seen in figure 7, a reduction in total toxin estimates as calculated by HPLC resulted in lower fish mortality.

Summary

The purpose of this research was to examine the effectiveness of ozone to reduce the toxins associated with Florida's red tide organism, *G. breve*. The results of the *G. breve* toxin experiments showed a three log cycle reduction in the total toxin recovered after 10 minutes (135 ppm) of ozone exposure. In the first experiment, toxins were extracted and reintroduced into the sea water media. The second experiment exposed whole cell *G. breve* culture to ozone treatment. Samples from both experiments displayed approximately the same three log cycle reduction in six major toxin associated with *G. breve*. The reduction in toxin concentration as mesearured by HPLC analysis, displayed a positive correlation with reduction of toxicity as determined by a fish (*Cyprinodon variegatus*) bioassay. Despite large doses of ozone, as compared to levels that might be found at a commercial ozonation facility, some toxins were still recoverable by HPLC analysis.

Results of further studies could help lessen the amount of mariculture revenues lost following red tide blooms. Moreover, both live and shucked shellfish

markets may benefit from ozone processing, by reducing the amount of toxin and bacterial content of the product. Information gained in further ozone-seawater research may also lead to an effective method for water detoxification that would beneficial in water treatment for aquaculture facilities. Future research could generate information that could be used by FDA when considering ozone as an acceptable depuration method.

Acknowledgements

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