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Experimental Chemical Treatments for the Control of Dinoflagellate *Cochlodinium polykrikoides* in the Land-based Culture of Olive Flounder *Paralichthys olivaceus*

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넙치 육상수조 양식에 있어 편조류 *Cochlodinium polykrikoides*의 구제를 위한 화학적 처리

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When *Cochlodinium polykrikoides* came into the culture tanks through influent cultivated water during the red tides, hundred thousands of commercial flounders were concomitantly killed and many culturists suffered from a great deal of financial loss in the east coast of Korea. It is characterized by high sinking rate after sunset and the formation of clump which results in oxygen deficiency by its respiration at tank bottom under dark condition. We investigated the efficacy of hydrogen peroxide and chlorine dioxide, known to form free radicals, for extermination of red tide organism *C. polykrikoides*. When *C. polykrikoides* seawater with a density of 6,000 cells/mℓ was treated with 14, 28 and 42 ㎎/ℓ of hydrogen peroxide, its survival rate was markedly decreased to 9.8, 0.8 and 0.3% respectively immediately after 6 hours of treatments whereas when it was treated with 1.5, 2.1 and 3.0 ㎎/ℓ chlorine dioxide, its survival rate showed 87.7, 81.3 and 80.1% respectively at the same treatment time. Hydrogen peroxide was the effective agent since it has scarcely injured the cultured olive flounder when exposed to the tested concentration range of 14~28 ㎎/ℓ with the extermination of almost3 *C. polykrikoides* during the experimental period of 5 days and has shown the oxygen increase of approximately 1.23 ㎎/ℓ 2 hours immediately after the treatment of same concentration. We propose that the main mechanism to kill cultured olive flounder by *C. polykrikoides* in the land-based culture tank is assumed to be not by the toxicity of itself but by oxygen deficiency from the rapid respiration of dinoflagellate clump sunken to the tank bottom.

Key words : *Cochlodinium polykrikoides*, Oxygen deficiency, Hydrogen peroxide, Chlorine dioxide

Introduction

Phytoplankton plays an important role as a primary producer in the coastal ecosystem whereas blooms of harmful phytoplankton species pose serious problems for fish and

shellfish farming (Cembella et al., 1988 ; Kim et al., 1993). The flagellate genus *Cochlodinium* is composed of a solitary cell which is ellipsoid, slightly flattened dorsoventrally, 39~40 μm long, 20~30 μm wide and chain cells consisting of less than 8 cells (rarely of 16 cells) (Fukuyo et al., 1990). *C. polykrikoides* is a flagellated phytoplankton, causing red tide, which is harmful to olive flounder *Paralichthys olivaceus* in the industrically land-based culture tanks with low light intensity of 0.6~3 $\mu E\ m^{-2}s^{-1}$. It has killed cultured finfish including olive flounder, red sea bream, rock fish and yellowtail in Japan, China, and Korea (Qi et al.. 1993 ; Onoue et al.. 1985 ; Kim et al., 1997). Several species of the genus *Cochlodinium* are known to cause fish kills in various sites of the world (Onoue et al., 1985 ; Yuki and Yosimatsu, 1989 ; Ichikawa et al., 1993). During September to October, 1995~1997, blooms of a harmful dinoflagellate *Cochlodinium polykrikoides* have progressively occurred in the southern coastal area where various kinds of culture grounds were intensified in Korea. In September 1995, an expansion of red tide to eastern coastal area by a tidal current resulted in the unprecedented mass mortality of wild and cultured fishes in this region. Lee (1996) suggested that live *C. polykrikoides* collected from Yokji island coastal waters, Kyeongnam, Korea was toxic to fish, however, the water soluble and chloroform soluble fraction of their methanol extracts did not shown ichthyotoxicity (5 ㎎/㎖) and toxicity to mice (50 ㎎, i.p.), and these fractions did not show any peaks corresponding to paralytic shellfish toxins on the fluorometric HPLC chromatograms. Yuki and Yosimatsu (1989) have suggested that it be necessary to monitor the occurrence of *C. polykrikoides* in the fish culture grounds since it is toxic to fish. Marking et al., (1994) identified hydrogen peroxide as a promising fungicide which has been granted low regulatory priority drug status by the FDA. Chlorine dioxide and hydrogen peroxide have been used as chemical agents to treat bleaching cellulose, paper-pulp and textiles and to purify and taste of water, and is currently being consumed as bactericide for bacterial gill disease and antiseptic for ectoparasite (Budavari et al., 1989). Hydrogen peroxide has been utilized to control sea lice in Norway (Thomassen, 1993) and to investigate the effect on fungal infected eggs of rainbow trout (Schreier et al., 1996). Therefore, effects of chemical agents such as hydrogen peroxide, chlorine dioxide were investigated for the control of dinoflagellate *C. polykrikoides* contained in the influent cultivated seawater of the land-based culture tank of olive flounder *Paralichthys olivaceus* to minimize an financial damage to the olive flounder farmer.

Materials and Methods

Culture

The strain of *Cochlodinium polykrikoides* was obtained during the harmful algal bloom in the coastal area near Pohang-city, Kyŏngsangbuk-do, Korea. For mass culture, *C. polykrikoides* with a four celled-chain were isolated from natural seawater containing red tide organisms under a stereomicroscope with a modified pasteur pipet. Cul-

tures of the dinoflagellate were carried out in a tissue culture well plate on modified F/2 medium (Guillard and Ryther, 1962). Culture has been carried out in a multithermo incubator (EYELA MTI201) to know optimal culture condition such as light, temperature and modified medium.

Culture technique

Cochlodinium polykrikoides was cultured in 20 ℓ autoclaved nalgen bottle at 23℃ using modified F/2 medium adjusted to pH 8.1. The aeration for mass culture was done through two glass filters to 0.45 μm membrane filter holder to decrease bacterial contamination by the air, and the light was illuminated using 120 μE $m^{-2}s^{-1}$ of continuous illumination. Mass culture has been carried out in a constant temperature cabinet with a 14L : 10D cycle at 23℃.

Measurement of dissolved oxygen concentration and pH.

A circular chamber was sealed with a circular rubber between a lid and a chamber. The values of dissolved oxygen were measured at saturated condition after falling far below the concentrations which one would expect to be the high oxygen value in red tide seawater. DO and DO% were measured at the chamber filled with 20ℓ *C. polykrikoides* seawater constaining with a density of 6,000 cells/mℓ and recorded using an unattended program of a multifunction parameter (YSI 6000). These data were used to investigate the rate of decrease of oxygen concentrations depending on the water depth of the sealed circular chamber under dark-treated condition. Twenty eight mg/ℓ of hydrogen peroxide was added to a chamber containing the red tide water of 6,000 cells/mℓ in order to know the change of dissolved oxygen and pH.

Effects of different concentrations of chlorine dioxide and hydrogen peroxide on dinoflagellate *C. polykrikoides*.

The efficacy study included two chemical regimes. Chemical agents of 28% hydrogen peroxide and 3% chlorine dioxide were prepared as stock solution and stored at about 4℃, respectively. The effectiveness of chlorine dioxide for red tide seawater was evaluated at treatment concentrations of 0.3, 0.9, 1.5, 2.1 and 3 mg/ℓ. The efficacy of hydrogen peroxide was studied at treatment concentrations of 2.8, 8.4, 14, 28 and 42 mg/ℓ. Each concentration of treatment of chemical agents was delivered with a pipet aid into red tide seawater and then mixed with a magnetic bar. Treatment seawater was sampled to determine the concentration for each test chemical. Concentrations of chlorine dioxide and hydrogen peroxide were analyzed using a spectrophotometer and a YSI 9100 photometer immediately after treatment began. Samples for cell counts were preserved in the lugol solution. Cells were counted with a Sedgwick-Rafter chamber under a microscope. This experiment was done triplicately.

Bioassay for protection of juvenile olive flounder from harmful *C. polykrikoides* cells by chemical treatment.

Cultured olive flounder fish of approximately 12~15 cm length were transferred to the laboratory of East Sea Regional Fishe-

ries Research Institute and acclimated in a water bath at 23℃ without feeding for 2 days. One ton seawater containing *C. polykrikoides* during red tide was sampled from the coastal area near Pohang, Kyŏngsangbuk-do. Sampled seawater was sieved with 50 μm müller gauze in the laboratory to get rid of some zooplankton. To investigate the effect of the chemical treatments to the red tide organism on the olive flounders, ten flounders were placed in each of ten 40 ℓ rectangular plastic aquaria (37cm×51cm×30cm). Each aquarium included 40 ℓ seawater containing 6,000 cells/ml of *C. polykrikoides* and ten olive flounder fish for bioassay by chemical treatments and control experiment. The efficacy of chlorine dioxide was evaluated at treatment concentrations of 1.5 and 3 ㎎/ℓ, respectively. The efficacy of hydrogen peroxide was evaluated at the range of 14 and 28 ㎎/ℓ hydrogen peroxide, respectively. Each aquarium was adjusted to 7.0~7.2 ㎎/ℓ at the value of dissolved oxygen and illuminated using 100 μE $m^{-2}s^{-1}$ of continuous illumination. After treatment of chemical agents, survival rate of olive flounder were counted for 5 days.

Results and Discussion

Measurement of oxygen consumption

C. polykrikoides was recognized to show high sinking rate in the coast after sunset and in the land-based culture tank for olive flounder with a light density of 0.6~3 μE $m^{-2}s^{-1}$. They formed clump at the tank bottom of land-based farming (Fig. 1A). Oxygen deficiency by rapid respiration of fish and *C. polykrikoides* was observed at the

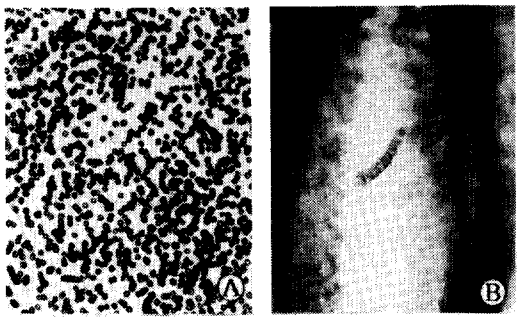

Fig. 1. *C. polykrikoides* cells observed in the land-based culture tank of olive flounder, *Paralichthys olivaceus*. Light microscopic views of : (A) numerous dinoflagellates, containing 12,000 cells/㎖, sunken to the bottom of the land-based culture tank of olive flounder. 40 × ; (B) eight celled-red tide organism attached to the gill lamellae of an olive flounder. 100 ×

tank bottom under dark condition and then mass olive flounders were killed soon after showing swirling swimming behavior. Therefore, from these observations *in situ*, we assumed that mass mortality of oliver flounder seems to be due to the oxygen deficiency resulted from the rapid competitive respiration of fish and dinoflagellate clump sunken and accmulated to the tank bottom (Fig. 1 B). According to the present study, dissolved oxygen in a circular chamber sealed was significantly different at the depth of 5 and 30 ㎝ in the culture column with the lapse of time in dinoflagellate seawater. The variation of dissolved oxygen values in the red tide water of the culture column was highly dependent on the water depth. The value of the dissolved oxygen at the deeper depth decreased sharply with the lapse of time after stocking of *C. polykrikoides* in the culture column (Fig. 2 and Fig. 3). DO values of the dinoflagellate *C. polykrikoides* seaw-

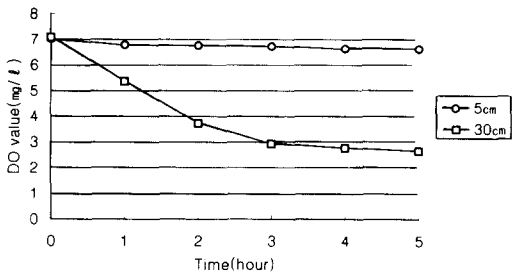

Fig. 2. Periodic variation of dissolved oxygen depending on depth in *C. polykrikoides* seawater in an dark-treated circular chamber without aeration. An aquarium was filled with *C. polykrikoides* seawater of the density of 6,000 cells/ml at the temperature of 23±0.3℃ and the salinity of 33.1±0.2‰.

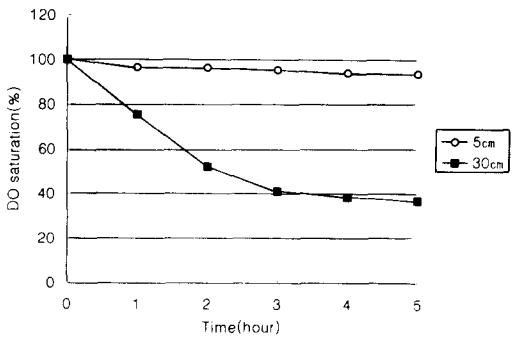

Fig. 3. Periodic variation of dissolved oxygen saturation(%) depending on depth in *C. polykrikoides* seawater in an dark-treated circular chamber without aeration. An aquarium was filled with *C. polykrikoides* seawater of the density of 6,000 cells/ml at the temperature of 23±0.3℃ and the salinity of 33.1±0.2‰.

ater maintained in the dark-treated chamber without aeration reached to 7.08, 5.35, 3.72, 2.93, 2.76 and 2.65 ㎎/ℓ immediately after initial, 1, 2, 3, 4 and 5 hours at 30 ㎝ depth when compared with 7.01, 6.79, 6.76, 6.73, 6.64 and 6.63 ㎎/ℓ immediately after initial, 1, 2, 3, 4 and 5 hours at 5 ㎝ depth (Fig. 2). DO percent of the dinoflagellate *C. polykrikoides* seawater also reached to 100, 75.5, 52.3, 41.2, 38.7 and 37.1% immediately after initial, 1, 2, 3, 4 and 5 hours at 30 ㎝ depth when compared with the values of 100, 96.7, 96.6, 95.6, 94.3 and 94.0% immediately after initial, 1, 2, 3, 4 and 5 hours at 5 ㎝ depth (Fig. 3). From these results, it comes to the conclusion that it is very dangerous for *C. polykrikoides* seawater to inflow to the land-based culture tank rearing mass olive flounders because the light intensity of land-based tank is commonly very low ranging from 0.6~3 μE $m^{-2}s^{-1}$ and olive flounders are reared at the bottom of culture tanks. Changes in cell bouyancy, mainly due to light, seawater density and nutrient concentration, could modify the sinking rate of the dinoflagellate. Therefore, measurement of dissolved oxygen consumption depending on the seawater depth in the present study was carried out under the condition of dark-treatment, artificial nutrient concentration and same natural seawater. Akinina (1969) has confirmed that the dinoflagellates *Prorocentrum micans* and *Gymnodinium kovalevskii* were found to sink most rapidly at night, when the division rate is lowest, and most slowly during the morning when division rate is high. It is obvious that sinking rate of dinoflagellates is different depending on light-dark cycle. Gran (1929) explained that the ability of all flagellates to migrate actively is an important advantage in obtaining sufficient nutrients and light. Blasco (1978) showed that motile cells, such as dinoflagellates, are able to perform vertical migrations of a phototactic nature and to choose an optimum depth. Sweeney (1984) also reported that both *Gonyaulax polyedra* and *Cachonina niei* migrated to the surface of the tank cul-

tured the dinoflagellate, respectively when it was illuminated during the day and down by active swimming during darkness. He suggested that migratiom is not always under the control of a circadian clock because both species started downward migration before the lights went off and at least *C. niei* started to ascend before the beginning of the light period. In the present study, it is likely that light intensity in the dinoflagellate *C. polykrikoides* can play a major role in determining the vertical migration since sinking rate of the dinoflagellate *C. polykrikoides* showing positive phototactic reaction was high after dark treatment although nutrient concentration and seawater density was constant.

When the dinoflagellate *C. polykrikoides* seawater was exposed to 28 ㎎/ℓ hydrogen peroxide, dissolved oxygen increased 5.96 ㎎/ℓ at initial concentration into 6.76 and 7.19 ㎎/ℓ immediately after 1 and 2 hours. However, there were not significant differences in pH between the initial value and 2 hours immediately after the treatment at the chamber containing dinoflagellate seawater when treated with 28 ㎎/ℓ hydrogen peroxide (Fig. 4).

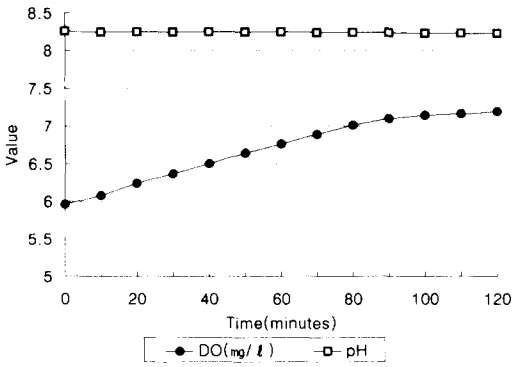

Fig. 4. Periodic variation of dissolved oxygen and pH with the lapse of time in *C. polykrikoides* seawater treated with 28 ㎎/ℓ hydrogen peroxide. A chamber was filled with *C. polykrikoides* seawater of the density of 6,000 cells/ml at the temperature of 23± 0.3℃ and the salinity of 33.1±0.2‰.

Effects of different concentrations of chlorine dioxide and hydrogen peroxide on dinoflagellate *C. polykrikoides*.

The sensitivity of dinoflagellate *C. polykrikoides* to chemical agents was greatly influenced by the changes of concentrations in hydrogen peroxide treatment group but no significant difference in survival rate was found in several concentration groups treated with chlorine dioxide. When dinoflagellate *C. polykrikoides* was exposed to various concentrations of 0.3, 0.9, 1.5, 2.1 and 3 ㎎/ℓ chlorine dioxide, its survival rate was 96.2, 94.1, 93.4, 91.4 to 86.3%, respectively 1 hour immediately after chemical treatment and then 89.1, 88.5, 87.7, 81.3 to 80.5% 6 hours after chemical treatment (Fig. 5). There were no significant differences in survival rate of *C. polykrikoides* among all chlorine dioxide treatment. The dinoflagellate response to chlorine dioxide treatment were not much more sensitive than the hydrogen peroxide agent tested. Groups treated with hydrogen peroxide on *C. polykrikoides* proportionately decreased its survival rate as the treatment concentrations increased. When dinoflagellate *C. polykrikoides* was exposed to various concentrations of 2.8, 8.4, 14, 28 and 42 ㎎/ℓ hydrogen peroxide, its survival rate was from 95.1, 68.4, 48.6, 29.4 to 21.8% 1 hour immediately after chemical treatmentand and then decreased to be 72.5, 20.9, 9.8, 0.8 to 0.3% 6 hours after chemical trea-

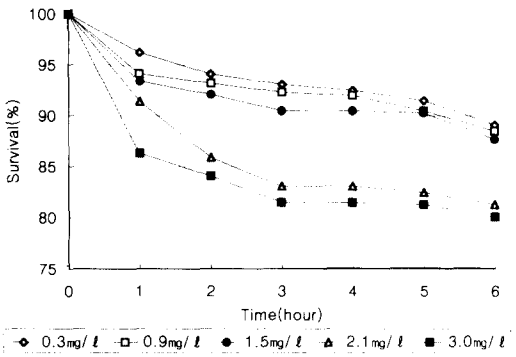

Fig. 5. Survival of *C. polykrikoides* treated with 0.3, 0.9, 1.5, 2.1 and 3 mg/ℓ chlorine dioxide in each chamber filled with 20ℓ dinoflagellate seawater. Initial concentration of dinoflagellate in each chamber, adjusted to the temperature of 23℃, was 6,000 cells/mℓ.

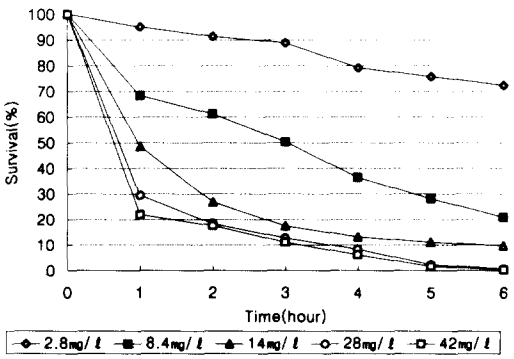

Fig. 6. Survival of *C. polykrikoides* treated with 2.8, 8.4, 14, 28 and 42 mg/ℓ hydrogen peroxide in each chamber filled with 20ℓ dinoflagellate seawater. Initial concentration of dinoflagellate in each aquarium, adjusted to the temperature of 23℃, was 6,000 cells/mℓ.

tment (Fig. 6). Ichikawa et al. (1992) explained that the cysts of *Polykrikos schwartzii* were exterminated after exposure to 100 mg/ℓ hydrogen peroxide for 24 hours and all cysts of *Alexandrium tamarense* exposed to 30 mg/ℓ hydrogen peroxide for 48 hours showed protoplasm contraction and decolorization. The present study shows that hydrogen peroxide treatment groups have seriously influenced on the survival of the dinoflagellate even at the concentration of 14 mg/ℓ after two hours of treatment (2 hrs : 26.8% ; 3 hrs : 17.4% ; 4 hrs : 13.2% ; 5 hrs : 11.1% ; 6 hrs : 9.8%). Howe et al.(1994) have reported that variability of pH and water temperature can increase or decrease the toxicity of some chemicals such as trichlorfon and 4-nitrophenol to the amphipod *Gammarus pseudolimnaeus* and rainbow trout. Therefore our tests were conducted using the dinoflagellate *C. polykrikoides* cultured from same nutrient and at constant pH, temperature and light level.

Microscopic observation was carried to know the morphological changes of *C. polykrikoides* cells by the 28 mg/ℓ treatment of hydrogen peroxide. The dinoflagellate *C. polykrikoides* has showed several cell type from one cell to four cells and eight cells in the favorable condition without chemical treatment. However, when the former chemical agents of 28 mg/ℓ concentrations was added to dinoflagellate solutions, the dinoflagellate of four or eight cells changed normal chain shape into ovoid type similar to weakly reniform hypnocyst of *Gonyaulax excavata*. They were rapidly divided into one cell type and then formed a temporary cyst-like cell which is similar to the typical mature coccoid cyst of *G. excavata* reported by Anderson and Wall (1978). Cyst-like cells were composed of pale nucleus, microgranular yellow-brown pigments, microgranular cytoplasm and a number of refractive starch grains. After that, destruction of pigments in the cytoplasm and swelling of cell wall

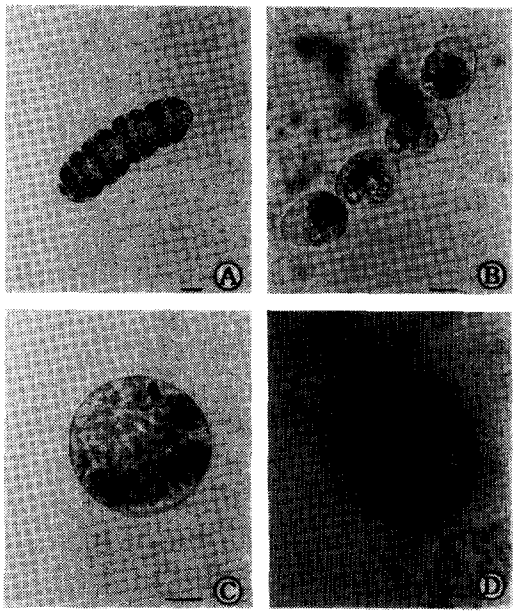

Fig. 7. Morphological changes of *C. polykrikoides* when treated with the chemical agent of 28 ㎎/ℓ hydrogen peroxide. A, Intact *C. polykrikoides* consisted of 4 cells with the length of 20~40 ㎛ ; B, *C. polykrikoides* immediately before the detachment to one cell from 4 cells ; C, *C. polykrikoides* detached to one cell from 4 cells ; D, *C. polykrikoides* destructed by swelling of cell wall and pigmental disintegration within the cell. Scale bar=10 ㎛.

have been presumed to occur at the same time and then to be swollen to death (Fig. 7).

Bioassay for protection of juvenile olive flounder from harmful *C. polykrikoides* cells by chemical treatment.

The sensitivity of olive flounder after the treatment of chemical agents in the dinoflagellate *C. polykrikoides* seawater was greatly influenced depending on the concentrations in chlorine dioxide treatment group but no significant effects on the survival rate of the tested olive flounder was found in several concentration groups treated with hydrogen peroxide. When olive flounder was exposed to various concentrations of 14, 28 and 42 ㎎/ℓ hydrogen peroxide, they have showed 100% survival rate until 5 days after chemical treatment except for the group treated with 42 ㎎/ℓ hydrogen peroxide (Fig. 8). On the contrary, significant effects on the survival rates of tested olive flounder was found in all the chlorine dioxide treatment experiments. The response of olive flounder to chlorine dioxide treatment was much more sensitive than the hydrogen peroxide agent tested. Groups treated with chlorine dioxide gradually decreased survival rate as the treatment concentrations increased. Ho and Zubkoff (1979) explained that relatively low mortalities of 12% in *Crassostrea virginica* larvae were observed in the *Cochlodinium heterolobatum* water which had low dinoflagellate densities of 205 cells/㎖ when treated with calcium whereas most larvae died within 50 hours for those with more than 1,000 cells/ ㎖. When olive flounder was exposed to the alloted concentrations of 0.3, 1.5 and 3.0 ㎎/ℓ chlorine dioxide, its survival rate showed from 100, 50, to 10% respectively 2 days after chemical treatment and then all the fish died of the toxicity of chlorine dioxide 5 days after treatment except for the lowest concentration of chemical treatment of 0.3 ㎎/ℓ.

But hydrogen peroxide has not affected the young flounder even at the concentration as high as 28 ㎎/ℓ during 5 days. If hydrogen peroxide is used in the olive flounder culture tank during red tide season at optimal concentration without affecting fish, it will be a promising candidate chemical treatment since it is resolved into oxygen

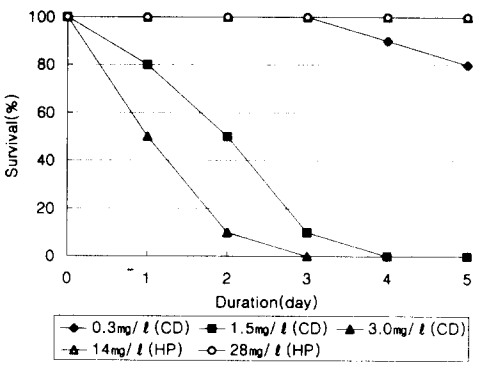

Fig. 8. Survival of the olive flounder *Paralichthys olivaceus* treated with two chemical agents in each aquarium filled with 40 ℓ red tide seawater during 5 days. Initial concentration of dinoflagellate, adjusted to the temperature of 23℃, was 6,000 cells/mℓ. CD ; chlorine dioxide, HP ; hydrogen peroxide.

and water. The present result treated with 28 mg/ℓ concentration of hydrogen peroxide has been consistent with what has been reported in the experiment on all cysts of *Alexandrium tamarense* exposed to 30 mg/ℓ hydrogen peroxide by Ichikawa et al. (1993). Survival rate of the flounder in the group treated with chlorine dioxide was significantly different from the group treated with hydrogen peroxide when exposed for 5 days to the concentrations as high as 28 mg/ℓ. When the olive flounders were tested with chlorine dioxide, they showed much more detrimental response to the chlorine dioxide than the compared group treated with hydrogen peroxide. Murata et al. (1989) reported that hydrogen peroxide as removal agent of a red tide plankton *Chattonella marina* seemed to be the most prospective agent because it destroyed the plankton above 15 mg/ℓ concentration and scarely injured the cultured fish below 50 mg/ℓ concentration. The FDA has classified hydrogen peroxide as a low regulatory priority drug if it is used at the concentration up to 0.5 mℓ/ℓ in fishery industry to control fungal infections on all life stages of all fish species, including eggs (Schreier et al., 1996). This study demonstrated that hydrogen peroxide treatment of 28 mg/ℓ appeared to be the most effective for the protection of juvenile olive flounder from harmful *C. polykrikoides* cells. However, additional toxicological and efficacy test of hydrogen peroxide on fingering fish, adult olive flounder and marine plankton should be conducted to evaluate the safety before the use for all the life stages of olive flounder.

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