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Histology and Histopathology

Diazo-reaction positive substance observed in the cortex of *Chattonella antiqua*

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Summary. In order to investigate the causative factors responsible for removal of mucous coat from the gill lamellae of young yellowlail, ***Seriola quinqueradiata*** by red tide, diazo-reactions were employed for planktons and their media. The concentration of NO_2^- in the medium containing the raphidophyceae, ***Chatonella antiqua*** (ca ***2000*** cells/ml), was 0.70 ± 0.05 (p g/ml ± SEM). In addition, diazo-reaction positive substances (NO_2) which may degenerate the mucous, was highly concentrated in the cortex (perikaryon) of ***Chattonella antiqua.*** Morphologically, mucocysts, and chloroplats were likewise present in the cortex. Mucocysts were packed with fine fibrous content. Histochemically, the mucocysts were stained with PAS and had an abundance of nitrogen oxides (NO_2). We observed discharge of the fibrous material from the mucocysts. These results suggest that when ***Chattonella antiqua*** is passing between the gill lamellae, NO_x discharged from the mucocysts may act on the mucous, leading to the degeneration and concomitant removal of the mucous coat from gill lamellae.

Key words: Red tide - ***Chattonella antiqua*** - Diazo-reaction - Young yellowtail

Introduction

In the previous work, we found that the disappearance of the mucous eoat, significant loss of mucous goblet cells on the afferent gill ridges, and hyaline degeneration of the mucous cell membrane, and intact pavement cells covering gill lamellae of the young yellowtail had been occured during the exposure to red tide (Shimada et al., 1982, 1983). These morphological changes on the respiratory system may be similar to the features of the mammalian lung after inharation of such substances as ozone and nitrogen oxides (Harris et al., 1971; Roehm et al., 1971; Mustafa and Tierney, 1978). Because of this similarity in morphological changes, it is reasonable to suggest that these substances may be candidates for the degeneration process. The present work was designed to investigate the causative factors responsible for removal of the mucous coat from the gill surface. It is a well-known observation that red tide often causes great damage at dawn to the fish farms, especially to young yellowtails, and less damage in the daytime. In Japan, red tide caused by ***Chattonella*** and ***Heterosigma*** is most common. As the plankton has an abundant chloroplast in the perikaryon, ozone may be produced in the chloroplast by photosynthesis during the daytime. Therefore, if the ozone contributes to these mechanisms, fish death may occur during the daytime. Based primarily on these findings, ozone is not likely related to the fish death. On the other hand, nitrate salt is one of the essential nutritions for these planktons. They use it for the ammonia production by the nitrate reduction, i.e., ammonia is necessary for the nitrate assimilation. Namely, nitrate salt (NO_3^-) is converted to nitrite (NO_2^-) by the nitrate reductase. Then, the nitrate might be further metabolized to nitrogen monoxide (NO-) by the nitrite reductase leading to hydroxyamine (NH, OH). Therefore, in the present work, concentrations of nitrite ion in the medium were estimated by diazo-reaction, and at the same time ***Chattonella antiqua*** stained with azo dye was observed with a light microscope and TEM. Consequently, the structures showing diazo-reaction positive substances were identified from the histological and histochemical point of view and causative factors responsible for the removal of the mucous coat from the gill lamellae were considered.

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Materials and methods

Diazo-reactions

In the present work, diazo-reactions were employed for the estimation of nitrite ions (NO,-) in the medium (sea water) using a modification of Griess's method (Snell and Snell, 1944), commonly used in the field of chemical qualitative and quantitative analysis. The nitrite was determined by diazotizing sulphanic acid and coupling with N-(1-naphtyl) -ethylenediamine to form a highly coupled azo dye, which has a characteristic absorption band at 520 nm. The procedure was as follows: 0.6 g of sulphanic acid was dissolved in 70 ml of hot water. After cooling, 20 ml of 35% HCI was added and total volume was adjusted to 100ml with dist. water. One hundred µl of this solution was added to 1.0 ml of the medium. Thereafter, 0.6 g of N-(1-naphtyl) -ethylenediamine was dissolved in 80 ml of dist. water containing 1.0 ml of 35% HCI. This solution was diluted to 100 ml with dist. water and stored in a brown bottle for a maximum of one week. One hundred µl was added to the medium solution and was allowed to stand for 30 min at 20°C in order to form azo dye, violet in color. Nitrite concentrations were measured with a specrophotometer (Hitachi 320, Japan), after centrifuging at 15,000 rpm for 10 min. The concentration of nitrite ions in the medium of 35 plakton species was measured in the present work. All species examined were incubated for 3 weeks using ESM (Erd-Schreiber Modified) -culture media ($NaNO_3$ 120mg. K_2HPO_4 5mg, Soil extract 50ml, Vitamine B, 100 pg, Vitamine H 1 pg, Fe-EDTA 259 ug, Mn-EDTA 332 µg, and tris (hydroxymethyl) aminomethane 1 g were dissolved into one litter of sea water and the solution was ajusted to pH 8.0.). It was impossible to differentiate nitrogen monoxide (NO-) from nitrite ion (NO,-) by this method. Therefore, we presented the diazo-reaction positive substance as NO, in the present work.

Histological and histochemical preparations for light microscopy

Because of the very thin cortical (pellicular) membrane in ***Chattonella antiqua,*** it is easily ruptured when using 10% formalin (Ono and Takano, 1980). Therefore, after slow centrifugation (ca. 200 rpm) precipitated plankton was transferred into the fixative developed by Yokote and Honjo (1983). This fixative contains 37% formalin, 20% ethanol, 5% Ficol 400 and 4% CaCl,. The plankton was stained with hematoxyline and eosin (H-E) and alcian blue (AB) (pH 2.6) counterstained with PAS. In order to diffentiate chloroplasts from mucocysts in the cytoplasm, a Leitz fluorescence microscope (Orthoplan, Ploemoak 2. L2-1 and K2 were used for the filter combination) was used after fixation to demonstrate the strong auto-fluorescence exhibited by the chloroplasts. Precipitated plakton after diazo-reaction were directly observed with a light microscope without further fixation and staining. Therefore, this method is quite different from the azo-coupling stain usually used in the field of histochemistry in order to detect such amino acids as tryptophan, tyrosine, and histidine in the protein.

Preparation for transmission electron microscopy (TEM)

Chattonella antiqua was fixed with 3.5% glutaraldehyde in 0.1M phosphate buffer at pH 7.0 for 3 hours at 0°C and post-fixed for one day in 1% osmium tetroxide in 0.1M phosphate buffer at pH 7.0 for 2 hours at O°C, dehydrated in graded ethanol solutions and then embedded with Epon resin (Taab Epon 872, England). For the fixation of mucocysts containing glycoprotein, the plankton was fixed with the method of Yokote and Honjo (1983), that is, immediately after adding 1% AB (pH 2.5) to the plankton, fixative containing 3% glutaraldehyde, 5% $CaCl_2$, and 2% $0s0_4$ in 0.1M collidine buffer at pH 7.4 was further added (Roehm et al., 1971). 1% AB was used for the rapid pre-fixation of glycoprotein. We speculate that this dye may prevent the discharge of fibrous material from the mucocysts. Precipitated planktons after diazo-reaction were also observed with TEM following fixation with 3.5% glutaraldehyde and post-fixed in 1% osmium tetroxide.

Results

Diazo-reaction

Table 1 shows the concentration of nitrite ion in the medium and diazo-reaction of the cytoplasm in 35 specie. examined in the present work. The concentration of NO_2- in 1.0 ml of the medium containing ***Chattonella antiqua*** (ca. 2000 cells/ml) was 0.70 ± 0.05 (µg/ml ± SEM). Among the rest, the medium in the ***Gonyaulax polyedra,*** another of the species causing red tide, showed the highest concentration in the nitrite ion.

Histological observations

From the preparations stained with H-E, numerous club-shaped structures were found in the cortex of the ***Chattonella antiqua*** (Fig. 1). At the same time, some other ***Chattonella antiqua*** showed the fibrous content and concomitant disappearance of club-shaped structures in the cortex (Fig. 2). Electron microscopically, the cortex of ***Chattonella antiqua*** fixed with AB, glutaraldehyde and post-fixed with osmium tetroxide contained the mucoapts having a fine fibrous content, many large vacant spaces, and chloroplasts (Fig. 4). On the other hand, no mucocyst could be seen in the preparations fixed with glutaraldehyde and post-fixed with OsO_4 (Fig. 3).

Histochemical observations

Followi..g diazo-reaction, the rod-shaped structures showing high electron density were found around the chloroplasts (Fig. 5) by TEM preparation. Relevant to the diazo-reaction, the cortex (perikaryon) of ***Chattonella antiqua*** was most deeply stained with azo dye (Fig. 6). With fluorescence microscopy, the chloroplats showed a strong red fluorescence, while no fluorescence appeared in the club-shaped structures (Fig. 7a, b). On the other hand, the cortex of ***Chattonella antiqua*** was weakly stained blue-red by the AB (pH 2.6) and PAS stain (Fig. 8). In the cortex, oval structures showing highly PAS positive staining were present (Fig. 8). In these preparations the oval structures were observed discharging the fibrous material, which was weakly stained with blue-red (Fig. 9).

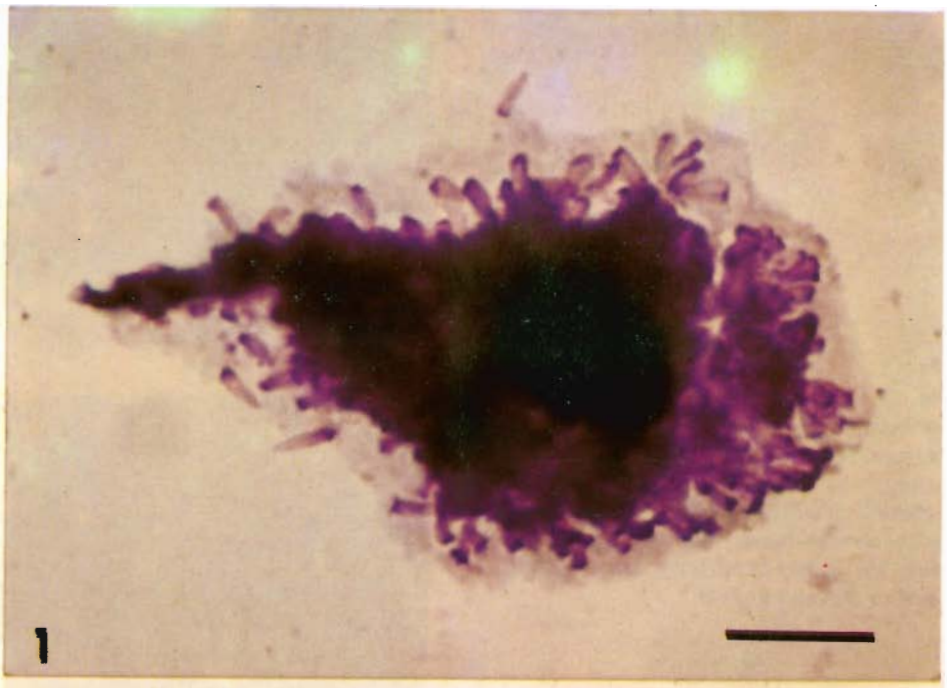


Fig 1. *Chattonella antiqua* stained with H-E. Bar indicates 10 µm. Note numerous club-shaped structure (mucocysts) in the cortex.

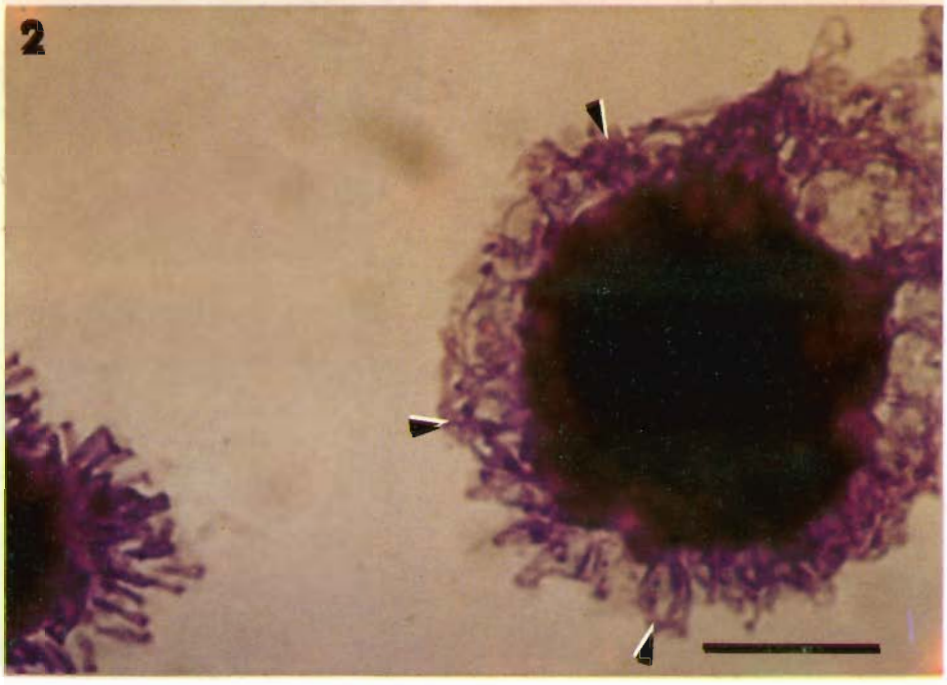


Fig. 2. *Chattonella antiqua* stained wit H-E. Bar indicates 10µ m. Note the fibrous content (arrows) and concomitant disappearance of club-shaped mucocysts in the cortex.

Rg. 3. Electron micrograph of *Chattonella antiqua* fixed with glutaraldehyde and post-fixed with osmium tetroxide. Bar indicates 10 µm. Arrows indicate the chloroplasts. Nup.: Nucleus

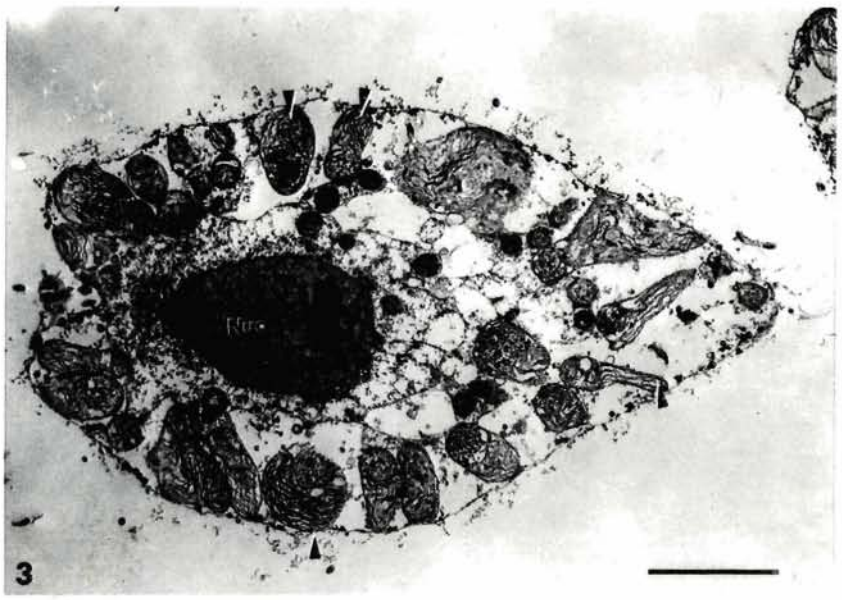


Fig. 4. Electron micrograph of *Chattonella antiqua* fixed with 1% alcian blue, glutaraldehyde and post-fixed with osmium tetroxide. See details in materials and methods. Bar indicates 10 µm. Short arrow heads indicate the mucoysts fixed with 1% alcian blue. Long arrow heads present the chloroplast.

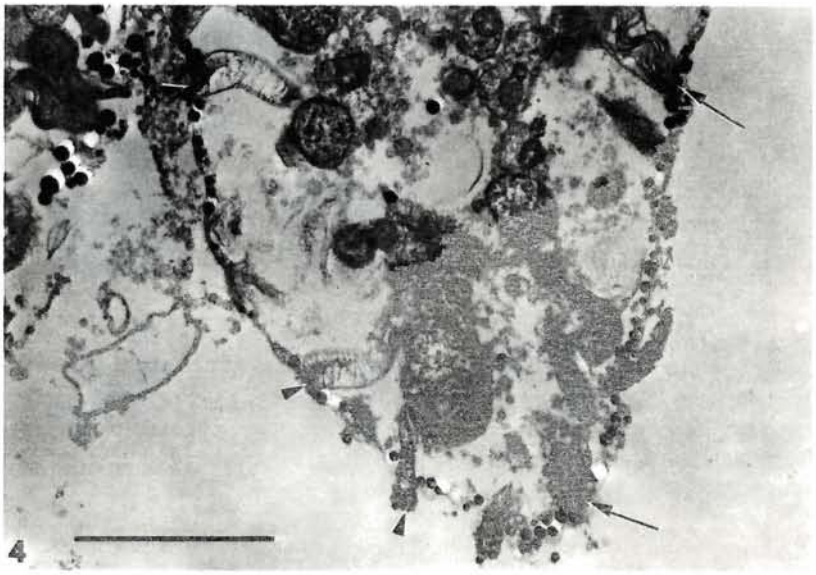
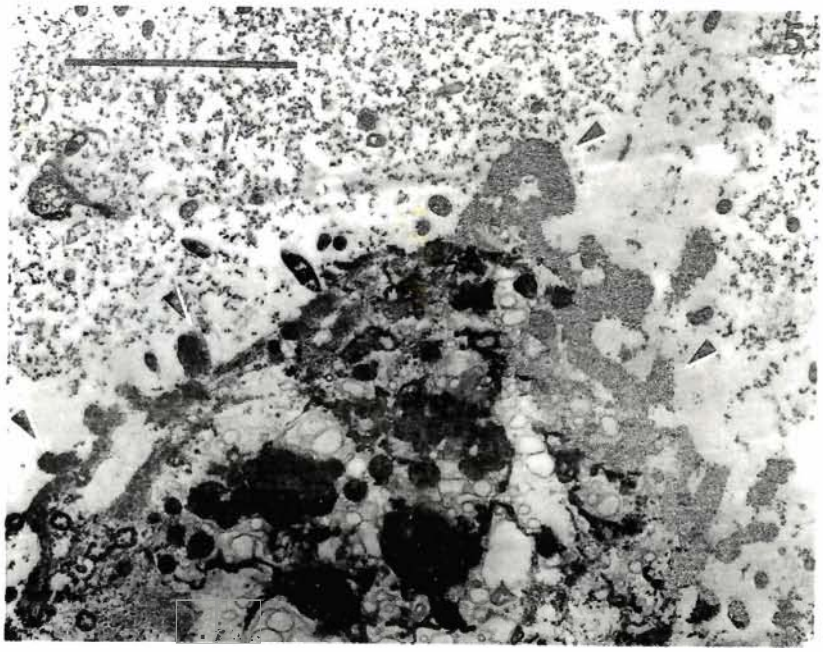


Fig. 6. Electron micrograph of the plankton treated with diazo-reaction. Bar, indicates 10 µm. Note the mucocysts (short arrow heads) showing high electron density.

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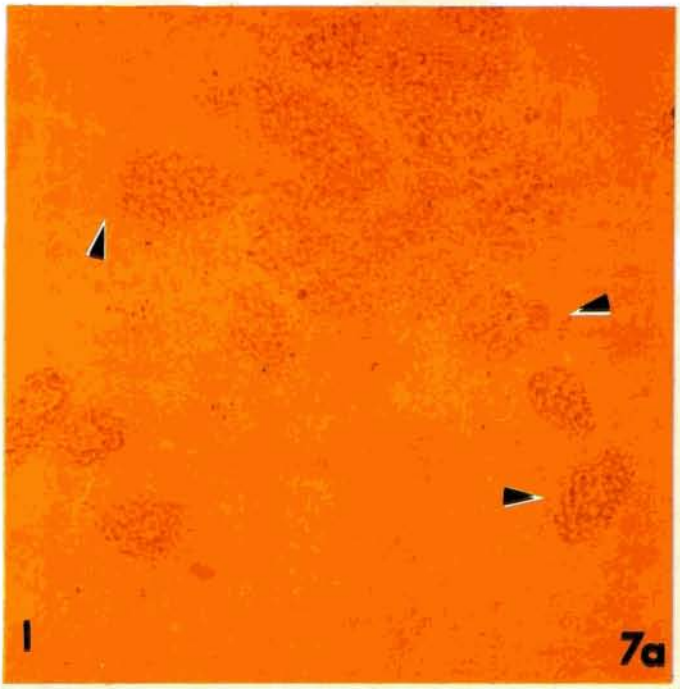
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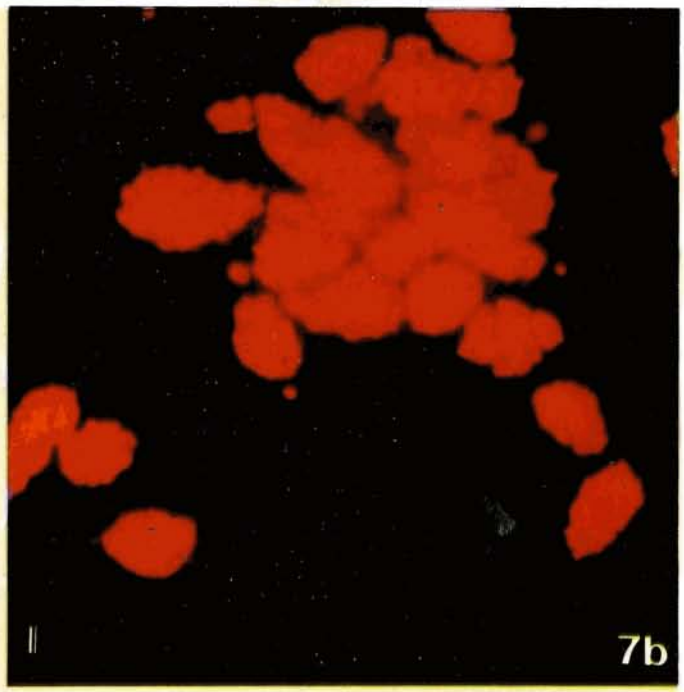
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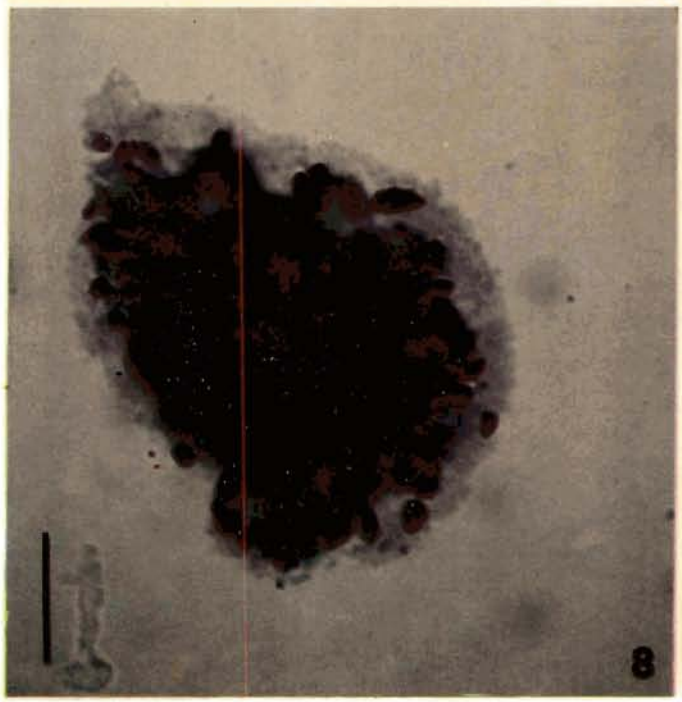
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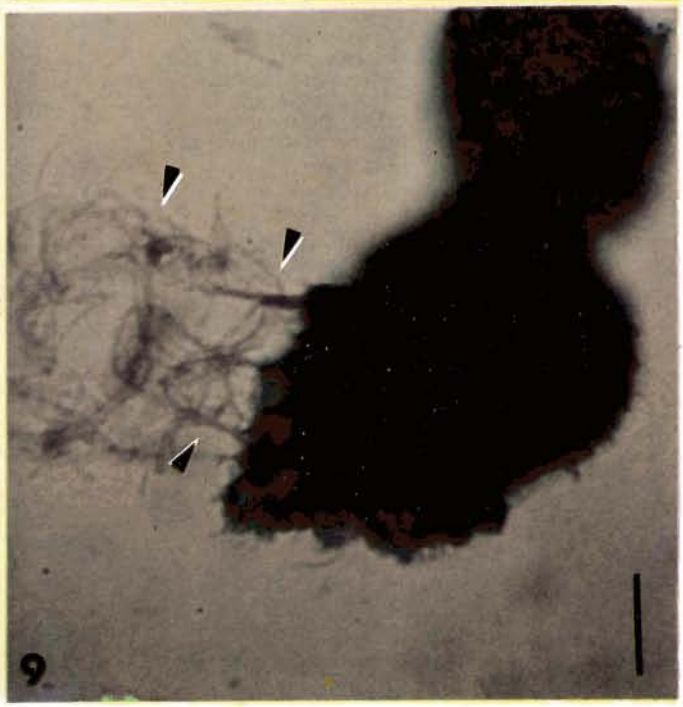
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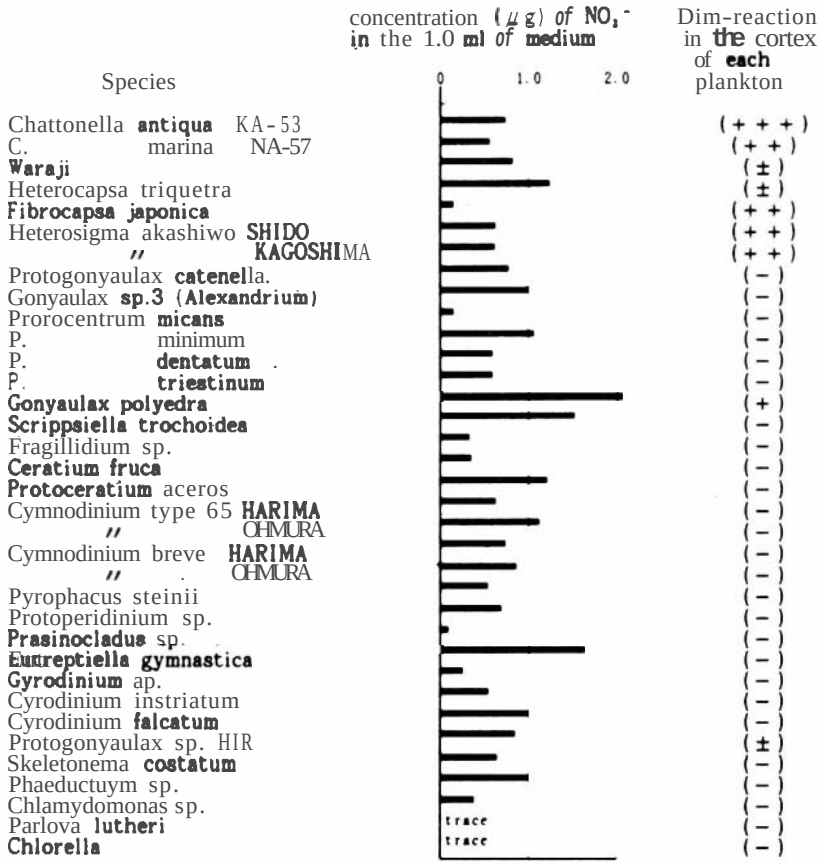
Fig. 6. Planktons (***Chattonella antiqua***) stained with diazo-reaction. Bar indicates 10 **µm.** Note the strong positive reaction (red in color) in the cortex.

Rg. 7. a. Planktons and club-shaped **mucocysts** (arrows). Bar indicates 10 **pm. b.** Fluorescencemicrograph of figure **7a.** Bar indictes 10 **µm.** Note the strong red fluorescence in the chloroplast, while can not be seen fluorescence in the club-shaped mucocysts.

Fig. 8. Planktons stained with alcian blue (pH 2.6) and PAS. Bar indicates 10 µ m. Note the oval structures showing PAS positive staining in the cortex.

Fig. 9. Planktons stained with alcian blue (pH 2.6) and PAS. Bar indicates 10 µ m. Arrow heads indicate the fibrous materials discharging from the oval structures.

Table 1. Concentrations of nitrite ion in the medium and azo-reaction in the cortex of 35 plankton species.



All planktons were incubated at **December** 26. 1983 and the concentrations of NO_2^- were measured at January 19. 1984. There were 3 **determinations** for **each** species.

NO_2^- concentration in the sea **water** containing *Chattonella antiqua* KA-53 were 0.70 ± 0.05(µg/ml ± SEM).

(+ + +) : intensively stained with diazo-reaction in the **cortex.**
(+ +) : somewhat strongly stained with "
(+) : moderately stained with "
(±) : weakly stained with "
(-) : not stained with "

Discussion

Twothousand cells of *Chattonella antiqua* per ml provides a sufficient cell density for killing young yellowtails within one hour (Shimada et al., 1983). High concentrations of nitrite ion (NO, -) were detected in the medium and the cortex of these planktons by the method of diazo-reaction used in the present work. On the other hand, the cortex of *Chattonella antiqua* contained mucocysts, and chloroplasts. To our knowledge, this is the first demonstration of normal structure in preparations of *Chattonella antiqua* by TEM. Following diazo-reaction, club-shaped structures showing high electron density were found around the chloroplast. These structures were undetectable in the preparations untreated with diazo-coupling. Therefore, club-shaped structures are supposed to be mucocysts from the morphological point of view. It was demonstrated by fluorescence microscopy that the chloroplast showed a strong red fluorescence, while no fluorescence appeared in the club-shaped structures present in the cortex. Therefore, the numerous club-shaped structures found in the cortex by the H-E stain might be undischarged mucocysts which are a saclike, membrane bound structures containing crystalline materials (Tokuyasu and Scherbaum, 1965, Wunderlich and Speth, 1972, Mignot, 1976). Histochemically, the cortex (perikaryon) may contain both acid and neutral glycoproteins. In regard to the mucocysts, most of them were club-shaped, as seen in the H-E preparations whereas some were spherical and highly PAS positive. The latter appeared in fewer numbers compared with the club-shaped structures. From these spherical mucocysts a fine fibrous material was discharged. According to Bresslan, discharge can be induced by addition of different stains, pH or temperature changes (Satir et al., 1973). Events of discharge occur within a few hundred miliseconds (Satir et al., 1973). Once it was discharged, the material was difficult to discern as it had almost the same density as the background and could no longer be identified by H-E or PAS staining. We speculate that during this process NO, may be also discharged from the mucocyst. From these data, it is reasonable to suggest that when *Chattonella antiqua* is passing between the gill lamellae, NO, discharged from the mucocysts may act on the gill mucous,, resulting in the degeneration of mucous coat. The medium in the *Gonyaulax polyedra* showed the highest concentration in the nitrite ion and the cortex was moderately stained with diazo-reaction. Therefore, it is easily suggested that this plankton may cause great damage to the the fish farm. Fortunately, red tide caused by this plankton has not yet been found in Japan.

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References

Harris M.J., Herp A. and Pigmen W. (1971). Depolymerization of polysaccharides through the generation of free radicals at a platinum surface: Free radical oxidation. Arch. Biochem. Biophys. 142, 615-622.

Hausmann K. (1973). Spindeltrichocysten und Mucocysten in Schragbeschatteten und negativ-kontrastierten Suspensionspreparaten. Ann. Stat. Biol. Bess-en-Chandesse, 7, 331-354.

Mignot J-P. (1976). Complements a L'etude des Chloromonadines Ultrastructures de Chattonella subsala Biecheler Flagelle D'eau Saumatre. Protistologia 2, 279-293.

Mustafe M.G. and Tierney D.F. (1978). Biochemical and metbolic changes in the lung with oxygen, ozone, and nitrogen oxide toxicity. Amer. Rev. Resp. Dis. 118, 1061-1090.

Ono C. and Takano H. (1980). Chattonella antiqua (HADA) comb. nov., and its occurrence of the Japanese coast. Bull. Tokai Reg. Fish. Res. Lab. 102, 93-100.

Roehm J.N., Hadley, J.G. and Menzel, D.B. (1971). Oxidation of unsaturated fatty acids by ozone and nitrogen dioxide. Arch Environ. Health 23, 142-148.

Satir B., Schooley C. and Satir P. (1973). Membrane fusion in a model system. Mucocyst secretion in *Tetrahymena.* J. Cell Biol. 56, 153-196.

Shimada M., Murakami T.H., Doi A., Abe S., Okaichi T. and Watanabe M. (1982), Morphological and histochemical study on gill primary lamellae of the teleost, *Seriola quinqueradiata,* exposed to sea bloom. Acta histochem. cytochem. 15, 497-507.

Shimada M., Murakami T.H., Imahayashi T., Ozaki H.S., Toyoshima T. and Okaichi T. (1983). Effects of sea bloom, *Chattonella antiqua,* on gill primary lamellae of the young yellowtail, *Seriola quinqueradiata.* Acta histochem. cytochem. 16, 232-244.

Snell F.P. and Snell C.T. (1944). Colorimetric methods of analysis. Vol. 1, Van Nostrand, pp 644-745.

Tokuyasu K. and Scherbaum O.H. (1965). Ultrastructure of mucocysts and pellicle of Tetrahymena pyriformis. J. Cell Biol. 27, 67-81.

Wunderlich, F. and Speth, V. (1972). Membranes in *Tetrahymena.* I. The cortical patten. J. Urtrastructure Res. 41, 258-268.

Yokote M. and Honjo N. (1983). Clycocalyx in the cell surface of *Chattonellaantiqua.* Japan. Soc. Sci. Fisch. Abst. pp. 57.

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