Histological Analysis of the Mechanisms of *Chattonella*-Induced Hypoxemia in Yellowtail[†]

Atsushi Ishimatsu,^{*1,††} Mamoru Sameshima,^{*1,†††} Akihiko Tamura,^{*1}
and Tatsuya Oda^{*2}

^{*1}Nomo Fisheries Station, Nagasaki University, Nomoaki, Nagasaki 851-05, Japan

^{*2}Faculty of Fisheries, Nagasaki University, Bunkyo, Nagasaki 852, Japan

(Received May 29, 1995)

The effect of *Chattonella* exposure on gill histology of yellowtail was examined to clarify the following questions: (1) Is the branchial edema, previously reported as the main branchial lesion in response to *Chattonella* exposure, truly caused by *Chattonella* cells or a secondary histological alteration by fatal hypoxemia during the exposure? (2) Is the branchial edema responsible for the rapid decline of blood oxygen levels during the early stages of *Chattonella* exposure? Fish dying from *Chattonella* exposure showed many types of lesions, especially severe epithelial separation in the lamellae and filaments. In contrast, the gills of fish dying from environmental hypoxia showed very few lesions and were histologically hardly discernible from the control gills. This demonstrated that the branchial edema was induced by *Chattonella* and not by hypoxemia. On the other hand, the epithelial separation had not developed when the tissues were sampled immediately after the onset of hypoxemia. Therefore, the edema cannot be the cause of hypoxemia. The only histological change observed in the gills sampled at blood oxygen drop was blockade of interfilamental spaces by mucus. Based on these results and the previous findings of oxygen radical production by *Chattonella*, we hypothesized that oxygen radicals, released from *Chattonella* cells, stimulated mucus cells in the gills, and the secreted mucus, possibly plus *Chattonella* cells trapped within the mucus, destroyed the gas exchange capacity of the gills by shunting respiratory water current away from the lamellae.

Key words: *Chattonella*, yellowtail, gill, histology, mucus, edema, epithelial separation

Blooming of toxic phytoplankton species, *Chattonella* spp., has repeatedly caused massive mortalities of cultured fish, particularly the yellowtail *Seriola quinqueradiata*, during the last two decades in Japan.¹⁾ Suffocation is generally supposed to be the direct cause of the fish death by this plankton species,^{2–6)} although limited information is available regarding the effects of *Chattonella* on the oxygen transport system of fish. We previously studied the effects of *Chattonella* on the physiological state of the yellowtail, and concluded that the rapid drop of arterial oxygen partial pressure (P_{aO_2}) occurring at the early phase of exposure was the likely proximate cause of fish mortality.^{7–9)} However, how *Chattonella* induce hypoxemia remains unknown.

A few papers have reported histological changes of the gills induced by *Chattonella*. Shimada *et al.*¹⁰⁾ found the apparent disappearance of mucus cells in the yellowtail gill exposed to *C. antiqua*. They proposed that the loss of mucus layers impaired osmoregulatory function. The resultant osmotic imbalance would lead to edema formation which in turn reduces the gas exchange capacity of the gills. Subsequently, Toyoshima *et al.*¹¹⁾ observed that *C. antiqua* caused histological alterations of chloride cells in the yellowtail gill. They also related the finding to edema formation. However, the cause-and-effect relationship be-

tween these histological and physiological findings has never been ascertained. There are two unanswered questions that we think are crucial in considering the mechanisms of fish kill by *Chattonella*. First, it is not entirely clear if the observed histological changes are truly attributable to the direct effect of *Chattonella* exposure, or induced secondarily by fatal hypoxemia. Second, it is not known if the branchial edema is truly responsible for the drop of blood oxygen levels during *Chattonella* exposure.

The present study was designed to clarify the above two questions. To answer the first question, we compared the gills of fish dying from *Chattonella* exposure and environmental hypoxia. The second question was investigated by taking gill tissue samples from chronically cannulated yellowtail as soon as P_{aO_2} started to decline after the onset of *Chattonella* exposure. If the branchial edema does cause hypoxemia, then the edema must be present when P_{aO_2} is lowered during the exposure (sometimes within 15 min, see Tsuchiyama *et al.*⁹⁾). Speare and Ferguson¹²⁾ demonstrated that artifacts like epithelial separation (an histological manifestation of edema) can quickly develop in fish gill, depending on sampling methods and fixatives used. Great care was therefore taken to preserve the histology of the gills at the moment of sampling.

The results demonstrated that (1) epithelial separation

[†] Contribution from Nomo Fisheries Station, Nagasaki University, No. 138.

^{††} Send reprint requests to: Dr. Atsushi Ishimatsu, Nomo Fisheries Station, Nagasaki University, Nomo 714, Nomoaki, Nagasaki 851-05, Japan.

^{†††} Present address: Kumamoto Prefectural Fisheries Research Center, Ohayo 2450-2, Amakusa, Kumamoto 869-36, Japan.

and other morphological changes, observed in fish dying from *Chattonella* exposure, were clearly induced by *Chattonella* and not by severe hypoxemia. More importantly, (2) these changes were not observed at the time of P_{ao_2} drop and cannot therefore be the cause of hypoxemia. The only histological alteration detectable at the time of P_{ao_2} drop was a considerable accumulation of mucus between the filaments and lamellae. Based on these findings, we propose blockade of the respiratory flow through the gills by mucus to be the main mechanism for the rapid depletion of blood oxygen during the early phase of *Chattonella* exposure.

Materials and Methods

Experimental Fish

Fish used in this study were mostly obtained from a local aquaculturist, but some fish used in experiment 2 were also kindly offered by Aquaculture Laboratory, Nagasaki Prefectural Institute of Fisheries. No distinction was made between the fish from the two sources. Fish were maintained in net pens and fed minced sardines every other day. Two days before the experiments, fish were transferred to a 1,000 l indoor tank and kept without feeding as previously described.⁷⁾ Sea water temperature was kept at $25 \pm 1^\circ\text{C}$. Means and standard errors (S.E.) of body weights were 276 ± 14 g for experiment 1 and $1,134 \pm 73$ g for experiment 2 (see below), respectively.

Plankton Culture

Chattonella sp. isolated in Kumamoto in 1990 were used in experiment 1. *Chattonella marina* isolated in Kagoshima in 1985 were used in experiment 2. Both strains were batch-cultured using ESM medium (Erd-Schreiber modified; NaNO_3 120 mg, K_2HPO_4 5 mg, vitamin B_{12} 1 μg , biotin 1 μg , thiamine HCl 100 μg , Fe-EDTA 259 μg , Mn-EDTA 332 μg , Tris (hydroxymethyl) aminomethane 1 g, soil extract 50 ml, sea water 950 ml, pH 8.0) as previously described.⁷⁾ These two strains showed the same growth characteristics, morphology and toxicity to yellowtails.

Experimental Protocols

1. Experiment 1

This experiment was designed to investigate whether the gill lesions previously reported during *Chattonella* exposure (see Introduction) were truly caused by the algae or by hypoxemia. For this purpose, we compared the gill histology of fish dying from *Chattonella* exposure and from environmental hypoxia. Fish were transferred to 100 l polycarbonate tanks with flow-through sea water supply 12 h before the experiments. Each tank contained six fish. Sea water supply was stopped immediately before the experiments. The fish were subjected to the following three treatments. To the first tank, 10 liters of ESM medium was added at the onset of an experiment (control). To the second tank, 10 liters of *Chattonella*-containing medium (approximately 40,000 cells/ml) were added to attain a final cell concentration of 4,000 cells/ml. Cell concentration was determined at 10, 30, and 60 min by counting cell numbers under a microscope. Dissolved oxygen levels of these two tanks were maintained at near saturation levels by bubbling the water with pure oxygen. To the third tank, 10

liters of ESM medium was also added, and dissolved oxygen level was lowered by nitrogen bubbling such that the time to death by the treatment was comparable to that in the *Chattonella*-exposed group. Fish were killed by a sharp blow on the head when they were moribund (the *Chattonella*-exposed and the hypoxia groups) or at the end of an experiment (8 h, control). The first gill arch on the left side was immediately cut, rinsed in diluted isotonic sea water, and fixed in Bouin's fixative. The time from killing to fixation was less than 20 s. Samples were discarded if sampling was delayed. Water temperature was kept at $25 \pm 1^\circ\text{C}$. Light intensity was 5,000 to 8,000 lx. Water pH was adjusted to 8.2 by adding 0.1 N NaOH when necessary.

2. Experiment 2

This experiment was designed to clarify the gill histology of the fish when blood oxygen level was lowered by *Chattonella* exposure. Fish used in this experiment ($N=6$ for both control and *Chattonella*-exposed groups) were anesthetized and fitted with chronic dorsal aortic cannulae as previously described.⁷⁾ Fish were allowed to recover for 24 h in a darkened Plexiglass chamber continuously supplied with sea water (for the details of the setup used in experiment 2, see Ishimatsu *et al.*⁷⁾). Water temperature was kept at $25 \pm 1^\circ\text{C}$ as above. For control, gills were sampled after blood gas measurements under resting conditions. Fish were quickly lifted out of the chamber with the aid of a plastic net placed inside it and killed by piercing the cranium with an eyeleteer. The heart was cut to prevent contamination of the gills with blood. The first gill arch on the left side was trimmed off and immediately placed in Bouin's fixative without rinsing. The time after fish removal from the chamber to fixation was within 20 s. Samples were discarded when sampling was delayed or the gills were covered with excessive amounts of blood. *Chattonella* exposure was conducted using the same protocol as previously described.⁷⁾ Plankton concentration was kept at 4,000 cells/ml. Gills in this group were sampled when P_{ao_2} fell below 30 mmHg, at which value secondary physiological disorders became manifest during *Chattonella* exposure.⁷⁾ The P_{ao_2} and arterial pH (pHa) were measured by an IL-213 blood gas analyzer (Instrumentation Laboratory, Mass.) thermostatted to 25°C .

Morphometric Evaluation

1. Experiment 1

The gills were fixed in Bouin's solution for 15 to 17 h, transferred to 80% ethanol, and left for 26 to 50 h. They were then placed in 5% EDTA (pH 7.0) for 24 h for decalcification, dehydrated, and embedded in paraffin. Sections (4 μm thick) were made on a plane parallel to the long axis of the filament and perpendicular to the surface of the filament (plane 1). These were then stained with hematoxylin and eosin. Six middle portions of five to six filaments were randomly chosen for each fish, photographed with an Olympus light microscope, and analyzed for the following types of lesions. Epithelial separation (intraepithelial edema) of the secondary lamellae was defined after Speare and Ferguson¹²⁾ and Skidmore and Tovell.¹³⁾ The extent of epithelial separation was semi-quantified as follows. First, the ratio of epithelial portion where the epithelium was separated from the underlying basal lamina-pillar cell com-

plex to the total perimeter of a lamella was calculated. Second, percentage frequencies of the lamellae with less than 5%, 5–20%, 20–80% and higher than 80% of epithelial separation were calculated for each fish. The part where the epithelium was lost (epithelial rupture) was included in this category (epithelial separation), but was also assessed independently. The extent of epithelial rupture was expressed as the ratio of the number of the lamellae which showed this type of lesion to the total number of lamellae observed. Erythrocyte numbers in the secondary lamellae were estimated by counting the number of nuclei in a section as an index for branchial congestion. Hypertrophy and hyperplasia were evaluated according to Speare and Ferguson,¹²⁾ and their extent was evaluated as for epithelial rupture. Mean values of these morphometric parameters were obtained for each fish, and overall means and S.E.s (means and S.E.s of the individual mean values) were calculated. Numbers of lamellae analyzed were 1,027 from 6 fish in the control, 835 from 5 fish in the *Chattonella*-exposed group, and 769 from 5 fish in the hypoxia group.

2. Experiment 2

Sectioning was made on three planes in this experiment. Plane 1 was the same plane as in experiment 1. Plane 2 was perpendicular to both the long axis of the filament and the surface of the filament (cross section of the filament). Plane 3 was parallel to both the long axis of the filament and the surface of the primary filament. Middle portions of 36 filaments taken from the middle part of the gill arches were analyzed in plane 1 for each fish. Sections cut in plane 2 were stained with either Alcian blue and the periodic acid-Schiffs reagent (PAS) or Alcian blue, PAS, hematoxylin and eosin, and used to observe mucus cells on the afferent (trailing) and efferent (leading) edges of the filament and mucus substances secreted from the cells (44 to 50 filaments and interfilamental spaces were observed for each fish). We defined mucus blockade of interfilamental spaces as the state where mucus spans the full distance between the neighboring filaments when viewed in plane 2. Ratios of the number of interfilamental spaces blocked by mucus to the total number of spaces observed were calculated for both the afferent and efferent edges. Those cut in plane 3 were stained with hematoxylin and eosin, and used to observe adductor and abductor muscles and the afferent and efferent filamental arteries at their origins from the branchial arteries. The extent of epithelial separation and erythrocyte number per lamellae were estimated as in experiment 1.

Statistics

One-way ANOVA followed by Bonferroni test for pairwise comparison was applied in experiment 1. Bartlett's test was conducted to check the homogeneity of variance. A Student *t*-test or Cochran-Cox test was also performed in experiment 1 and 2 where appropriate. Data are expressed as mean $\pm$ S.E. wherever possible.

Results

Experiment 1

All fish in the control group survived 8 h of experiment and showed no signs of distress. All fish in the *Chattonella*-exposed group died with a mean death time of

Fig. 1. Gill filaments of yellowtail in the control (A), the *Chattonella*-exposed group (B), and the hypoxia-exposed group (C) in experiment 1.

Note a considerable degree of epithelial separation in (B) and its absence in (A) and (C). Gill samples (B) and (C) in this experiment were taken near the death. Bars represent 100 μ m.

37.0 $\pm$ 5.0 min. Sea water oxygen level in the hypoxia group fell to 50% of the saturation level at 10 min, 25% at 25 min, and stabilized at 15% at 60 min and thereafter. Fish in the hypoxia group died with a mean death time of 44.8 $\pm$ 3.5 min. There was no significant difference between death time of the two groups ($p > 0.05$, *t*-test).

Figure 1 shows microscopic photographs of the gills sampled in this experiment. A marked difference in gill morphology is obvious between the control (Fig. 1A) and the

Chattonella-exposed fish (Fig. 1B), while the gills of the hypoxia-exposed fish (Fig. 1C) were quite similar to those of the control. In the *Chattonella*-exposed fish (Fig. 1B), lesions were not restricted to the lamellae. Filamental epithelium also showed a considerable degree of epithelial separation and most cells in the filamental epithelium, including chloride cells, appeared to be necrotic.

Morphometric analysis supported the above qualitative evaluation between the gills of the three groups. Figure 2A compares percentage frequencies of the secondary lamellae that were classified into 4 degrees of epithelial separation between the groups. Apparently, the pattern was similar between the control and the hypoxia groups in that most lamellae showed less than 5% of separation ($95.4 \pm 0.7\%$ for the control; $83.7 \pm 6.1\%$ for the hypoxia group), although there was a moderate increase in the extent of epithelial separation in the latter. In contrast, fish in the *Chattonella*-exposed group showed a very high incidence ($61.5 \pm 7.2\%$) of lamellae with $>80\%$ of epithelial separation. The distribution pattern of erythrocyte numbers within a lamella in the *Chattonella*-exposed group was also quite different from those in the control and the hypoxia groups (Fig. 3). Mean erythrocyte numbers were 17.8 ± 0.6 cells/lamella in the control and 14.8 ± 0.6 in the hypoxia group, whereas lamellae in the *Chattonella*-exposed group showed a wider distribution pattern ranging from less than 10 to more than 150 cells/lamella. The extent of epithelial rupture in the *Chattonella*-exposed group ($33.4 \pm 6.5\%$) was significantly higher than that in the control ($7.6 \pm 2.7\%$) and the hypoxia groups ($2.3 \pm 0.9\%$) ($p < 0.01$, Bonferroni test). Hypertrophy in the control fish showed a small but significantly higher incidence than in the *Chattonella*-exposed group ($p < 0.05$, Bonferroni test). There was no significant difference in the extent of hyperplasia between the groups.

Experiment 2

Arterial oxygen pressure (P_{aO_2}) rapidly declined after the onset of *Chattonella* exposure (Fig. 4). Mean time for the P_{aO_2} to decrease to below 30 mmHg was 27.5 ± 4.6 min ($N=6$). The pH_a was not significantly different between the pre-exposure and the last samples (7.863 ± 0.017 for the pre-exposure, 7.844 ± 0.028 for the last sample, $p > 0.05$, paired t -test).

When the gills cut in the plane I were compared between the control (Fig. 5A) and the *Chattonella*-exposed groups (Fig. 5B), they appeared almost indistinguishable from each other, if it were not for *Chattonella* cells occasionally trapped between the filaments or lamellae in the latter group. The gills of the control fish showed low occurrence of lesions, indicating that the cannulation procedure only minimally affected the gill histology of these fish (cf. Figs. 1A and 5A). The percentage frequency of lamellae with epithelial separation in the control fish in this experiment was higher than that of the control fish in experiment 1, but lamellae with less than 5% of separation still occupied $82.0 \pm 4.3\%$ of the total (Fig. 2B). The lamellae of the *Chattonella*-exposed fish sampled at the time of P_{aO_2} drop showed an almost identical pattern of percentage frequency of epithelial separation to that in the control fish of this experiment (Fig. 2B). There was no significant difference in erythrocyte number within a lamella between the groups (22.6 ± 2.1 for control and 21.9 ± 2.3 for *Chattonella*-exposed, $p > 0.05$, t -test). Epithelial rupture, hypertrophy and nuclear pyknosis were seldom observed in the gills of the two groups. There were no apparent lesions in both the abductor and adductor muscles in the gills of the *Chattonella*-exposed group, nor was there any abnormality (vasoconstriction or vasodilation) observed in the filamental arteries at their basal portions.

A marked difference in gill morphology was evident

Fig. 2. A comparison of percentage frequencies of the secondary lamellae showing epithelial separation in less than 5%, 5–20%, 20–80%, and higher than 80% of the total perimeter of a lamella in experiment 1 (A) and experiment 2 (B).

Vertical bars represent S.E. N is 6 for the control, 5 for the *Chattonella*-exposed and the hypoxia-exposed groups in experiment 1, and is 6 for both the control and the *Chattonella*-exposed groups in experiment 2. For details, see the text.

Fig. 3. Histograms of erythrocyte numbers per lamella in the control (A), the *Chattonella*-exposed (B) and the hypoxia-exposed groups (C) (experiment 1).

Vertical bars represent S.E. N is 6 for the control, 5 for the *Chattonella*-exposed and the hypoxia-exposed groups. For details, see the text.

Fig. 4. Changes in arterial P_{O_2} during *Chattonella* exposure in experiment 2.

Gills were sampled when arterial P_{O_2} fell below 30 mmHg (marked by asterisks).

Fig. 5. Gill filaments of yellowtail in the control (A), and the *Chattonella*-exposed group (B) in experiment 2.

Gill samples of the *Chattonella*-exposed group were taken as soon as the arterial P_{O_2} fell to 30 mmHg. Arrowheads indicate *Chattonella* cells. Note the absence of epithelial separation in (B). Bars represent 100 μm.

when the gills cut in plane 2 were compared between the groups (cf. Fig. 6A for the control and Fig. 6B for the *Chattonella*-exposed fish). Frequent blockade of the interfilamental spaces was found in the gills of the *Chattonella*-exposed fish, whereas less occurrence of blockade was evident in the control gill. Table 1 compares the occurrence of mucus blockade of the interfilamental spaces in the control and the *Chattonella*-exposed fish. More than 50% of the interlamellar spaces on the afferent side were blocked by mucus in three fish in the *Chattonella*-exposed group. The other three fish showed 3, 35, and 49%. Three control fish showed no incidence of mucus blockade on the afferent side at all, two fish showed 20–30%, and a value of 76% was found in one fish. In contrast, there were mostly less than 10% of mucus blockade on the efferent side in both the control and the *Chattonella*-exposed fish. *Chattonella* cells were occasionally trapped within the mucus (Fig. 6C), although we did not quantitatively analyze the gill samples in this respect.

Discussion

Critiques of the Technique

Histological analysis should form an integral part of the

Fig. 6. Cross sections of yellowtail gill filaments obtained in the control (A) and the *Chattonella*-exposed group (B) in experiment 2.

Note that mucus blocked interfilamental spaces on the afferent side in (B). Micrograph (C) shows several *Chattonella* cells (arrowheads) in a mass of mucus trapped between the filaments of the *Chattonella*-exposed group in experiment 2. Bars represent 500 μm in (A) and (B), and 100 μm in (C).

investigation of the mechanisms of fish kill by *Chattonella*. Several investigators therefore reported histological changes induced in fish gills by *Chattonella* exposure (see Introduction for references). The mechanisms of fish kill may not be easily postulated from the results of these histological studies because of the following two uncertainties. First, a detailed account was not always given as to the

method of sample processing, and it is known that fish gills easily incur artifactual alterations.¹²⁻¹⁵ Epithelial separation is the most commonly observed histological change, but it seems possible that the reported frequent occurrence of epithelial capillary separation is possibly due to poor handling of the samples. Second, the timing of tissue sampling has never been related to physiological measurements.

In the present study, we treated the samples in a strictly consistent way, keeping the delay from killing the animals to fixing to less than 20 s. Epithelial capillary separation and other types of lesions were observed with only a low percentage in the control samples of experiment 1, indicating that artifacts were kept minimal, if any (Fig. 2A). Speare and Ferguson¹² also demonstrated that if the time delay between killing the fish and fixation was set to 20 s, epithelial separation and other types of lesions rarely occurred. The cannulation procedure also did not affect gill histology to any extent (cf. Fig. 2A and 2B, see also Figs. 1A and 5A). We did not find frequent lamellar dilation as reported for trout killed by a sharp blow on the head or decapitation.¹⁶

Comparison of the Gills in Moribund Chattonella-exposed and Hypoxia-exposed Fish (Experiment 1)

Striking histological changes were found in fish dying from *Chattonella* exposure as compared with the control fish (Figs. 1-3). In contrast, the gills of the hypoxia-exposed fish were histologically almost indiscernible from the control gills. These facts indicate that the observed histological alterations by *Chattonella* were not caused by hypoxic death but by direct influences of the algae.

Extensive epithelial capillary separation and necrosis of both the filamental and lamellar epithelia were consistent with the results of Toyoshima *et al.*¹¹ where the gill samples were taken immediately after death. Endo *et al.*⁴ also stated that the gills taken from moribund fish that had been exposed to *Chattonella* at a concentration of 4,000 cells/ml for 60 min showed edema and hypertrophy of the epithelia in many secondary lamellae. We also noticed numerous necrotic chloride cells as reported by Toyoshima *et al.*¹¹ Thus, our results on the *Chattonella*-exposed fish in experiment 1 were generally in good agreement with those previous findings in this respect. These lesions were clearly caused by *Chattonella*, although their significance in fish kill mechanism by the algae remains unclear. Soivio and Tuurala¹⁷ found that environmental hypoxia induced vascular distention, shortening of diffusion distance and swelling of red blood cells in rainbow trout. Branchial lesions as found in the *Chattonella*-exposed fish in experiment 1 were absent from the trout gills. Secondary lamellae of the hypoxic yellowtail appeared to be thinner than the control ones, though resolution of our light microscopic observation precluded a definite statement (Fig. 1). A marked difference was also observed for red blood cell count per lamella (Fig. 3). Ferguson¹⁴ ascribed aneurysm of the lamellae to acute gill injury from chemical or physical trauma. It is conceivable that the lamellar engorgement resulted from cardiovascular disorder that developed toward the death.⁷

Morphology of the Gills at the Time of Po_2 Decline (Ex-

Table 1. Mucus blockade of the interfilamental spaces in the control and the *Chattonella*-exposed fish when P_{aO_2} decreased to 30 mmHg (Experiment 2)

Control	Fish #4	Fish #5	Fish #8	Fish #9	Fish #24	Fish #25
Afferent side	0.0	0.0	75.6	0.0	29.2	21.1
Efferent side	0.0	0.0	0.0	0.0	2.0	10.5
<i>Chattonella</i> -exposed	Fish #16	Fish #17	Fish #18	Fish #19	Fish #20	Fish #23
Afferent side	35.2	95.2	2.8	66.1	48.4	75.0
Efferent side	7.1	12.8	0.0	4.4	6.7	3.7

Values represent percentages of blocked interfilamental spaces. Afferent and efferent sides are the side where the afferent and efferent filamental arteries run within the filament, respectively.

periment 2)

In sharp contrast to the gill histology found in the *Chattonella*-exposed fish in experiment 1, the gills sampled at the time of P_{aO_2} decline were almost indistinguishable from the control gills (Fig. 5). There was a very low incidence of epithelial separation and other types of histological alterations. Erythrocyte numbers in the secondary lamellae were almost identical between the *Chattonella*-exposed and the control fish in this experiment. These results clearly indicate that epithelial separation (edema) in the secondary lamellae is not the cause of blood oxygen depletion as previously proposed.^{10,11} Blood oxygen measurements evidenced that the gas exchange capacity of the gills had already been severely impaired at the time of tissue sampling.

Piiper and Scheid¹⁸) and Cameron¹⁹) theoretically analyzed respiratory gas exchange in fish gills and proposed a number of possibilities that would lower P_{aO_2} . These include: (1) a decrease in ambient water P_{O_2} , (2) a decrease in mixed venous blood P_{O_2} , (3) a decrease in ventilatory flow, (4) an increase in cardiac output, (5) ventilatory shunting, and (6) vascular shunting. The ambient water P_{O_2} was maintained at near saturation level in this study so that this possibility should be discarded. We did not measure mixed venous P_{O_2} in this and previous studies. We also did not measure ventilatory flow rates during *Chattonella* exposure, but Matsusato and Kobayashi²⁾ reported a preliminary data that showed an increase in ventilatory flow after exposure. Ishimatsu *et al.*⁷⁾ reported a sharp increase in ventilatory pressures, in response to P_{aO_2} decline after *Chattonella* exposure. There are no data available as to the effect of *Chattonella* on cardiac output. The heart rate did not change during the early phase of exposure but decreased toward the death.⁷⁾ Without detailed information about the performance of the gas transport system during *Chattonella* exposure, it is difficult to speculate how *Chattonella* impairs the system, although the present finding on gill histology at the time of P_{aO_2} drop may shed some light on this problem.

The only histological difference from the control gills, found at the time of P_{aO_2} decline, was more frequent occurrence of mucus that apparently blocked interfilamental respiratory water channels. If such blockade does occur, a counter-current flow disposition of water and blood that is widely accepted to reside in fish gill should no longer be functional. A high resistance to flow imparted by mucus would shunt water flow away from close contact with the blood in the secondary lamellae. Secretion of mucus in

response to *Chattonella* exposure has in fact been repeatedly reported.^{2,4,10,20} Shimada *et al.*¹⁰⁾ found that loss of mucus from the afferent side of the filament was more pronounced than that on the efferent side. We also noticed such difference between the two sides (Table 1). Kobayashi²⁰⁾ pointed out that mucus could be secreted from the gill within 15 min after *Chattonella* exposure. However, these results may have been flawed to some extent as the method used was not specifically aimed at preservation of mucus that was only loosely attached onto the surface of the gill. Half of the control fish showed mucus blockade on the afferent side, while one fish in the *Chattonella*-exposed group showed almost no mucus blockade in spite of a severe decrease in P_{aO_2} (Table 1). Thus, some mucus was possibly secreted from the mucus cells during tissue processing or, on the contrary, some secreted mucus could have been lost during tissue processing. There are a few papers specifically devoted to the histological preservation of mucous coat of the gills.²¹⁻²⁴⁾

The Mechanism of Fish Kill by *Chattonella*

We propose that the initial P_{aO_2} drop during *Chattonella* exposure results from blockade of respiratory water flow through the gills by massive secretion of mucus and not from edema formation as previously proposed. This contention is based on the findings that (1) interfilamental spaces were often blocked by mucus at the time of P_{aO_2} drop, but (2) branchial edema was not observed in the gills sampled at that time. Mucus covering the surface of the lamellae acts as a diffusion barrier as its diffusion constant is comparable to that of water, and would therefore constitute an additional unstirred layer through which oxygen must diffuse.²⁵⁾ It may be more important for the secreted mucus to impair water flow through the lamellae by blocking the interfilamental spaces, thereby shunting inflowing respiratory water away from the gas exchange surface. It is true that *Chattonella* severely damaged the gills, causing epithelial separation in both secondary lamellae and filaments as well as several other types of lesions as demonstrated in experiment 1. However, these alterations were hardly detectable at the time of P_{aO_2} drop and therefore cannot be the cause of hypoxemia. Ishimatsu *et al.*^{7,8)} found other physiological perturbations, such as acidosis and an elevation of plasma ions, becoming manifest toward the death. These may be related to destruction of the ion transport capacity caused by chloride cell dysfunctioning.

We currently speculate that oxygen radicals are involved

in the release of mucus from the gills. There is increasing evidence for the production of oxygen radicals by *Chattonella* in resting conditions.²⁶⁻³² Oda *et al.*³⁰ reported that the concentration of hydrogen peroxide in medium increased 10 to 15-fold after disruption of *C. marina* cells. Shimada *et al.*²⁷ and Tanaka *et al.*³² reported evidence that superoxide (O_2^-) was generated in small particles or "verruciform protrusions," located on the surface of *C. antiqua* cells. These structures were released from the cells when mucus from yellowtail gill was added to sea water. They interpreted the results as an indication of enhanced generation of O_2^- in response to stimulation. Oxygen radicals are known to induce mucin secretion from mucus cells in guinea pig gallbladder³³ and rat gastric mucosa.³⁴ Thus, it is possible that oxygen radicals released from *Chattonella* cells are responsible for mucus secretion from gill mucus cells observed in the present study. The release of oxygen radicals might be enhanced when cells are inhaled into the mouth and contact the surface of the gills or are mechanically stimulated by colliding against the branchial surface. *Chattonella* cells trapped within the mucus (Fig. 6C) may further aggravate the situation. It is also possible that part of the mucus was derived from *Chattonella* cells. Further studies are needed to test the hypothesis.

Acknowledgments We are grateful to the staff of the Aquaculture Laboratory, Nagasaki Prefectural Institute of Fisheries for their help in conducting this study, which was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (04660208).

References

- 1) T. Okaichi: Red tide problems in the Seto Inland Sea, Japan, in "Red tides: biology, environmental science, and toxicology" (ed. by T. Okaichi, D. M. Anderson, and T. Nemoto), Elsevier, New York, 1989, pp. 137-142.
- 2) T. Matsusato and H. Kobayashi: Studies on death of fish caused by red tide. *Bull. Nansei Reg. Fish. Res. Lab.*, **7**, 43-67 (1974).
- 3) K. Yamaguchi, K. Ogawa, N. Takeda, K. Hashimoto, and T. Okaichi: Oxygen equilibria of hemoglobins of cultured sea fishes, with special reference to red tide-associated mass mortality of yellowtail. *Nippon Suisan Gakkaishi*, **47**, 403-409 (1981).
- 4) M. Endo, T. Sakai, and A. Kuroki: Histological and histochemical changes in the gills of the yellowtail *Seriola quinqueradiata* exposed to the Raphidophyceae flagellate *Chattonella marina*. *Mar. Biol.*, **87**, 193-197 (1985).
- 5) M. Endo, R. Foscarini, and A. Kuroki: Electrocardiogram of a marine fish, *Pagrus major*, exposed to red tide plankton, *Chattonella marina*. *Mar. Biol.*, **97**, 477-481 (1988).
- 6) T. Sakai, K. Yamamoto, M. Endo, A. Kuroki, K. Kumanda, K. Takeda, and T. Aramaki: Changes in the gill carbonic anhydrase activity of fish exposed to *Chattonella marina* red tide, with special reference to the mortality. *Nippon Suisan Gakkaishi*, **52**, 1351-1354 (1986).
- 7) A. Ishimatsu, H. Maruta, T. Tsuchiyama, and M. Ozaki: Respiratory, ionoregulatory and cardiovascular responses of the yellowtail *Seriola quinqueradiata* to exposure to the red tide plankton *Chattonella*. *Nippon Suisan Gakkaishi*, **56**, 189-199 (1990).
- 8) A. Ishimatsu, T. Tsuchiyama, M. Yoshida, M. Sameshima, M. Pawluk, and T. Oda: Effect of *Chattonella* exposure on acid-base status of the yellowtail. *Nippon Suisan Gakkaishi*, **57**, 2115-2120 (1991).
- 9) T. Tsuchiyama, A. Ishimatsu, T. Oda, S. Uchida, and M. Ozaki: Effect of *Chattonella* exposure on plasma catecholamine levels in the yellowtail. *Nippon Suisan Gakkaishi*, **58**, 207-211 (1992).
- 10) M. Shimada, T. H. Murakami, T. Imahayashi, H. S. Ozaki, T. Toyoshima, and T. Okaichi: Effects of sea bloom, *Chattonella antiqua*, on gill primary lamellae of the young yellowtail, *Seriola quinqueradiata*. *Acta Histochem. Cytochem.*, **16**, 232-244 (1983).
- 11) T. Toyoshima, H. S. Ozaki, M. Shimada, T. Okaichi, and T. H. Murakami: Ultrastructural alterations on chloride cells of the yellowtail *Seriola quinqueradiata*, following exposure to the red tide species *Chattonella antiqua*. *Mar. Biol.*, **38**, 101-108 (1985).
- 12) D. J. Speare and H. W. Ferguson: Fixation artifacts in rainbow trout (*Salmo gairdneri*) gills: A morphometric evaluation. *Can. J. Fish. Aquat. Sci.*, **46**, 780-785 (1989).
- 13) J. F. Skidmore and P. W. A. Tovell: Toxic effects of zinc sulphate on the gills of rainbow trout. *Wat. Res.*, **6**, 217-230 (1972).
- 14) H. W. Ferguson: Systemic pathology of fish, Iowa State University Press, Iowa 1989, p. 263.
- 15) J. Mallatt: Fish gill structural changes induced by toxicants and other irritants: A statistical review. *Can. J. Fish. Aquat. Sci.*, **42**, 630-648 (1985).
- 16) S. Crespo, F. Padros, R. Sala, and M. J. Marlasca: Gill structure of cultured *Salmo trutta fario* related to sampling technique. *Dis. Aquat. Org.*, **4**, 219-221 (1988).
- 17) A. Soivio and H. Tuurala: Structural and circulatory responses to hypoxia in the secondary lamellae of *Salmo gairdneri* gills at two temperatures. *J. comp. Phys.*, **145**, 37-43 (1981).
- 18) J. Piiper and P. Scheid: Physical principles of respiratory gas exchange in fish gills, in "Gills" (ed. by D. F. Houlihan, J. C. Rankin, and T. J. Shuttleworth), Cambridge University Press, Cambridge, 1982, pp. 45-61.
- 19) J. N. Cameron: Models of gas exchange, in "The respiratory physiology of animals" (ed. by J. N. Cameron), Oxford University Press, New York, 1989, pp. 135-154.
- 20) H. Kobayashi: Some aspects on the secretion of gill mucus in red sea bream *Pagrus major* exposed to the red tide. *Nippon Suisan Gakkaishi*, **55**, 553-557 (1989).
- 21) R. D. Handy and F. B. Eddy: The absence of mucus on the secondary lamellae of unstressed rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J. Fish Biol.*, **38**, 153-155 (1991).
- 22) M. D. Powell, D. J. Speare, and J. F. Burka: Fixation of mucus on rainbow trout (*Oncorhynchus mykiss* Walbaum) gills for light and electron microscopy. *J. Fish Biol.*, **41**, 813-824 (1992).
- 23) M. D. Powell, D. J. Speare, and G. M. Wright: Comparative ultrastructural morphology of lamellar epithelial, chloride and mucous cell glycocalyx of the rainbow trout (*Oncorhynchus mykiss*) gill. *J. Fish Biol.*, **44**, 725-730 (1994).
- 24) J. S. Lumsden, H. W. Ferguson, V. E. Ostland, and P. J. Byrne: The mucous coat on gill lamellae of rainbow trout (*Oncorhynchus mykiss*). *Cell Tiss. Res.*, **275**, 187-193 (1994).
- 25) G. R. Ultsch and G. Gros: Mucus as a diffusion barrier to oxygen: possible role in O_2 uptake at low pH in carp (*Cyprinus carpio*) gills. *Comp. Biochem. Physiol.*, **62**, 685-689 (1979).
- 26) M. Shimada, N. Nakai, H. Goto, M. Watanabe, H. Watanabe, M. Nakanishi, S. Yoshimatsu, and C. Ono: Free radical production by the red tide alga, *Chattonella antiqua*. *Histochemical Journal*, **23**, 362-365 (1991).
- 27) M. Shimada, S. Kawamoto, Y. Nakatsuka, and M. Watanabe: Localization of superoxide anion in the red tide alga *Chattonella antiqua*. *J. Histochem. Cytochem.*, **41**, 507-511 (1993).
- 28) T. Oda, T. Akaike, K. Sato, A. Ishimatsu, S. Takeshita, T. Muramatsu, and H. Maeda: Hydroxyl radical generation by red tide algae. *Arch. Biochem. Biophys.*, **294**, 38-43 (1992).
- 29) T. Oda, A. Ishimatsu, M. Shimada, S. Takeshita, and T. Muramatsu: Oxygen-radical-mediated toxic effects of the red tide flagellate *Chattonella marina* on *Vibrio alginolyticus*. *Mar. Biol.*, **112**, 505-509 (1992).
- 30) T. Oda, A. Ishimatsu, S. Takeshita, and T. Muramatsu: Hydrogen peroxide production by the red-tide flagellate *Chattonella marina*. *Biosci. Biotech. Biochem.*, **58**, 957-958 (1994).
- 31) K. Tanaka, S. Yoshimatsu, and M. Shimada: Generation of superoxide anions by *Chattonella antiqua*: Possible causes for fish death by 'Red Tide'. *Experientia*, **48**, 888-890 (1992).
- 32) K. Tanaka, Y. Muto, and M. Shimada: Generation of superoxide anion radicals by the marine phytoplankton organism, *Chattonella*

- antiqua*. *J. Plankt. Res.*, **16**, 161–169 (1994).
- 33) J. T. LaMont: Oxygen radicals stimulate gallbladder glycoprotein secretion, in “Mucus and related topics” (ed. by E. Chantler and N. A. Ratcliffe), The Company of Biologists Ltd, Cambridge, 1989, pp. 273–278.
- 34) H. Hiraishi, A. Terano, S. Ota, H. Mutoh, T. Sugimoto, M. Razandi, and K. J. Ivey: Oxygen metabolites stimulate mucous glycoprotein secretion from cultured rat gastric mucous cells. *Am. J. Phys.*, **261**, G662–G668 (1991).