Treatments for Hinge Ligament Disease in Juvenile Pacific Oysters (*Crassostrea gigas*)

By

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TREATMENTS FOR HINGE LIGAMENT DISEASE IN JUVENILE PACIFIC OYSTERS (*CRASSOSTREA GIGAS*)

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ABSTRACT

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Kimberly S. Rich

Cytophaga-like bacteria are the known etiological agent responsible for hinge ligament disease in juvenile Pacific oysters (*Crassostrea gigas*). An economically viable, natural way to manage this disease has not been documented. *In vitro* efficacy of hydrogen peroxide, CitroBio and ozone were determined for one strain (C1B-2) of *Cytophaga*-like bacteria. *In vitro* results showed that the CLB strain had the greatest sensitivity to CitroBio at 1000 ppm after 30 seconds. *In vivo* trials with hydrogen peroxide and extensive histological work failed to show a decrease in hinge ligament erosion. Approximately 71-74% of juvenile *C. gigas* were observed with ligament erosion at the conclusion of field trials. No serious pathological conditions resulting from a weakened hinge ligament were observed. No mortality nor negative effects on growth were noted as a result of treatment with hydrogen peroxide. *In vitro* results suggest further testing with CitroBio would be of benefit.

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INTRODUCTION

Hinge ligament disease is an ubiquitous, non-notifiable disease that occurs in many juvenile bivalves (Elston 1984, Dungan and Elston 1988, Elston 1990, Elston 2003, personal communication). *Cytophaga*-like bacteria (CLB) are the known etiological agents associated with the disease (Dungan 1987). The bacteria have caused high mortalities (25-90%) in intensive nursery facilities producing cultchless Pacific oyster (*Crassostrea gigas)* seed (Elston et al. 1982, Dungan and Elston 1988, Kuiper 2003, personal communication). Typically, smaller oysters from settlement to 10 mm are most susceptible (Elston 1990). Treatments to reduce hinge ligament disease in juvenile *C. gigas* are lacking.

Cytophaga-like bacteria have been described as gram-negative, oxidase positive, yellow pigmented, gliding organisms that are capable of hydrolyzing starch, casein, chitin and gelatin (Dungan et al. 1989). The hinge ligament disease in juvenile *C. gigas* is distinguished by homogenous, dense populations of long rod-shaped bacteria that invade and subsequently erode the hinge ligament causing death of the oyster and economic losses for the farmer (Dungan and Elston 1988).

The hinge ligament of an oyster is located between the anterior margins of the valves (Figure 1). The ligament is comprised of two components, the central resilium and the lateral tensilium (Galstoff 1964). The resilium is compressed when the adductor muscle is contracted, closing the valves. As the adductor muscle releases its tension, the resilium forces the valves apart (Trueman 1951, Galstoff 1964).

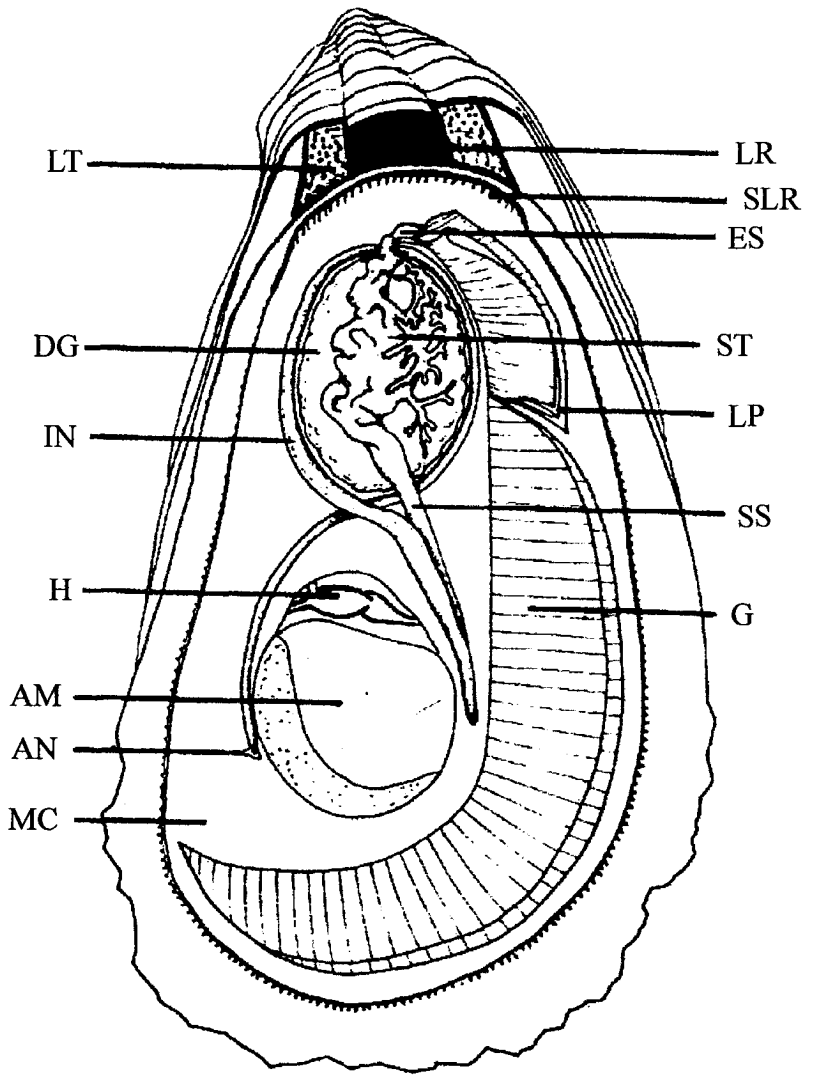

Figure 1. Basic oyster anatomy in sagittal view. Anatomical features are labeled as follows, ligament tensilium (LT), ligament resilium (LR), subligamental ridge (SLR), esophagus (ES), digestive gland (DG), stomach (ST), intestine (IN), labial palps (LP), style sac (SS), heart (H), gills (G), adductor muscle (AM), anus (AN), mantle cavity (MC). This diagramtic representation has been modified from Galstoff (1964) and Dungan (1987).

The strength of the resilium is attributed to its chemical composition. It is made up of approximately 30-67% calcium carbonate in the Eastern oyster (*Crassostrea virginica*) (Galstoff 1964). The tensilium, which ensures proper alignment of the valves during adduction, is only partially calcified (5.3-8.5% calcified) and consists primarily of quinone-tanned proteins that withstand significant stretching (Trueman 1951, Galstoff 1964, Carriker 1996). The resilium and tensilium work simultaneously against the adductor muscle. This allows the oyster to maintain the perfect gape required for basic functions such as, feeding and release of fecal matter and gametes (Dungan 1987). In addition, a healthy hinge ligament acts as a barrier against undesirable organisms and poor environmental conditions (Galstoff 1964, Dungan 1987). The subligamental ridge consists of a single layer of tall, undulating, narrow epithelial cells that contain secretory granules that are responsible for the creation of the hinge ligament. Freshly secreted layers of the ligament are in contact with the subligamental ridge while the older, nonfunctional, distal layers are exposed to the environment. The outer hinge ligament layers can become cracked, dried and exposed to many organisms, including bacteria (Galstoff 1964, Carriker 1996).

In past studies *Cytophaga*-like bacteria have been grouped with the myxobacteria, flexibacteria or flavobacteria (Christensen 1977). Currently, the Order Cytophagales is considered part of the *Bacteriodes-Flavobacterium-Cytophaga* branch (Paster et al. 1985, Woese et al. 1985, Reichenbach 1999, Kirchman 2002). The family *Cytophagaceae* includes several genera, including those pertinent to this study. Genera include *Cytophaga, Flexibacter* and an

unnamed *Cytophaga*-like bacteria (Reichenbach 1999, Madigan et al. 2003). Bacteria in the *Cytophagacea* family are ubiquitous in terrestrial, freshwater and marine systems and are known for their ability to breakdown resistant polysaccharides such as cellulose, chitin, agar, and keratin (Murchelano and Bishop 1969, Christensen 1977, Dungan et al. 1989, Madigan et al. 2003). Several members of the Order Cytophagales including *Flavobacterium columnare* (*Cytophaga columnaris*) and *Flexibacter psychrophilus* (*Cytophaga psychrophila*) are known freshwater fish pathogens (Noga 2000, Madigan et al. 2003). Marine *Cytophaga*-like bacteria have been associated with skin lesions of pen-reared Atlantic salmon (*Salmo salar*) (Kent et al. 1988) and carapace lesions in American lobsters (*Homarus americanus*) (Becker et al. 2004).

There is little published information on treatment and prevention of *Cytophaga*-like bacteria in Pacific oysters using antimicrobial agents. Current suggestions for treatment of diseased oysters are sodium hypochlorite (common household bleach) or antibiotics including penicillin, novobiocin, and tetracycline. These antibiotics are only known to inhibit some strains of the bacteria and are not recommended for routine use (Elston 1990).

Hydrogen peroxide is a compound of low regulatory priority, as established by the U.S. Food and Drug Administration (FDA). When used at maximum concentrations of 500 mg/L for 60 minutes, the compound is considered safe for use on all species and life stages of fish in the treatment of fungal infections (Marking et al. 1994, Arndt and Wagner 1997, Rach et al. 1997).

It has been used as an antimicrobial agent in a variety of applications including cheese production, packaged foods and water treatment. Bacteria, yeasts, and viruses can also be inhibited by its use (Marking et al. 1994). Static treatments with 6% hydrogen peroxide at 200 mg/L reduced saddle–patch and fin necrosis in juvenile rainbow trout (*Oncorhynchus mykiss*) when given twice weekly to treat *Flavobacterium columnare* infections (Speare and Arsenault 1997). The causative agent of bacterial gill disease (BGD), *Flavobacterium branchiophilium*, was reduced in trials using naturally infected fingerling brown trout (*Salmo trutta*), chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout by using 35% food grade hydrogen peroxide (Lumsden et al. 1998, Rach et al. 2000). Hydrogen peroxide is also used in aquaculture as an antiseptic in the treatment of skin parasites including protozoans, sea lice and monogenetic trematodes (Marking et al. 1994). Gill samples of Pacific threadfin (*Polydactylus sexfilis*), after treatment with 75-150 mg/L hydrogen peroxide for 30 minutes, were free of the ectoparasite *Amyloodinum ocellatum* (Montgomery-Brock et al. 2001). Thirty-two isolates recovered from the gills of *O. mykiss* at the Humboldt State University Fish Hatchery were shown to be affected by 3×10^3 ppm hydrogen peroxide during chemical sensitivity tests (Menghini 2000). Saturated discs of hydrogen peroxide were placed in Cytophaga agar and *Cytophaga pectinovora* and *Cytophaga aquatilis* showed no growth, with a zone of inhibition of 30 and 34 mm. Other isolates within the study had inhibition zones which ranged from 6 to 40 mm. The ability of hydrogen peroxide to revert back to water and oxygen and its minimal impact on water quality make this compound a suitable candidate for

aquaculture (Marking et al. 1994, Arndt and Wagner 1997).

Ozone has been used as a disinfectant to reduce contaminants found in water supplies, industrial effluents and within recirculating aquaculture systems (Summerfelt and Hochheimer 1997, Tango and Gagnon 2003). It is an attractive alternative for aquaculture because it is a powerful oxidizing agent that can inactivate a variety of waterborne microorganisms including those that may cause disease (Summerfelt and Hochheimer 1997). Ozone has a half life of 15 minutes and produces oxygen as a reaction end product (Colberg and Lingg 1978, Summerfelt and Hochheimer 1997). In field trials, mortalities due to *Ceratomyxa shasta* in steelhead and cutthroat trout were reduced when water was treated with ozone at the Cowlitz (Washington) State Fish Hatchery (Tipping 1988). Bullock et al. (1997) attempted to use ozone to reduce bacterial gill disease in a recirculating system for rainbow trout. The authors met with some success. Further outbreaks were prevented, but the causative agent, *Flavobacterium branchiophilum*, still colonized gill tissue. In-vitro work with several marine and freshwater fish pathogens such as *Aeromonas salmonicida*, *Yersinia ruckeri* and *Pseudomonas fluorescens* have shown three to four log reductions in viable counts when ozone is used at 0.1 mg O_3/L (Colberg and Lingg 1978, Sugita et al. 1992, Liltved et al. 1995). In shellfish hatcheries, ozone applied to seawater was effective against *Vibrio*, *Flavobacterium* and *Achromobacter* at 0.56 mg O_3/L for 2 hours (Blogoslawski et al. 1978). In France and Australia, ozone is commonly used in depuration of adult oysters. Although currently not approved by the U.S. Food and Drug Administration (FDA) as a depuration aide, ozone has been used

to reduce fecal coliform levels before animals are considered acceptable for human consumption (Burkhardt et al. 1992).

CitroBio is a commercially available product made from whole oranges, glycerin, citric acid and alpha-tocopherol (CitroBio, Inc. 2004). The active ingredient in CitroBio is a citrus extract that is formed by many elements including, ascorbic acid, palmatic acid, oleic acid, glycerides and peptin. Under current FDA regulations, the product is "generally regarded as safe". The agriculture industry, veterinarians and the fishing industry use CitroBio to disinfect equipment, water and food products. The CitroBio company claims that the product prevents or inhibits growth of *Escherichia coli*, *Salmonella*, *Staphylococcus*, *Clostridium*, *Listeria*, *Corynebacteria*, *Proteus*, *Brucella*, *Klebsiella*, *Pasteurella* and *Pseudomonas* (CitroBio, Inc. 2004). Lancaster Laboratories (Lancaster, PA) determined that a 3% concentration resulted in a 23 mm zone of inhibition for *Listeria* spp. (CitroBio, Inc. 2004). *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus* and *Micrococcus luteus* were inhibited by a 0.005% solution of CitroBio. CitroBio produced average zones of inhibition of 10-24 mm (CitroBio, Inc. 2004). Shrimp farmers in Sonora, Mexico, struggling with white spot disease have reported a higher survival rate of shrimp in ponds treated with this product (CitroBio, Inc. 2004).

Intensive culture of *C. gigas* juveniles will be improved by studying treatments to prevent mortalities due to hinge ligament disease. Not only will those who culture oysters benefit from an inexpensive and readily available treatment method for hinge ligament disease, but the bivalve industry, and the

scientific community will also gain more insights into the prevention and management of these bacteria. The objective of the study was to find an economically viable, therapeutic agent that inhibits growth of *Cytophaga*-like bacteria in juvenile *C. gigas*.

MATERIALS AND METHODS

Field Study

Juvenile Pacific oysters, approximately 1 mm in size, were obtained from a private aquaculture facility in Humboldt County, California. Oysters were reared in small scale, 10 cm diameter polyvinyl chloride (PVC) forced flow upweller units (18.92 L buckets). Upweller units were fitted at the bottom with a 500 μm screen to hold oysters. Water was pumped from Mad River Slough, Humboldt Bay, California to a 5678.11 L holding tank, which fed an internal 378.54 L head tank. PVC pipe (5 cm in diameter), leading from the head tank, fed upwelling units with slough water. A 1.91 cm ball valve on each upwelling unit regulated flow. Flow was kept at approximately 2-4 L/min depending on size of oysters.

Trial 1 was conducted from July 8 to July 29, 2003 using 60,000 juvenile, diploid oysters. Trial 2 was conducted from August 4 to August 25, 2003 using 27,000 juvenile, triploid oysters. In each trial, juvenile *C. gigas* were divided into six equal groups. Test dilutions 0 (control), 100 and 1000 ppm of hydrogen peroxide were prepared for specified groups using fresh water. The fresh well water used had the following properties: 13-15°C; 170 mg/L hardness; 40,000 μg calcium; 850 μg iron; 18,000 μg magnesium; 850 μg manganese. Each hydrogen peroxide concentration was tested for 15 or 30 minutes twice per week in designated exposure containers. Trials were terminated the day after the sixth

treatment. Upon completion of treatments, oysters were removed from designated exposure containers and returned to slough water.

Upwellers with oysters were rinsed with freshwater twice daily as part of the normal health and maintenance of the animals, with the exception of weekends when one rinse was given. Disinfection of the complete upwelling system including: pipes, holding containers and head tanks took place once per week. Mortality was assessed weekly. Temperature and salinity were determined daily with a standard thermometer and a Brix refractometer, respectively. Observations of the shell surface were made weekly, prior to and after treatment, with a Leica G26 dissecting microscope.

Each week, shell height of a random sample (approximately 50) of oysters was measured to the nearest 0.01 mm with digital calipers. Oysters were fixed in their shells with Davidson's fixative (Shaw and Battle 1957) and decalcified with a formic acid/sodium citrate solution (Luna 1968). Approximately 10 oysters were embedded in each block of JB-4 glycol methacrylate (Polysciences, Inc; Warrington, PA). Sections were cut at 5μm using a tungsten carbide knife mounted on an 820 HistoStat Reichert rotary microtome. Sections were mounted on slides. Slides were stained in methylene blue with a basic fuchsin counter stain. Permount was used to affix coverslips to the slides. Oyster sections were surveyed for the presence of *Cytophaga*-like bacteria erosive lesions in the hinge ligament. A consistent subligamental ridge depth was chosen for evaluation so that histological observations were comparable. All oysters were then scored by the intensity of the lesion. Categories used for scoring included, normal (0%

erosion), mild (20% erosion), moderate (40% erosion) and severe (greater than 60% erosion). The ratio of remaining (non-eroded) resilium depth to subligamental ridge depth was measured to determine the severity of the erosion with regard to functionality. Resilium and subligamental ridge depth was measured using an ocular micrometer.

At the termination of trial 1 and trial 2 a random sample (n=250) of oysters from each upweller was placed in floating trays in Humboldt Bay. Trays were made of polyethylene, with mesh inserts of approximately 750 μm. Tray culture lasted for approximately two weeks. Fifty oysters from each group were then randomly selected for measurements of shell height and 100 oysters were counted from each experimental group for bag and rack culture. Bags were constructed using 0.64 cm polyethylene with 30.48 cm x 121.9 cm fir board inserts creating four compartments. Rack culture for Trial 1a occurred from August 2003 to January 2004. Trial 2a extended from September 2003 to February 2004. Routine maintenance of bags, common within the shellfish industry, consisted of rolling the bags around to jostle the oysters and flipping the bags to expose a clean debris free surface to the water. This took place twice a month. Upon completion of the experiments, shell height was measured using a standard metric ruler.

Laboratory Study

The microorganism used was a *Cytophaga*–like bacterium. Stock cultures originated from degraded hinge ligaments of a high mortality population (62%

over 90 days) of juvenile *C. gigas*, having 20-30 mm shell height from a Washington state nursery (Dungan 2003, personal communication). The bacterium C1B-2 was isolated prior to 1987. It was shipped to me in February of 2004 to be used in this study. Seawater (50%) cytophaga agar (SWCA) and seawater cytophaga broth (SWCB) were used as growth media.

In preparation for experiments, a 1.0 ml aliquot of the C1B-2 culture was added to 250 ml of SWCB and incubated at 22°C. After 5 days an aliquot of 1.0 ml of bacterial broth was added to 9.0 ml of SWCB diluent. A Hach DR 850 colorimeter was used to ensure an initial turbidity reading of 5 NTU (9.3×10^7 CFU). Culture tubes were then treated with the designated theraputent.

Final concentrations of hydrogen peroxide added to the culture tube were 100 and 1000 ppm. CitroBio final concentrations were 10, 100 and 1000 ppm. All ozone exposures were done at 0.1 ppm. Samples (1.0 ml) were withdrawn at different time intervals: 30 seconds, 2, 5, 15, 20 and 30 minutes. Serial dilutions (1×10^7) were carried out in 0.1% sterile peptone broth, plated on SWCA, and then incubated at 22°C for 5 days. Colony forming units were quantified on the fifth day.

Ozone was generated from oxygen gas using an Ozotech model OZ4PC10 (Yreka, CA) ozone generator. The ozone was bubbled through an air-stone diffuser into a 250 ml glass beaker containing sterile seawater. Ozone concentration was measured prior to exposure of the bacteria with a Hach DR 850 colorimeter. Bacteria were added when 0.1 ppm ozone concentration was achieved.

Differences in growth rate and resilium depth between groups, over the trial period, were analyzed by ANOVA and Tukey-Kramer Multiple Comparison Test. Ratio and frequency data of resilium lesions were also evaluated.

RESULTS

Growth of juvenile diploid and triploid *C. gigas* was not affected by exposure to hydrogen peroxide (Tables 1, 2). No notable trends in growth were observed during treatment in trial 1 (Table 1). During the second and third weeks of trial 2, the group treated with 100 ppm for 30 minutes was significantly larger ($P<0.05$) than other groups (Table 2). Following transfer of juvenile oysters to rack and bag culture the long term growth effects of hydrogen peroxide treatment indicated no significant difference between groups in trial 1a (Table 3). Animals treated with 100 ppm for 30 minutes in trial 2a were significantly larger ($P<0.05$) than other groups (Table 4). No mortalities were associated with the administration of hydrogen peroxide. The temperature for trial 1 ranged from 18 to 21°C and increased in trial 2, to 21 - 24°C. Salinity was between 35 and 37 ppt during both trials (Figures 2, 3).

Multiple comparison tests showed no significant differences in measurements of resilium depth in trial 1 (July 8 to July 29, 2003, diploid oysters) or trial 2 (August 4 to August 25, 2003, triploid oysters) (Tables 5, 6). The ratio of resilium depth to subligamental ridge depth (trial 1) increased over time in the control groups and in those groups treated with 100 and 1000 ppm of hydrogen peroxide for 15 minutes (Table 7). In trial 2, there was an increase in one control group and in those groups treated with 100 and 1000 ppm for 30 minutes (Table 8). No significant difference was found between groups in either trial (Tables 7, 8).

Table 1. Mean shell height of juvenile diploid *Crassostrea gigas* before and during hydrogen peroxide treatments in trial 1. Treatments were conducted twice a week in July 2003, and included two controls, 15 and 30 minute treatments at 100 and 1000 parts per million (ppm) each. Control A was used for concentrations of 100 ppm and control B was used for concentrations of 1000 ppm. Random samples of approximatley 50 oysters were collected at the end of each week and measured in the laboratory.

	Control A	Control B	15/100	30/100	15/1000	30/1000	P-value
Variable	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	
Pretreatment	1.97[a] ± 0.52	2.04[a] ± 0.41	1.87[a] ± 0.44	1.98[a] ± 0.48	1.88[a] ± 0.51	1.90[a] ± 0.45	0.73
Week 1-Shell Height	2.79 ± 0.66	2.76 ± 0.55	2.48[a] ± 0.53	2.59 ± 0.54	2.36[a] ± 0.44	2.33[a] ± 0.61	0.49[b]
Week 2-Shell Height	3.82 ± 0.85	4.31 ± 1.06	3.91 ± 0.80	3.33[a] ± 0.87	3.19[a] ± 0.71	3.86 ± 1.14	0.00[c]
Week 3-Shell Height	5.65 ± 1.46	4.96[a] ± 1.29	5.97 ± 1.64	5.10[a] ± 1.41	4.58[a] ± 1.30	5.06[a] ± 1.46	0.00[c]

[a] Means sharing the same letter within a row did not differ ($P<0.05$) based on a Tukey-Kramer multiple comparison test.
[b] Concentration (ppm) is significantly different ($P<0.05$).
[c] Concentration (ppm) and exposure time are significantly different ($P<0.05$).

Table 2. Mean shell height of juvenile triploid *Crassostrea gigas* before and during hydrogen peroxide treatments in trial 2. Treatments were conducted twice a week in August 2003, and included two controls, 15 and 30 minute treatments at 100 and 1000 parts per million (ppm) each. Control A was used for concentrations of 100 ppm and control B was used for concentrations of 1000 ppm. Random samples of approximatley 50 oysters were collected at the end of each week and measured in the laboratory.

	Control A	Control B	15/100	30/100	15/1000	30/1000	P-value
Variable	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± 0.48	
Pretreatment	2.00[a] ± 0.51	2.00[a] ± 0.46	2.58 ± 0.50	2.02[a] ± 0.46	2.08[a] ± 0.53	1.91[a] ± 0.48	0.00[c]
Week 1-Shell Height	2.94[a] ± 0.75	3.09[a] ± 0.72	2.81[a] ± 0.54	2.94[a] ± 0.63	2.72[a] ± 0.59	2.66[a] ± 0.61	0.25
Week 2-Shell Height	3.56[a] ± 0.94	3.62[a] ± 0.98	3.27[a] ± 0.80	4.24 ± 1.22	3.65[a] ± 0.94	3.68[a] ± 1.08	0.00[c]
Week 3-Shell Height	4.42[a] ± 1.29	4.15[a] ± 1.27	4.34[a] ± 1.13	4.87 ± 1.10	4.13[a] ± 1.09	4.24[a] ± 1.20	0.00[c]

[a] Means sharing the same letter within a row did not differ ($P<0.05$) based on a Tukey-Kramer multiple comparison test.

[c] Concentration (ppm) and exposure time are significantly different ($P<0.05$).

Table 3. Long term effect (trial 1a) of treatments with hydrogen peroxide on mean shell height of juvenile diploid *Crassostrea gigas* from trial 1, July 2003. Initial measurement on August 14, 2003. Final measurement on January 16, 2004.

		Shell Height (mm)	
Control or Concentration (ppm)	Exposure Time (min)	Initial	Final
Control A		14.2[a]	47.9[a]
Control B		13.9[a]	48.8[a]
100	15	15.1[a]	43.1[a]
	30	13.3[a]	46.0[a]
1000	15	11.7[a]	46.9[a]
	30	12.9[a]	45.6[a]

[a] Means sharing the same letter within a column did not differ ($P<0.05$) based on a Tukey-Kramer Multiple Comparison Test.

Table 4. Long term effect (trial 2a) of treatments with hydrogen peroxide on mean shell height of juvenile triploid *Crassostrea gigas* from trial 2, August 2003. Initial measurement on September 12, 2003. Final measurement on February 18, 2004.

		Shell Height (mm)	
Control or Concentration (ppm)	Exposure Time (min)	Initial	Final
Control A		15.5[a]	33.2[a]
Control B		12.7[a]	33.6[a]
100	15	13.6[a]	35.7[a]
	30	14.3[a]	38.4
1000	15	15.3[a]	33.8[a]
	30	14.2[a]	35.3[a]

[a] Means sharing the same letter within a column did not differ ($P<0.05$) based on a Tukey-Kramer Multiple Comparison Test.

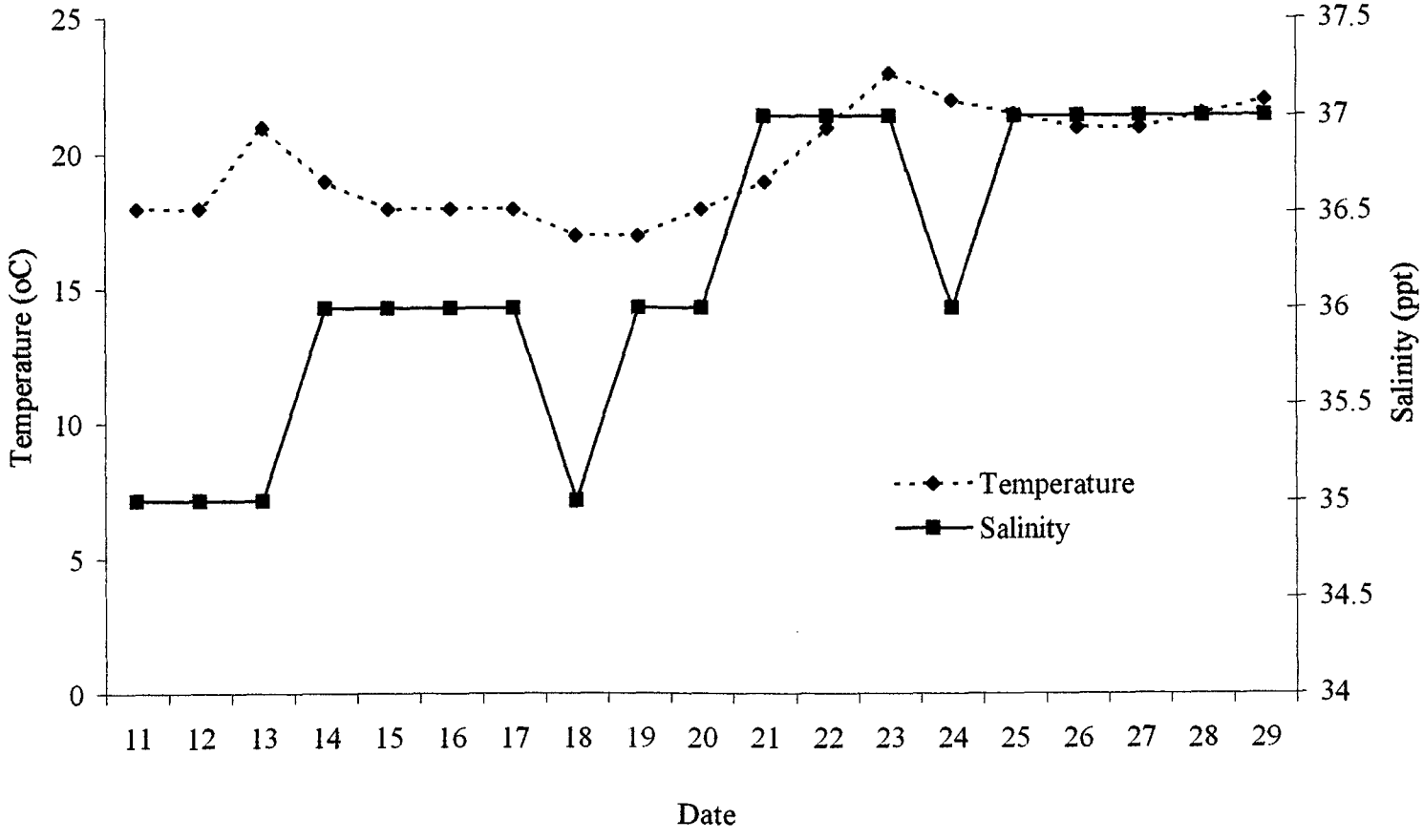

Figure 2. Daily temperature and salinity during trial 1, July 2003.

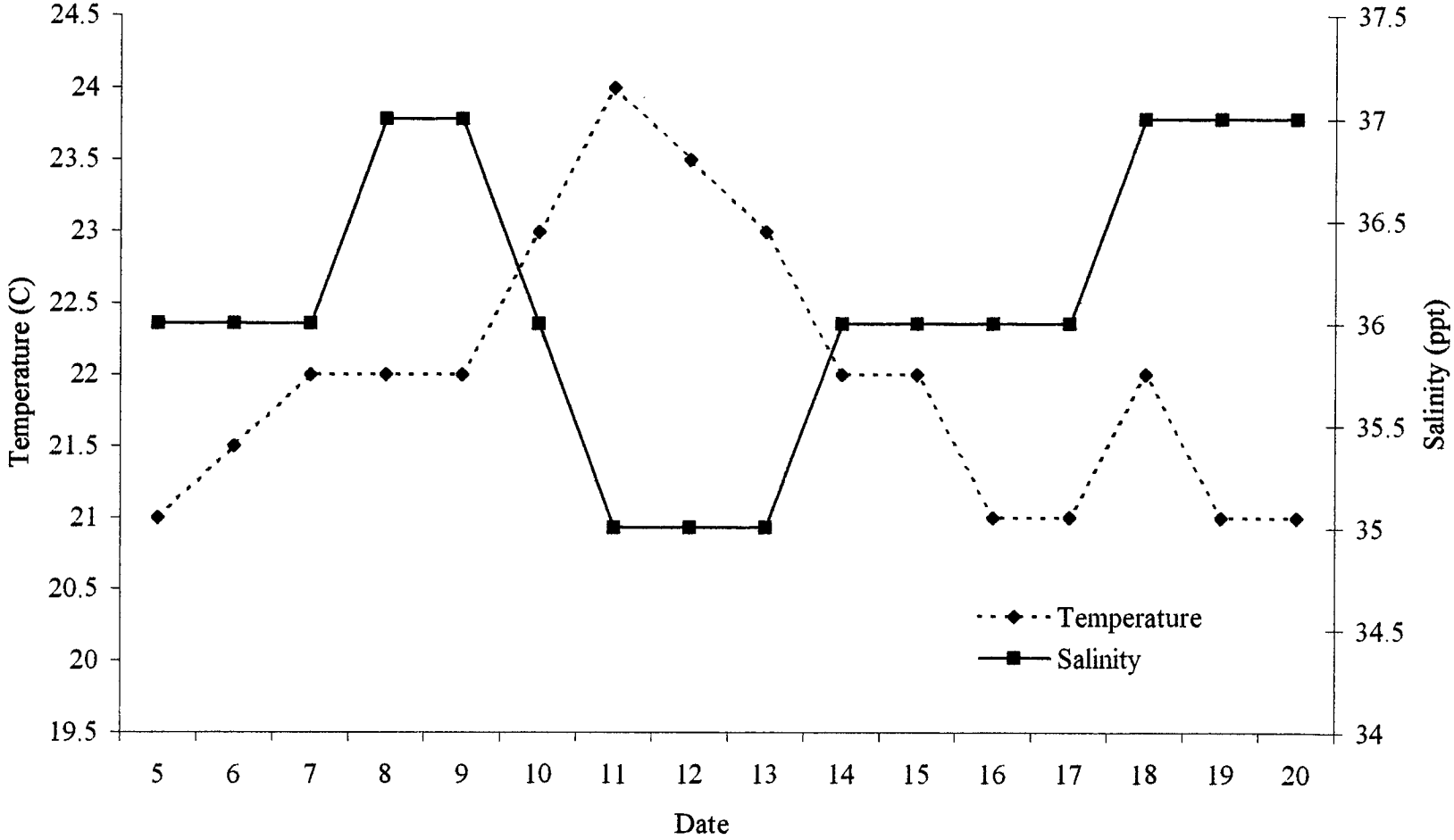

Figure 3. Daily temperature and salinity during trial 2, August 2003.

Table 5. Tukey -Kramer Multiple Comparison Test of mean resilium depth and the combined affect of treatment concentration and time. Resilium depth was measured from histological sections of juvenile diploid *Crassostrea gigas* after treatment in trial 1 with hydrogen peroxide for 15 or 30 minutes at 0, 100 or 1000 ppm. Degrees of freedom (DF), mean square (MS), sum of squares (SS) and f-ratio (F). A common line indicates no significant difference between the treatment groups. Trial 1, July 2003.

Response Variables -Trial 1	DF	MS	SS	F	P	Multiple Comparison
Week 0 - Resilium Depth[a]	2	0.00011	0.0002	0.95	0.39	15/100, 15/0, 30/100, 30/0, 30/1000, 15/1000
Week 1 - Resilium Depth	2	0.00007	0.0001	0.37	0.69	15/1000, 15/100, 30/0, 30/100, 15/0, 30/1000
Week 2 - Resilium Depth	2	0.00141	0.0020	2.02	0.14	15/100, 15/1000, 30/1000, 15/0, 30/0, 30/100
Week 3 - Resilium Depth[b]	2	0.00440	0.0080	3.31	0.04	30/1000, 15/100,30/0, 15/0, 30/100, 15/1000

[a]ppm = significant, $P < 0.05$

[b] Time and ppm = significant, $P < 0.05$

Table 6. Tukey -Kramer Multiple Comparison Test of mean resilium depth and the combined effect of treatment concentration and time. Resilium depth was measured from histological sections of juvenile triploid *Crassostrea gigas* after treatment in trial 2 with hydrogen peroxide for 15 or 30 minutes at 0, 100 or 1000 ppm. Degrees of freedom (DF), mean square (MS), sum of squares (SS) and f-ratio (F). A common line indicates no significant difference between the treatment groups. Trial 2, August 2003.

Response Variables -Trial 2	DF	MS	SS	F	P	Multiple Comparison
Week 0 - Resilium Depth	2	0.00008	0.0001	0.52	0.59	30/1000, 15/0, 15/100, 15/1000, 30/100, 30/0
Week 1 - Resilium Depth[b]	2	0.00113	0.0022	2.14	0.13	30/0, 30/100, 15/1000, 30/1000, 15/0, 15/100
Week 2 - Resilium Depth[b]	2	0.00124	0.0024	2.38	0.10	15/100, 15/1000, 15/0, 30/0, 30/1000, 30/100
Week 3 - Resilium Depth	2	0.00010	0.0002	0.12	0.88	15/0, 30/100, 15/100, 15/1000, 30/0, 30/1000

[b] Time = significant, $P < 0.05$

Table 7. Average ratio of resilium depth to sub-ligamental ridge depth in juvenile diploid *Crassostrea gigas* treated with hydrogen peroxide at 100 or 1000 ppm for 15 or 30 minutes in July 2003 (trial 1). Exposure time in parenthesis. Depth measured microscopically to the nearest 0.01mm.

	Pretreatment	Week 1	Week 2	Week 3
Control A	0.69[a]	0.81	0.98	1.21
Control B	0.51[a]	0.88	1.11	1.31
H100 (15)	0.60[a]	0.83	0.96	1.23
H100 (30)	0.62[a]	0.23	1.27	1.23
H1000 (15)	0.76[a]	1.05	1.13	1.82
H1000 (30)	0.70[a]	1.13	1.03	0.96

[a] Means sharing the same letter within a column did not differ ($P<0.05$) based on a Tukey-Kramer Multiple Comparison Test.

Table 8. Average ratio of resilium depth to sub-ligamental ridge depth in juvenile triploid *Crassostrea gigas* treated with hydrogen peroxide at 100 or 1000 ppm for 15 or 30 minutes in August 2003 (trial 2). Exposure time in parenthesis. Depth measured microscopically to the nearest 0.01mm.

	Pretreatment	Week 1	Week 2	Week 3
Control A	1.09[a]	1.01	0.91	0.95
Control B	0.83[a]	0.93	1.07	2.42
H100 (15)	0.95[a]	1.08	1.00	0.98
H100 (30)	0.89[a]	0.93	1.19	1.23
H1000 (15)	0.88[a]	0.96	0.98	0.98
H1000 (30)	0.78[a]	0.93	0.96	1.03

[a] Means sharing the same letter within a column did not differ ($P<0.05$) based on a Tukey-Kramer Multiple Comparison Test.

Normal hinge ligaments (Figure 4) were identified in approximately 30 % of all animals examined in the histological survey for both trials, while eroded ligaments (Figure 5) were found in approximately 70 % of juvenile oysters. The average frequency of bacterial lesions in the hinge ligament, during pretreatment through week three (trial 1, July 8 to July 29, 2003, diploid oysters), decreased in animals that were treated with concentrations of 100 and 1000 ppm hydrogen peroxide for 15 or 30 minutes. (Table 9). In trial 2 (August 4 to August 25, 2003, triploid oysters), oysters treated with 100 ppm for 30 minutes and 1000 ppm for 15 minutes showed a decrease in the frequency of bacterial lesions (Table 10). For all hydrogen peroxide treatments, lesions were placed into one of four categories; (0) normal (0% erosion), (1) mild (20% erosion), (2) moderate (40% erosion) and (3) severe (greater than 60% erosion). Over 60% of the resilium lesions in juvenile Pacific oysters were given ratings of 1 and 2. Twenty–seven percent were rated as normal, 23 % were rated as severe.

Counts of viable *Cytophaga*-like bacteria decreased as a result of hydrogen peroxide, CitroBio and ozone applications (Figure 6). *Cytophaga*-like bacteria were most sensitive to the CitroBio 1000 ppm (7.9 log reduction), followed by the CitroBio 10 ppm (2.5 log reduction) and the 0.1 mg/L ozone treatment (2.2 log reduction). Inactivation time for the three top treatments was 30 seconds (Figure 6).

During field observations of treated oysters, a decrease in external shell fouling organisms was observed. There was a reduction in ciliated folliculinid protozoans. This protozoa was found on the external shell surface during

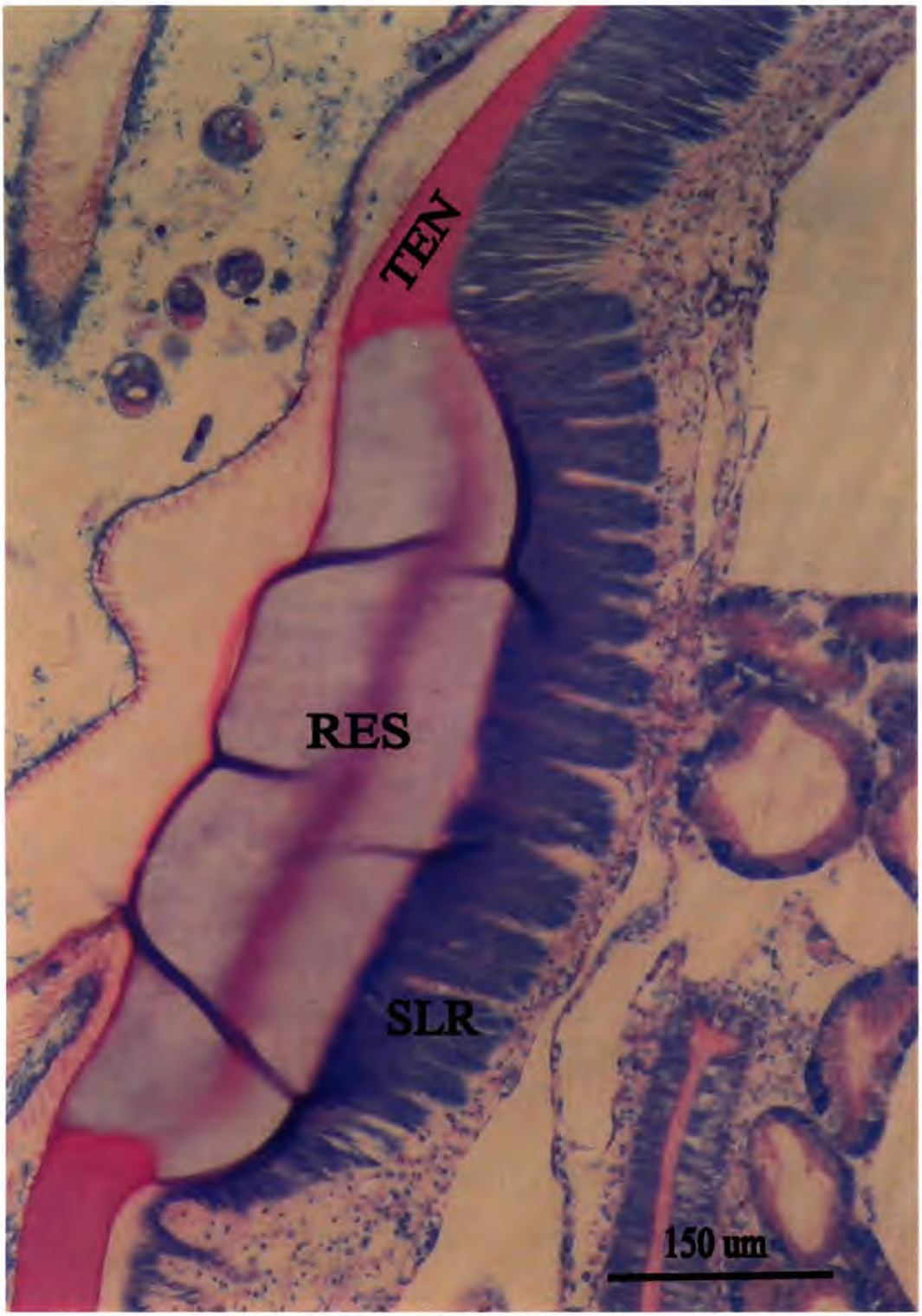

Figure 4. Sagittal section through a normal hinge ligament of a juvenile Pacific oyster (*Crassostrea gigas)*. The resilium (RES), which lies parallel to the subligamental ridge (SLR), is flanked by paired tensilia (TEN).

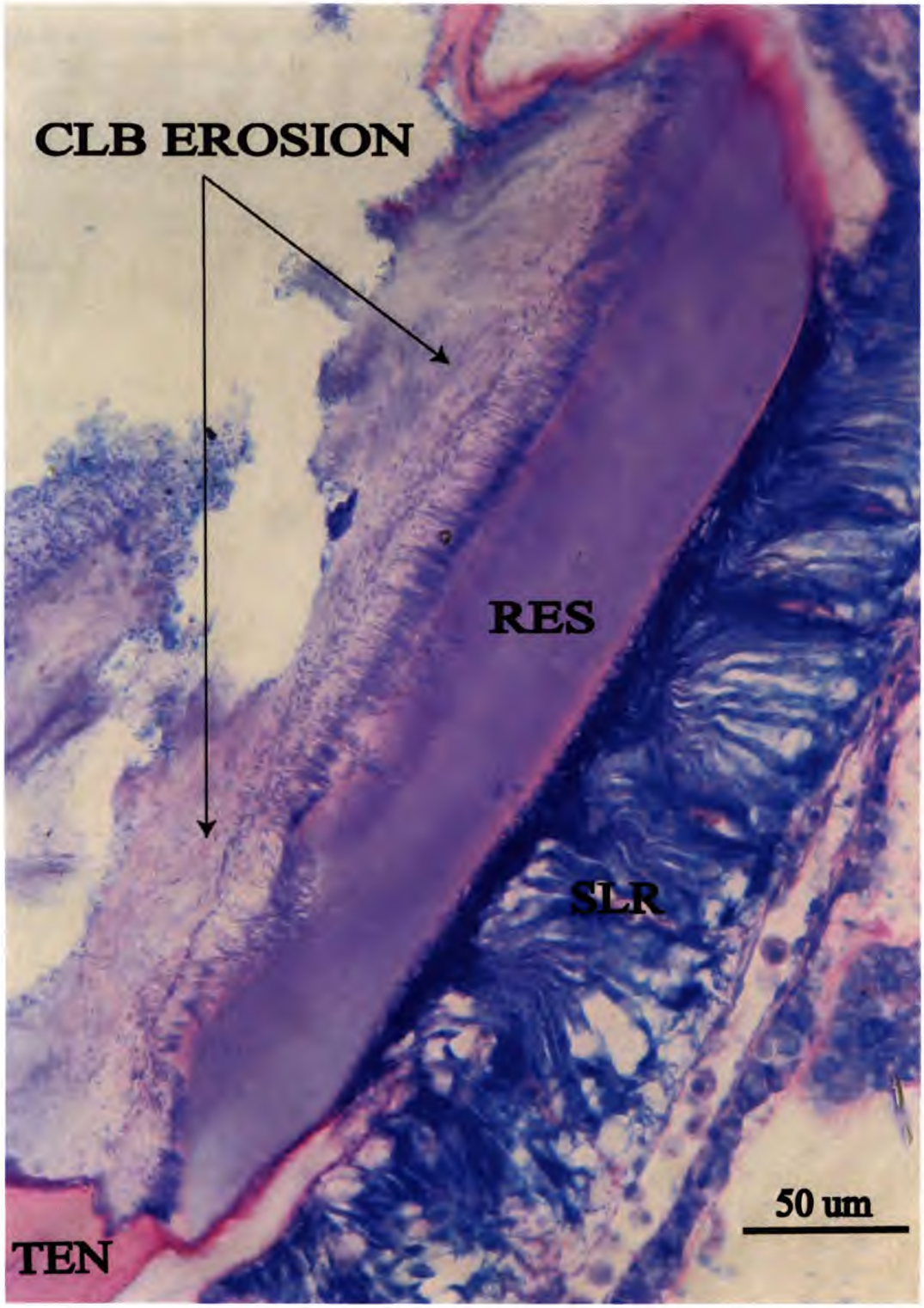

Figure 5. Sagittal section through an eroding hinge ligament of a juvenile Pacific oyster (*Crassostrea gigas)*. The resilium (RES), which lies parallel to the subligamental ridge (SLR), is flanked by paired tensilia (TEN). Arrows show the parallel alignment of cytophaga-like bacteria in the resilium.

Table 9. Frequency of bacterial lesions in juvenile diploid *Crassostrea gigas* treated with hydrogen peroxide in trial 1, July 2003. Hydrogen peroxide administered at 100 or 1000 ppm for 15 or 30 minutes. Exposure time in parenthesis. Oysters were sampled at the end of each week. Resilium and tensilium lesions may be found in the same oyster. Frequencies determined through survey of histological sections.

	Animals (*n*)	Normal hinge	Resilium lesion	Tensilium lesion
Pretreatment				
Control A				
n	7	2	5	1
%	100	28	71	14
Control B				
n	10	3	7	0
%	100	30	70	0
H100 (15)				
n	7	3	4	0
%	100	42	57	0
H100 (30)				
n	6	1	5	2
%	100	16	83	33
H1000 (15)				
n	7	2	5	2
%	100	28	71	28
H1000 (30)				
n	6	2	4	0
%	100	33	66	0
Week 1				
Control A				
n	5	3	2	0
%	100	60	40	0
Control B				
n	9	2	7	4
%	100	22	77	44
H100 (15)				
n	2	2	0	0
%	100	100	0	0
H100 (30)				
n	4	2	2	0
%	100	50	50	0
H1000 (15)				
n	8	5	3	0
%	100	62	37	0
H1000 (30)				
n	8	4	4	0
%	100	50	50	0

Table 9. Frequency of bacterial lesions in juvenile diploid *Crassostrea gigas* treated with hydrogen peroxide in trial 1, July 2003. Hydrogen peroxide administered at 100 or 1000 ppm for 15 or 30 minutes. Exposure time in parenthesis. Oysters were sampled at the end of each week. Resilium and tensilium lesions may be found in the same oyster. Frequencies determined through survey of histological sections (continued).

	Animals (*n*)	Normal hinge	Resilium lesion	Tensilium lesion
Week 2				
Control A				
n	6	0	6	3
%	100	0	100	50
Control B				
n	9	1	8	3
%	100	11	88	33
H100 (15)				
n	8	2	6	0
%	100	25	75	0
H100 (30)				
n	7	2	5	3
%	100	28	71	42
H1000 (15)				
n	10	1	9	6
%	100	10	90	60
H1000 (30)				
n	6	3	3	1
%	100	50	50	16
Week 3				
Control A				
n	7	0	7	3
%	100	0	100	42
Control B				
n	10	2	8	2
%	100	20	80	20
H100 (15)				
n	7	2	5	0
%	100	28	71	0
H100 (30)				
n	9	2	7	0
%	100	22	77	0
H1000 (15)				
n	3	0	3	0
%	100	0	100	0
H1000 (30)				
n	7	2	5	0
%	100	28	71	0

Table 10. Frequency of bacterial lesions in juvenile triploid *Crassostrea gigas* treated with hydrogen peroxide in trial 2, August 2003. Hydrogen peroxide administered at 100 or 1000 ppm for 15 or 30 minutes. Exposure time in parenthesis. Oysters were sampled at the end of each week. Resilium and tensilium lesions may be found in the same oyster. Frequencies determined through survey of histological sections.

	Animals (*n*)	Normal hinge	Resilium lesion	Tensilium lesion
Pretreatment				
Control A				
n	5	2	3	0
%	100	40	60	0
Control B				
n	1	0	1	0
%	100	0	100	0
H100 (15)				
n	9	3	6	0
%	100	33	66	0
H100 (30)				
n	8	2	6	2
%	100	25	75	25
H1000 (15)				
n	5	2	3	0
%	100	40	60	0
H1000 (30)				
n	6	1	5	3
%	100	16	83	50
Week 1				
Control A				
n	3	0	3	0
%	100	0	100	0
Control B				
n	3	3	0	0
%	100	100	0	0
H100 (15)				
n	8	1	7	2
%	100	12	87	25
H100 (30)				
n	6	2	4	3
%	100	25	75	50
H1000 (15)				
n	8	2	6	3
%	100	25	75	37
H1000 (30)				
n	5	3	2	1
%	100	60	40	20

Table 10. Frequency of bacterial lesions in juvenile triploid *Crassostrea gigas* treated with hydrogen peroxide in trial 2, August 2003. Hydrogen peroxide administered at 100 or 1000 ppm for 15 or 30 minutes. Exposure time in parenthesis. Oysters were sampled at the end of each week. Resilium and tensilium lesions may be found in the same oyster. Frequencies determined through survey of histological sections (continued).

	Animals (*n*)	Normal hinge	Resilium lesion	Tensilium lesion
Week 2				
Control A				
n	7	1	6	1
%	100	14	85	14
Control B				
n	7	0	7	1
%	100	0	100	14
H100 (15)				
n	11	1	10	6
%	100	9	90	54
H100 (30)				
n	8	3	5	2
%	100	37	62	25
H1000 (15)				
n	8	3	5	2
%	100	37	62	25
H1000 (30)				
n	8	2	6	1
%	100	25	75	12
Week 3				
Control A				
n	8	2	6	4
%	100	25	75	50
Control B				
n	9	1	8	1
%	100	11	88	11
H100 (15)				
n	10	1	9	1
%	100	10	90	10
H100 (30)				
n	8	3	5	2
%	100	37	62	25
H1000 (15)				
n	11	4	7	2
%	100	36	63	18
H1000 (30)				
n	4	0	4	0
%	100	0	100	0

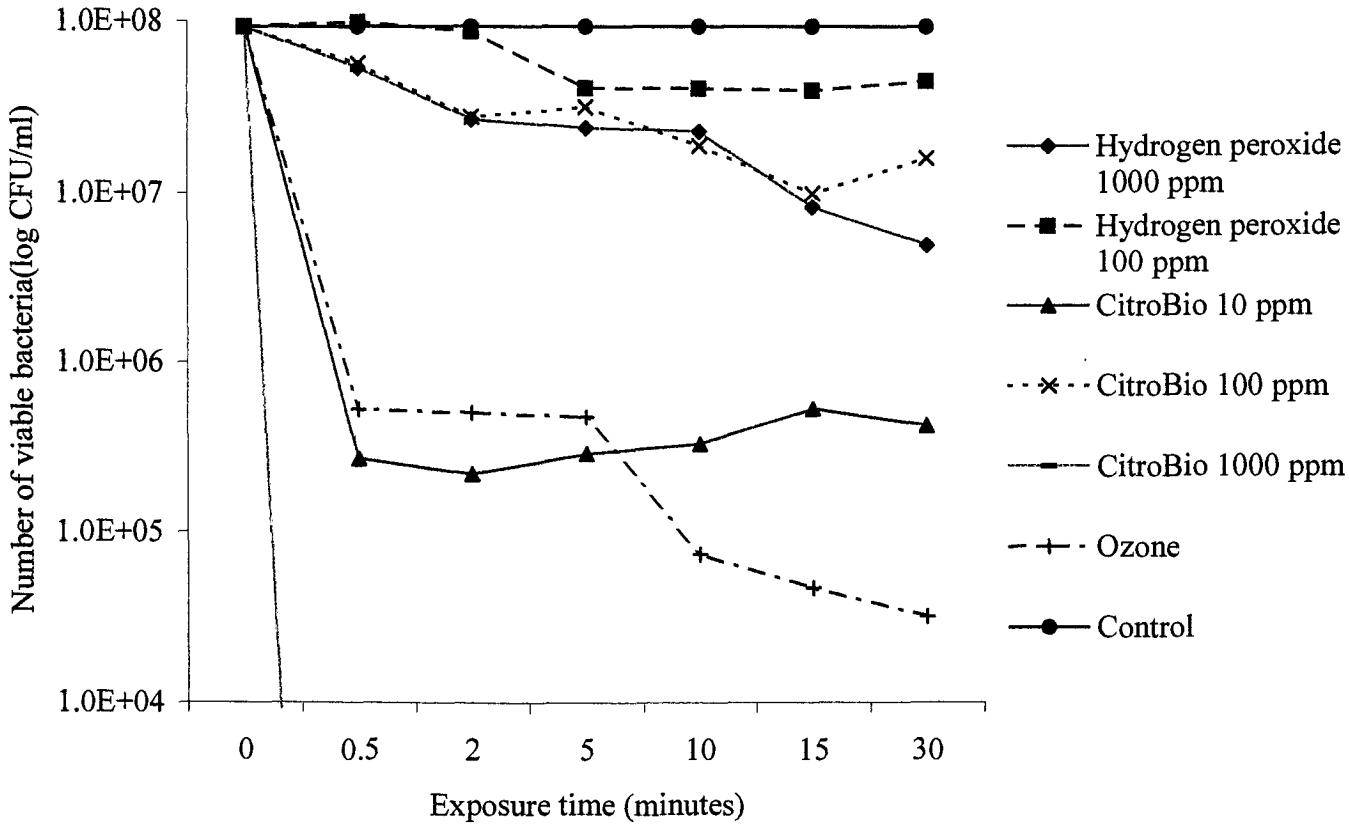

Figure 6. Inactivation of cytophaga-like bacteria during treatments with hydrogen peroxide, CitroBio, and ozone. Hydrogen peroxide concentrations were 100 and 1000 ppm. CitroBio concentrations were 10,100 and 1000 ppm and ozone was administered at 0.1 mg/L. Each point represents colony forming units counted at the specific time interval.

treatment. After the first week of treatment with hydrogen peroxide, this reduction was significant. Hydrogen peroxide reduced the number of folliculinids (trial 1 and 2) when administered at 100 ppm for 30 minutes and 1000 ppm for 15 or 30 minutes (Figures 7, 8).

Histological survey methods also revealed one other possible parasite within the body of the oyster. Small numbers (1-4 individuals) of a *Trichodina* sp. (Figure 9) were present in oysters in both trials. These organisms were located primarily in the gills and attached to the wall of the visceral body.

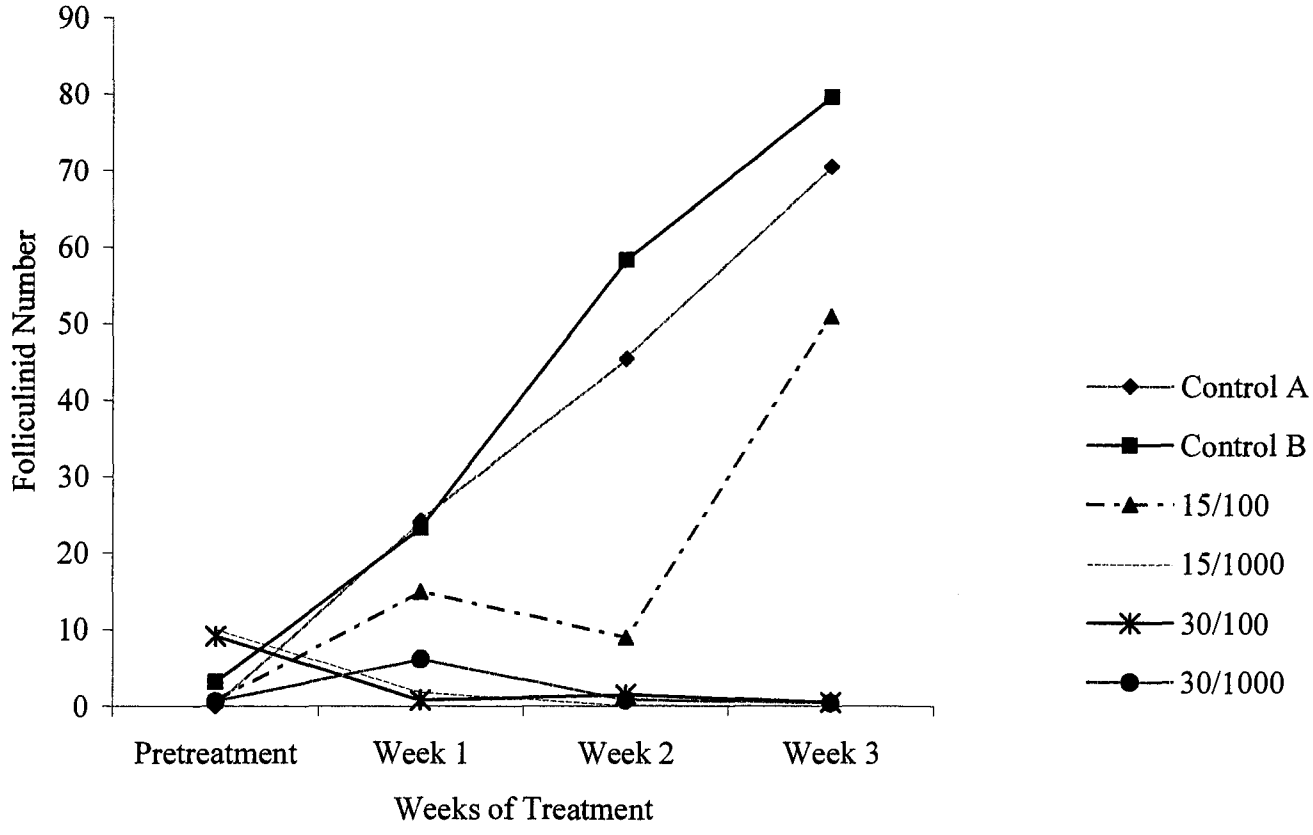

Figure 7. Mean number of folliculinids in trial 1, July 2003. Hydrogen peroxide was administered for 15 or 30 minutes at 100 and 1000 ppm. Controls received no treatment.

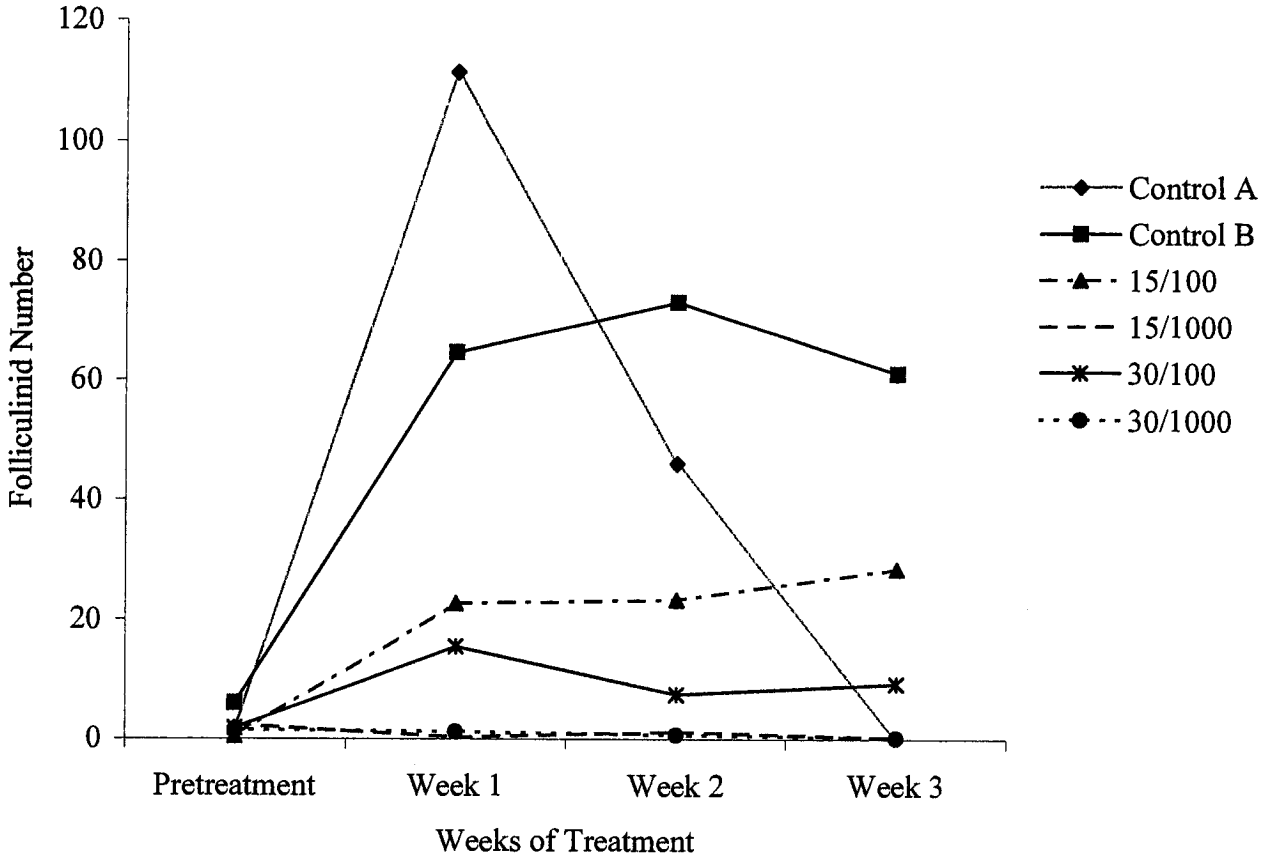

Figure 8. Mean number of folliculinids in trial 2, August 2003. Hydrogen peroxide was administered for 15 or 30 minutes at 100 and 1000 ppm. Controls received no treatment.

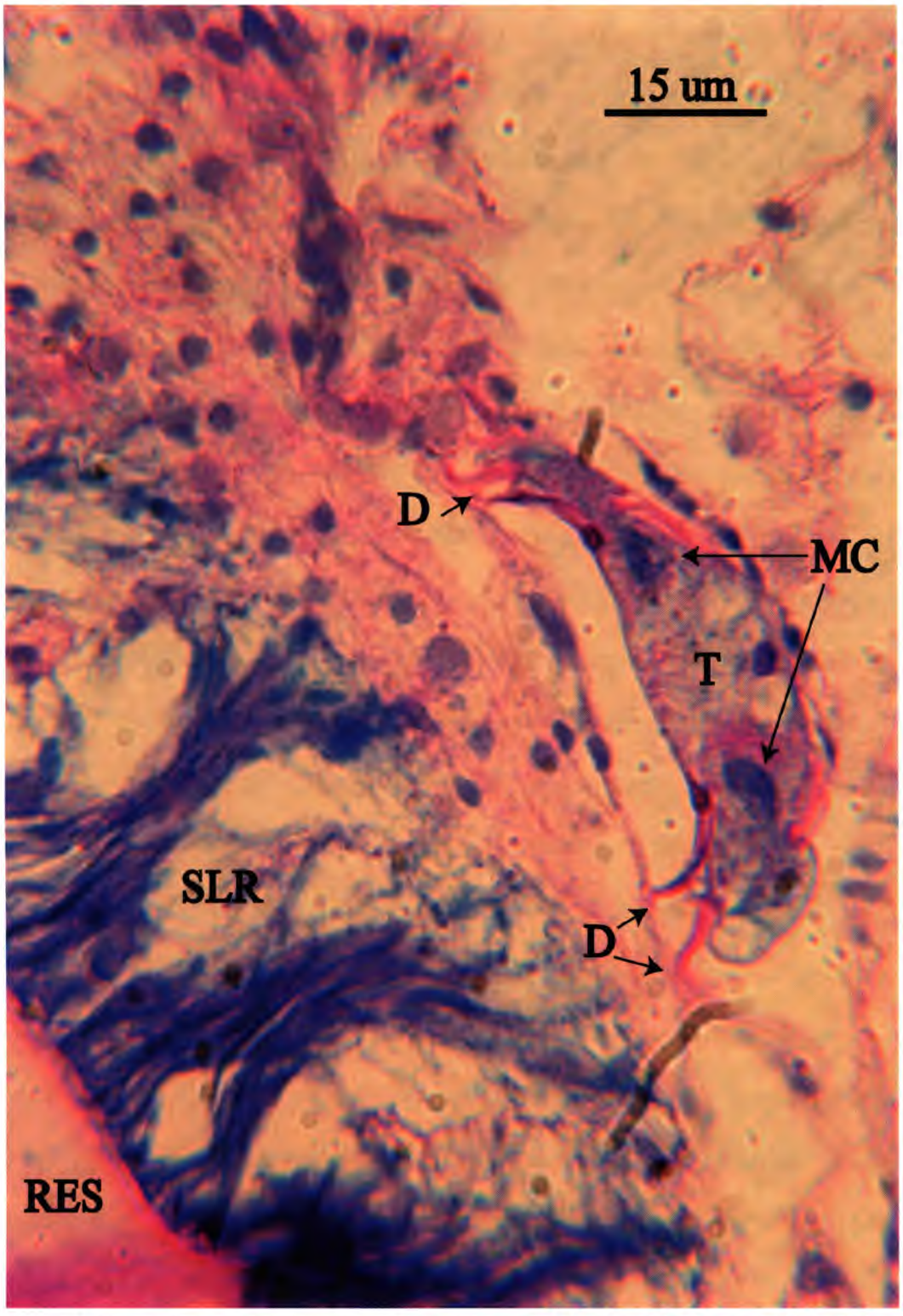

Figure 9. *Trichodina* sp. (T) found near the subligamental ridge (SLR) and resilium (R) of a juvenile Pacific oyster (*Crassostrea gigas*). This cross sectional view of the organism shows the horshoe shaped macronucleus (MC) and the dentricles (D).

DISCUSSION

My study shows that *Cytophaga*–like bacteria isolate C1B-2 is most susceptible to the commercial citrus product CitroBio. My laboratory study showed that submersion in 1000 ppm CitroBio for 30 seconds is sufficient for 100% bacterial reduction of C1B-2. CitroBio administered at 10 ppm was more effective at reducing bacteria than at 100 ppm. It is unclear why this was the case. However, it is possible that cell membrane structure limited permeability of CitroBio at a 100 ppm. For example, ethanol at concentrations of 50-70% will denature proteins and makes lipids soluble. However, if administered at lower or higher concentrations ethanol may have minimal or even no effect (Todar 2002, Madigan et al. 2003, Brenneman 2006, personal communication). Further study with CitroBio is suggested in order to completely understand its effectiveness.

There are several advantages to using CitroBio for disease control. First, a 360 ml bottle of CitroBio can be obtained for $19.95. It is even cheaper in 1, 5, and 275 gallon containers. Using the dosage recommended by the manufacturer, one 360 ml bottle will make 48 gallons (96 gallons if the aquaculturist follows guidelines specified in my study). Second, the product is safe for human use and has FDA approved ingredients. CitroBio is also acceptable for organic use according to the Organic Materials Review Institute (CitroBio Inc. 2004). The usage rate recommended by the CitroBio company is approximately 2000 ppm (7.4 ml/3.79 liters) for 5 to 10 minutes (CitroBio Inc. 2004). A 20,000 ppm treatment of CitroBio was applied to alfalfa seeds contaminated with *Escherichia*

coli and *Salmonella* (Fett and Cooke 2003). Results showed that CitroBio was effective only against *Salmonella*. Peer reviewed research with regard to CitroBio is scarce. My study shows that more science based research on this product in controlled settings may further elucidate potential beneficial qualities for use in shellfish and fish culture.

Ozone was also effective in controlling the *Cytophaga*-like bacteria isolate C1B-2. There was a sharp decline in bacterial numbers within the first 30 seconds of exposure to 0.1mg O_3/L ozone and again after 5 minutes. This downward trend led to an overall 3.5 log reduction of bacterial density over a 30 minute period. Based on the results of my study, ozone appears to be suitable for treatment of hinge ligament disease in oysters. However, there are several disadvantages to its use. The initial cost of the equipment and the price of operating and maintaining the machinery is high. Water quality at the site may limit the rate at which ozone is able to oxidize pathogens. Free radicals such as free ozone, ozonides and hyperperoxides are difficult to measure and potentially dangerous byproducts to both humans and fish. In addition, personnel using the machinery must be adequately trained and provided with adequate safety gear. Ozone can be damaging to the respiratory tract of humans at very low levels (Summerfelt and Hochheimer 1997). The Federal Occupational Safety and Health Administration (OSHA) allows an exposure level of less than 0.1 mg O_3/L on a time weighted average for an 8 hour work period (Summerfelt and Hochheimer 1997, Federal Occupational Safety and Health Administration 2006).

In-vitro studies with known fish pathogens, *Aeromonas salmonicida*, *Vibrio salmonicida*, *Vibrio anguillarium*, *Yersenia ruckeri* and infectious pancreatic necrosis virus (IPNV), have shown four log reductions in viable counts of pathogens within 3 minutes when ozone is used at 0.1 -0.2 mg O_3/L in either fresh, brackish or sea water (Liltved et al. 1995). Colberg and Lingg (1987) showed a greater than 99% reduction in bacterial fish pathogens such as, *Aeromonas salmonicida*, *A. liquefaciens*, *Pseudomonas flourescens* and *Yersenia ruckeri* when exposed to 1.0 and 0.1 mg O_3/L of ozone for 1 minute. Although work with ozone in shellfish hatcheries has been limited, ozone applied to seawater was effective in controlling against *Vibrio*, *Flavobacterium* and *Achromobacter* at 0.56 mg O_3/L for 2 hours (Blogoslawski et al. 1978). Based on the results of my study and the literature, it is reasonable to suggest that ozone administered at 0.1 mg O_3/L is sufficient to cause mortality in a variety of fresh and salt water pathogens including *Cytophaga*-like bacteria.

Based on my laboratory trials, hydrogen peroxide was the least effective of all treatments used. Hydrogen peroxide administered at 1000 ppm for 30 minutes showed a 1.3 log reduction in the viable count of the *Cytophaga*-like bacteria isolate C1B-2. A 0.3 log reduction in bacterial density was shown for a treatment of 100 ppm solution of hydrogen peroxide for 30 minutes. Although other treatments were significantly better, there remains a distinct advantage to its use. Hydrogen peroxide (3%) is inexpensive. A 473 ml bottle can be purchased over the counter for approximately $0.77. In addition, hydrogen peroxide easily reverts back to water and oxygen with no detectable affect on water quality

(Marking et al. 1994, Arndt and Wagner 1997). Some of the drawbacks to its use are again related to water quality. Hydrogen peroxide is similar to ozone in that its stability is dependent on a variety of water quality parameters. Tort et al. (2003) found that the highest rate of decomposition of hydrogen peroxide was in a static environment with organic matter present. Other processes that may initiate breakdown are light, heat and high pH (Tort et al. 2003). An advantage to using hydrogen peroxide is that it is readily available and used quite frequently in hatchery applications to treat a wide variety of fish pathogens. Varying concentrations of 3% to 35% hydrogen peroxide have been administered at 75-200 mg/L to successfully treat fish bacteria such as *Flavobacterium columnare* and *Flavobacterium branchiophilium* and parasites such as protozoans, sea lice and monogenetic trematodes (Marking et al. 1994, Speare and Arsenault 1997, Lumsden et al. 1998, Rach et al. 2000). Menghini (2000) showed that a 3×10^3 ppm hydrogen peroxide solution was effective in inhibiting the growth of the *Cytophaga pectinovora* and *Cytophaga aquatilis* in a freshwater hatchery. Based on results of my study, hydrogen peroxide is an economical treatment of *Cytophaga*-like bacteria; however, it is not the most effective treatment when administered at 100 or 1000 ppm. Further work with this theraputent should be carried out at higher dosages.

In the *in vivo* portion of my study, I attempted to determine the effects of hydrogen peroxide, as a theraputent, on hinge ligament erosion. I found that treatment with hydrogen peroxide did not cause mortality nor any negative effects on growth among juvenile *C. gigas*. However, erosive lesions in the functional

portions of the hinge ligament still persisted after treatment. Quantifying the effects of bacterial destruction on the hinge ligament proved difficult in this portion of my study. I tried to use the data collected as an indicator of the condition of the hinge ligament and subsequently of the health of the juvenile oysters.

Although hydrogen peroxide was very effective in removing biofouling organisms from the external shell of juvenile oysters in my study, it does not appear that a specific concentration (ppm) or time was responsible for the increase in resilium depth. Both control groups and treated groups showed similar results with respect to resilium depth increases over time. Therefore, it is more likely that the rapid growth of *C. gigas*, combined with the ability of the subligamental ridge to secrete the resilium matrix, allowed the oysters to avoid a pathological condition. Also, a lack of disease-free juvenile oysters for this study limited my ability to determine if hydrogen peroxide had an inhibitory effect on *Cytophaga*-like bacteria. All juvenile oysters utilized in this study were obtained from a domestic hatchery. As such, they had been exposed to *Cytophaga*-like bacteria prior to the start of the experiment. This is evidenced by the percentage (70%) of sampled *C. gigas* with eroded hinge ligaments prior to any treatment.

The severity of the hinge ligament lesion was determined by using a ratio of remaining resilium thickness to subligamental ridge height. This method was suggested by Dungan (2003, personal communication) in order to compare related microscopic structures at consistent depths and locations. The exact rate at which the subligamental ridge produces the resilium and tensilium has not been studied.

Dungan (1987) observed that the height of the subligamental ridge in ten and thirty-day post-set juvenile oysters increased with age. Results of my study indicate that as the subligamental ridge becomes larger, with the age of the oyster, the resilium is secreted faster. This is most evident when comparing trial 1 pretreatment groups with week three groups (Table 7). One hundred percent of pretreatment oysters in trial 1 had a resilium depth that was less than or equal to the size of the subligamental ridge. By week three, oysters had grown such that 75 % of oysters had a resilium depth that was greater than or equal to the size of the subligamental ridge. Eighty-one percent of pretreatment oysters in trial 2 had a resilium depth that was less than the subligamental ridge, while at week three, 58% of oysters had a resilium depth greater than or equal to the depth of the subligamental ridge. Trials 1 and 2 indicate that the subligamental ridge is capable of secreting the resilium-tensilium matrix at a rate faster than the ability of the *Cytophaga*-like bacteria to hydrolyze it. Based on my results, I believe that the ratio method employed in my study was useful in further understanding the relationship between the subligamental ridge and the resilium. To my knowledge, this method has not been employed with regard to juvenile oyster histology.

Further histological and field observations utilized in my study showed evidence of two ciliate species on both the interior and exterior surfaces of juvenile *C. gigas*. Heterotrich ciliates of the family Folliculinidae were observed on the exterior shell surface of oysters in this study. These animals form permanent attachments, as adults, to living or nonliving solid substrates (Andrews 1949, Pechenik 2000). The animal itself lies within a protective encasement (test

or lorica) and as adults; they are capable of dedifferentiating to a simplified, free swimming "larval" phase. They leave the lorica behind and relocate to a new site. In some instances, the animal will dedifferentiate, and the anterior portion of the animal will relocate, while the remaining half is left at the original location. Both then redifferentiate to new adults (Andrews 1949, Pechenik 2000). Adult folliculinids have a right and left feeding lobe, covered with longitudinal rows of cilia, which they extend outside of the lorica to feed. At the base of the lobes a ciliated funnel leads to the inner mouth (Andrews 1949). Andrews (1949) observed dense settlement of *Parafolliculina amphora* on oysters. The ciliates, *Folliculina viridis* and *Pebrilla paguri*, have also been described as epibionts on crustaceans in the Mediterranean (Fernandez-Leborans 2003). The folliculinid observed in this study is tentatively identified as a *Folliculina* sp. based on lorica morphology. Juvenile *C. gigas* (control groups) were intensively covered by this folliculinid species. Based on the continued growth of the juvenile oysters, *Folliculina* sp. did not appear to affect biological activities such as respiration and excretion. This folliculinid species has not been observed before in relation to aquaculture activities on Humboldt Bay. Therefore, it is suggested that the conditions under which this experiment was implemented were optimal for this ciliate species and suboptimal for juvenile *C. gigas* cultivation.

Ciliates of the genus *Trichodina*, observed in this study, have been reported in bivalves from various parts of the world (Lauckner 1983). These organisms primarily attach themselves to gills or mantle cavity and are characterized by their dome-like discoidal shape, C-shaped macronucleus and

basal disc bearing hooklets or denticles. Trichodinids feed on bacteria and are believed to be harmless commensals (Lauckner 1983). However, Boussaïd et al. (1999) reported that adult *C. gigas* with heavy gill infestation of trichodinids exhibited an inflammatory response of the gill along with deformed epithelium, and deformed hemocytes. Bower (1987) found that 23-100% of adult *C. gigas* were infected with an average of 11 ciliates per oyster. The author also found no evidence of pathology associated with the *Trichodina* spp., even in one oyster with 312 *Trichodina.* Cultivated scallops from Tongoy Bay, Chile, were parasitized in the gills (prevalence of 56%) by a trichodinid; however, no pathological changes were evident (Lohrmann et al. 2002). Histological sections of juvenile *C. gigas* in my study were only lightly infected with trichodinids. These ciliates may interfere with respiratory function of the gill increasing the chances of a pathological condition (Boussaïd et al. 1999). However, this was not evident in juvenile oysters from Humboldt Bay.

The prevalence of hinge ligament lesions in populations of juvenile *C. gigas*, that have experienced mortality in previous studies, have been reported as 78% and 93% (Elston 1984, Dungan and Elston 1988). Hinge ligament erosion in juvenile *C. gigas* in my study is equally high (71% in trial 1 and 74% in trial 2). However, no mortality was noted in my study. Out of the 71% of erosive lesions found in July (trial 1), only 17% had accompanying tensilium erosion. However, in August (trial 2), 22% of hinge ligament lesions had tensilium erosion. Oysters with an eroded resilium may or may not have tensilium erosion. No oysters were observed with tensilium lesions only. Based on similar resilium and tensilium

observations in juvenile oysters, Dungan (1987) suggested that the resilium was more vulnerable to *Cytophaga*-like bacteria erosion due to its physical or chemical properties.

Bacterial colonization of the hinge ligament may initially be benign (Dungan 2003, personal communication). *Cytophaga*-like bacteria colonize and erode the older, distal layers of the ligament that are exposed to the environment. This may actually be beneficial to the oysters. A pathological condition, however, may occur if the newly secreted interior resilium matrix is compromised thereby allowing the bacteria to penetrate. Signs of a pathological condition would be a disruption in basic physiological functions such as, feeding, respiration and excretion. In a previous study, oysters with extensive hinge ligament erosion had low profile (low cell height) digestive gland absorptive cells that suggested starvation (Elston 1982). Elston (1999) categorized the condition of the digestive gland tubular epithelium based on height of the absorptive cells. High epithelial height is considered normal and represents active ingestion and absorption. Medium height indicates a condition of reduced feeding and metabolism. Low epithelial height indicates non-feeding and may be considered a pathological condition (Elston 1999). The physical state of the digestive gland absorptive cells in juvenile oysters in my study did not indicate a pathological condition. However, my observations did suggest that a reduced feeding condition, as evidenced by a medium absorptive cell height, may have been present in a portion of the study population. This reduced feeding could be a result of a weakened

hinge ligament or lack of available food. However, the latter is unlikely and could not be verified.

In addition to maintaining physiological functions, the hinge ligament also acts as a physical barrier against opportunistic pathogens. Elston (1984) originally postulated that vibriosis in juvenile oysters was preceded by destruction of the hinge ligament barrier. Dungan and Elston (1988) attempted to test this hypothesis. If erosion of the hinge ligament was followed by bacterial infection in the mantle tissue, then it was hypothesized that those oysters with completely liquefied and perforated hinge ligaments would also have bacterial infections in other tissues. They reported that 7% of oysters surveyed had perforated hinge ligaments while 47% had mantle tissue infections. However, they were unable to provide evidence that hinge ligament erosion was the portal of entry for other bacteria. No bacteria were observed in the pallial space of juvenile *C. gigas* used in my study. This suggests that the hinge ligament barrier was not penetrated.

Temperature, salinity, pH, and poor management practices (overcrowding) may increase the likelihood of a pathological condition associated with *Cytophaga*-like bacteria. *In vitro* studies conducted by Dungan (1987) and Dungan et al. (1989) showed that liquefaction of the hinge ligament by *Cytophaga*-like bacteria occurs repeatedly in different strains at 15°C or higher. In my study, temperatures exceeded 15°C during all of trial 1 and 2. In addition, salinity ranged from 35-37 ppt. Regardless of temperature or salinity, my study shows that resilium depth continued to increase over time. This suggests that an unknown factor associated with poor management practices may play a critical

role in transforming a benign event into a pathological one. A more thorough examination of salinity and management practices such as overcrowding, water quality and cleanliness should be undertaken in future studies.

The efficacy of further experimental treatment regimes for hinge ligament disease will largely depend on the ability of the selected theraputent to penetrate the deepest erosive lesions. Colonization of *Cytophaga*-like bacteria begins on the exterior or outside surface of the hinge ligament. Therefore, it seemed appropriate to choose a treatment type that would disrupt external growth of the *Cytophaga*-like bacteria population. In my study, hydrogen peroxide was administered to juvenile oysters via freshwater baths. Juvenile *C. gigas* stop active pumping and seal their valves tightly when exposed to freshwater. I chose freshwater as the test medium based on a limited knowledge of the lethal concentration (LC_{50}) of hydrogen peroxide in saltwater. In addition, I was concerned over potential losses of juvenile oysters during an active production cycle. In this study, with the use of freshwater, hydrogen peroxide was unable to enter into the pallial cavity and was limited in its effectiveness on the exterior surface of the oyster. It is unknown whether entry into the pallial cavity by hydrogen peroxide or any other theraputent would inhibit *Cytophaga*-like bacteria activity. Hydrogen peroxide has been shown to be effective and non-lethal in saltwater, with short-lived or no side effects to Atlantic Salmon (*Salmo salar*), in the treatment of sea lice (Bowers et al. 2002) or to Pacific threadfin (*Polydactylus sexfilis*) in the treatment of *Amyloodinium ocellatum* (Montgomery-Brock 2001).

The results of my study have practical value for farmers who are struggling to maintain the health of their shellfish products. My study shows that natural alternatives exist that are both inexpensive and relatively harmless. However, further study is needed to clarify potential affects of their use under varying conditions (i.e. fresh vs. saltwater, temperature). Based on the successful results of *in vitro* use of CitroBio and the cost effectiveness of this product, it may be worthwhile, in the future, to perform field trials on *C. gigas* with this product. Additionally, bath treatments using hydrogen peroxide and CitroBio should be carried out in saltwater. Saltwater may be the correct delivery medium whereby treatment can effectively reach deep erosive lesions caused by *Cytophaga*-like bacteria.

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