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Microbial Flora of Pacific Oysters (*Crassostrea gigas*) Subjected to Ultraviolet-Irradiated Seawater[1]

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The ability of oysters to purge themselves of microbial contaminants was investigated by identifying the microorganisms retained by oysters after they have been subjected to ultraviolet (UV) light-treated seawater. A UV intensity of 960 μw per min per cm^2 reduced the microbial count of seawater from 263 to 13 per ml. The coliform multitube test (MPN) was reduced from a high of 17 to <0.18 per 100 ml. Over 75% of the microorganisms found in treated seawater were *Acinetobacter/Moraxella*, *Vibrio/Pseudomonas* type II, and *Flavobacterium/Cytophaga*. With the exception of coliforms, the microbial composition of oysters subjected to UV-treated seawater remained at levels comparable to the control oysters held in untreated seawater. Total counts ranged between 10^3 and 10^5/g. The microorganism most frequently encountered were *Flavobacterium/Cytophaga*, *Vibrio/Pseudomonas* type II, *Pseudomonas* type III or IV, *Acinetobacter/Moraxella*, gram-positive cocci and *Bacillus*. Together they comprised over 90% of the flora. Coagulase-positive, deoxyribonuclease-positive, and beta-hemolytic cocci were found in some samples, as were *V. parahaemolyticus*, *V. aliginolyticus*, and *Aeromonas* species.

The natural ability of oysters to cleanse themselves of contaminating microorganisms has been recognized for many years. As early as 1899, Herdman and Boyce (16) reported that oysters contaminated with *Salmonella typhi* could be rendered noninfectious if allowed to "drink" in a pollutant-free habitat. Later Wells (30, 31) found that oysters could be freed of "sewage" bacteria by suspending them in tanks containing seawater treated with calcium hypochlorite.

Shellfish purification with respect to enteric bacteria (2, 10, 11, 15) and virus (17, 20) has been studied. Little is known, however, about the responses of the natural microbial flora of oysters to purification. Colwell and Liston (7) reported the existence of a "commensal" microorganism in Pacific oysters. Later, Lovelace, Tubiash, and Colwell (19) observed that the composition of microbial flora of oysters merely reflected the geographical differences. This view of the passive nature of oyster microbial flora was supported by Murchelano and Brown (21).

The advantage claimed for ultraviolet (UV) light over that of chlorination is that it would least interfere with the normal pumping mechanism of oysters (13, 18, 28). The examination of microbial flora of oysters subjected to UV-treated seawater, therefore, would provide an insight into the dynamic relationship between microorganisms and shellfish.

MATERIALS AND METHODS

Purification system. The oyster purification facility of the Northwestern Water Hygiene Laboratory, Environmental Protection Agency, Gig Harbor, Wash., was used. Seawater was passed by gravity through a UV unit described by Kelly (18). The unit consisted of 13 30-w germicidal lamps arranged in a linear configuration. Each lamp emitted almost exclusively 253.7-nm radiation. One aquarium received irradiated water and another nonirradiated water (control). Dispersing manifolds located at the intake produced laminar flow with minimal transverse mixing in each aquarium.

The flow rate through the UV unit was adjusted to 15 liters/min which led to a retention time of 15 sec.

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This provided a UV intensity of 960 μw per min per cm^2. A portion of this water (12 liters/min) was introduced into the aquarium. The flow rate was in excess of the minimum required for shellfish (29) and satisfied the dissolved oxygen, turbidity, and temperature requirements established by Furfari (12) for Pacific oysters.

One day before the experiment, the UV unit was partially dismantled and cleaned with a chlorine solution. The wooden aquaria and the polyvinyl-chloride pipe fittings were subjected to the same rigorous cleaning and disinfection.

Oysters. Pacific oysters measuring 20 to 30 cm in length were harvested from a commercial oyster bed 2 hr before the experiment. The test animals were freed from fouling organisms, mud, and other debris adhering to the shell by scrubbing in fresh water. The shell stock was then divided into two lots of 25 oysters each and placed into the aquaria. Five oysters were immediately sacrificed and examined for 0-hr microbial content.

Five actively feeding oysters were selected at random from each aquarium at 12, 24, 48, and 72 hr. At the same time, both irradiated and control seawater samples were obtained from the influent ends of the aquaria.

Microbial isolation. Microorganisms from shellfish samples were isolated by the following procedure. Oysters were shucked aseptically according to procedures recommended for shellfish by the American Public Health Association (1). The contents from five oysters were then pooled and weighed aseptically. After the addition of Butterfield's phosphate buffer (26), the 1:1 mixture was homogenized for 90 sec in a Waring Blendor. Serial dilutions were made of the homogenate, and 0.1-ml portions were spread-plated, in triplicate, on agar which contained 0.5% peptone, 0.5% tryptone, 0.25% yeast extract, 0.5% NaCl, 0.1% glucose, and 2.0% agar (Difco).

Microorganisms in seawater were isolated by the membrane filter (Millipore Corp., Bedford, Mass.) technique with gridded membrane filters with a pore size of 0.45 μm. Ten-, 100-, and 1,000-ml amounts of seawater were filtered in duplicate through a sterile filter apparatus. After filtration, the membrane was removed with sterile forceps and rolled onto the agar plates. All plates were incubated at 25 C for 3 days.

In addition, bacteriological analysis included the standard multitube (MPN) test for coliform and fecal coliform bacteria and plate count at 35 C (1).

Microbial identification. After 3 days of incubation, plates from dilutions containing 100 to 300 colonies were selected, and each colony was identified by the computer-assisted replica plating method of Corlett, Lee, and Sinnhuber (8, 9).

The original identification scheme was modified to include *Vibrio* and *Cytophaga*, which the authors indicated would be grouped with *Pseudomonas* type II and *Flavobacterium*, respectively. *Achromobacter* in the original scheme was reclassified as *Acinetobacter/Moraxella*. Because of the taxonomic uncertainty of this group of bacteria (23, 27), no further distinction was attempted.

The gram-positive rods were identified as *Bacillus*, *Lactobacillus*, or "coryneforms" by the scheme of Shiflett, Lee, and Sinnhuber (25).

The gram-positive cocci were restreaked on Brain Heart Infusion agar (BHI) and incubated at 25 C for 3 days. The cultures were then examined for coagulase production on polymyxin-coagulase-mannitol agar (PCMA; reference 22), for beta-hemolysis on human blood-agar, and for deoxyribonuclease activity on DNAse agar (Difco).

Hemolytic vibrios. The starch-salt medium of Baross and Liston (3, 4) was employed for the initial isolation and presumptive confirmation of hemolytic vibrios. Oyster homogenates or seawater was spread-plated on starch-salt-agar and incubated anaerobically at 35 C in an Anaerojar (BBL) for 72 hr. All white, nonspreading, and discrete colonies, which showed starch hydrolysis, were transferred to triple sugar-iron-agar (TSI) containing 3% NaCl, seawater broth (4), Trypticase soy agar (BBL), human blood-agar, and motility agar (Difco). The gram-negative, motile, pleomorphic, beta-hemolytic, and H_2S-negative isolates, which gave acid butts and alkaline slants on TSI, were considered presumptive positives for hemolytic vibrios. They were then subjected to differential tests for *Vibrio* proposed by Gibbs and Shapton (14) and Shewan, Hobbs, and Hodgkiss (24).

RESULTS AND DISCUSSION

Microbial flora of seawater and oysters. The microbial counts of seawater monitored during the 72-hr period are presented in Fig. 1. The coliform counts, expressed in MPN, fluctuated between 0.8 and 17 in nonirradiated seawater but dropped to <0.18 after UV irradiation.

The 35 and 25 C counts were also reduced by a factor of 10 to 100 by UV treatment.

Figure 2 illustrates the microbial counts obtained from oysters. The coliform count was reduced by a factor of 10, but both 35 and 25 C counts remained relatively unchanged in oysters subjected to UV-treated seawater.

Since UV-treated seawater provided a smaller input of microorganisms, the failure to reflect this reduction in microbial count in oysters, with the exception of coliforms, could be due to the persistence of a stable microbial population in the oysters.

The difference between the composition of the microbial populations in treated seawater and the oysters further substantiates the semi-independent relationship of the two flora.

Table 1 shows the composite data obtained from all seawater samples taken during the 72-hr period. The microbial flora of untreated seawater contained larger percentages of *Pseudomonas* type I, *Vibrio/Pseudomonas* type II, *Acinetobacter/Moraxella*, and *Flavobacterium/Cytophaga*. After irradiation, only *Acinetobacter/Moraxella* remained predominant.

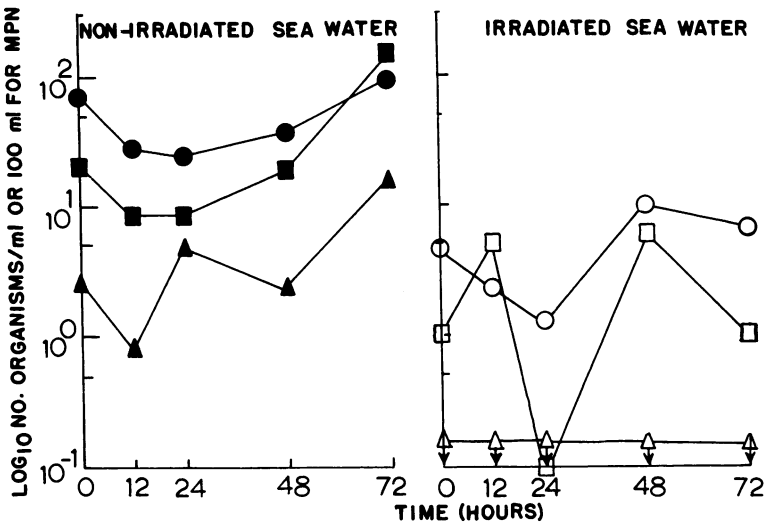

FIG. 1. *Microbial counts in seawater before and after exposure to UV. Each count shown is the average of two determinations. Symbols:* ● ○, *25 C plate count;* ■ □, *35 C plate count;* ▲ △, *coliform MPN. Arrows pointing downward indicate a value less than that indicated.*

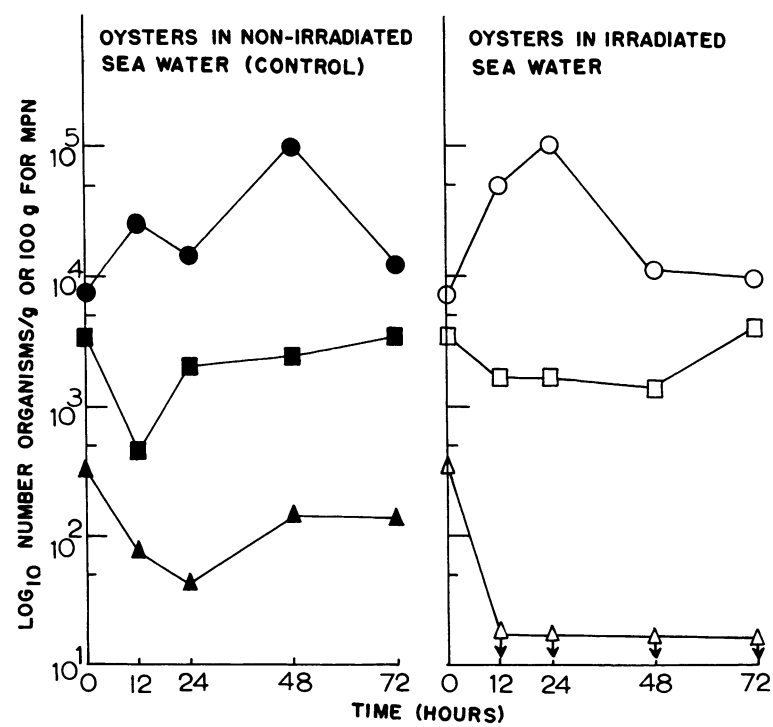

FIG. 2 *Microbial counts of oysters sampled during a 72-hr period. Each count shown is the average of two determinations. Symbols:* ● ○, *25 C plate count;* ■ □, *35 C plate count;* ▲ △, *coliform MPN. Arrows pointing downward indicate a value less than that indicated.*

A different picture is presented by the microbial flora of the oysters (Table 2). Regardless of UV treatment, the microorganisms commonly found in oysters were *Vibrio/Pseudomonas* type II, *Acinetobacter/Moraxella*, *Flavobacterium/Cytophaga*, *Pseudomonas* type III or IV, and gram-positive cocci. Their respective numbers in five oyster samples obtained during the 72-hr purification period are presented in Fig. 3. It is noteworthy that the microbial flora of the oysters generally resembled those of untreated seawater (Table 1).

Another experiment conducted with a UV intensity of 12,000 μw per min per cm^2 revealed that the increased UV intensity did not increase the degree of microbial inactivation.

TABLE 1. *Microbial flora of UV-irradiated seawater*

Microorganisms	Per cent distribution	
	Control	Treated
Pseudomonas type I	21.6	1.7
Vibrio/Pseudomonas type II	26.2	16.7
Pseudomonas types III or IV	2.2	0.2
Acinetobacter/Moraxella	19.0	57.1
Flavobacterium/Cytophaga	14.4	11.1
Bacillus	1.1	1.3
Lactobacillus	0	0
"Coryneforms"	0	0
Yeasts	0	0
Gram-positive cocci	4.6	0.9
Coliforms	8.7	<0.1
Unidentified	2.0	11.1
Microbial count/ml	263	13
No. of colonies identified	126	171

TABLE 2. *Microbial flora of oysters subjected to UV-treated seawater*[a]

Microorganisms	Per cent distribution	
	Control	Treated
Pseudomonas type I	0	0
Vibrio/Pseudomonas type II	31.0	21.4
Pseudomonas type III or IV	7.0	18.4
Acinetobacter/Moraxella	17.3	12.8
Flavobacterium/Cytophaga	27.0	29.5
Bacillus	4.0	2.7
Lactobacillus	3.0	0
"Coryneforms"	0	0
Gram-positive cocci	8.0	8.1
Yeasts	0.2	1.7
Coliforms	<0.2	<0.2
Fecal coliforms	<0.2	<0.2
Unidentified	3.0	5.4
Microbial count/g	3.8×10^4	4.8×10^4
No. of colonies identified	385	549

[a] Averages obtained from five determinations.

The microbial flora of seawater and those of oysters were essentially the same as those reported with 960 μw per min per cm^2 (Tables 1 and 2).

Gram-positive cocci. The level of gram-positive cocci in the test oysters appeared to be higher than that reported in the literature (6, 7, 19). Since this group might contain pathogenic *Staphylococcus aureus*, a series of tests were conducted to examine their potential pathogenicity.

Table 3 shows the sources, number of isolates tested, and their reactions on PCMA agar (21), DNAse agar (Difco), and human blood-agar.

In a total of 369 organisms tested, 57% produced deoxyribonuclease, 36% were beta-hemolytic and 20.5% were coagulase-positive; 18% of them exhibited positive reactions to all three tests.

Hemolytic vibrios. Recent reports by Baross and Liston (4) on the isolation of *Vibrio parahaemolyticus* from oysters and clams gathered from the same area prompted a search for this organism. The experiment was conducted during August to coincide with the reported high incidence of this organism in the estuary. Altogether, 20 oysters and an equal number of seawater samples were examined. The results of these examinations are summarized in Table 4.

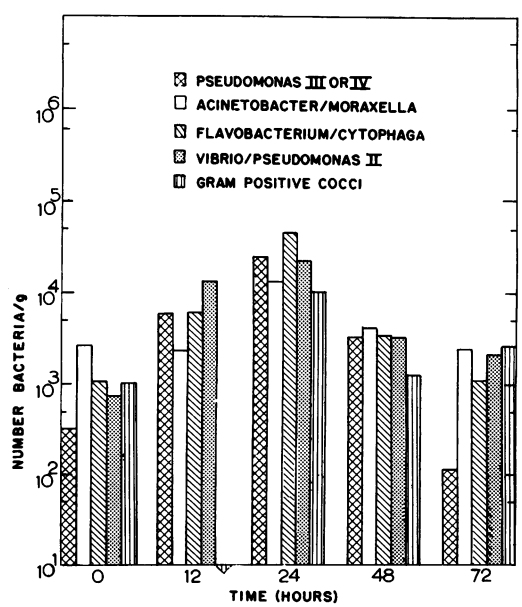

FIG. 3. *Microbial counts of the five most frequently found bacteria in oysters subjected to irradiated seawater.*

TABLE 3. *Potential pathogenicity exhibited by gram-positive cocci*

Source	Total no.	Per cent positive			
		Coagulase	Deoxyribo-nuclease	Beta hemolysis	To all three
Nonirradiated seawater	42	26	38	40	19
Irradiated seawater	32	15	81	28	18
Control oysters	155	23	62	40	23
Treated oysters	140	18	47	36	14

TABLE 4. *Identity of presumptive positive hemolytic vibrios*

Determination	No. presumptive positive[a]	*Vibrio parahaemolyticus*	*Vibrio alginolyticus*	*Vibrio* sp.	*Aeromonas* sp.	Unidentified
Seawater	6	0	2	2	2	0
Irradiated seawater	1	0	0	0	0	1
Control oysters	13	1	0	0	8	4
Treated oysters	5	1	0	0	1	3

[a] Gram-negative, pleomorphic, hemolytic, H_2S-negative rods which hydrolyzed starch-agar and gave alkaline slants and acid butts on TSI.

Based on biochemical reactions, two isolates from oysters were identified as *V. parahaemolyticus*. Among 18 presumptive positives, the two isolates were methyl red-positive, sucrose-positive, indole-positive, and cellobiose-negative, fermentative in Hugh Leifson's glucose medium, liquefied gelatin, and were arginine decarboxylase-negative and lysine decarboxylase-positive. They grew in 3 and 7% but not in 0 and 10% NaCl. Both were cytochrome oxidase-positive and sensitive to vibriostatic agent O/129 (2,4-diamino-6,7-diisopropyl pteridine). Four other vibrios were isolated from seawater, two of which were identified as *V. alginolyticus*. Both produced acetylmethylcarbinol and were fermentative in sucrose and cellobiose. They grew in the presence of 3, 7, and 10% but not in 0% NaCl.

Eleven isolates were identified as beta-hemolytic *Aeromonas* species. They were oxidase-positive, inositol-negative, and insensitive to vibriostatic agent O/129. Those isolates which were cytochrome oxidase-negative and oxidative in Hugh Leifson's medium could not be classified.

The similarity between the microbial flora of seawater and those of oysters seems to indicate that the composition of microbial flora in oysters reflected the environment. Yet the microbial flora did not turn over as readily as might be expected from the data obtained with indicator organisms.

Coliforms and some *Pseudomonas* species appeared to be eliminated easily from oysters, but some potentially hazardous microorganisms, such as gram-positive cocci and *Vibrio* species, tended to persist for longer periods of time. A high incidence of hemolytic *Vibrio* from oysters which showed negative coliform count has been reported by Barrow and Miller (5).

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