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Microbiological Quality of Oysters *(Crassostrea gigas)* and Water of Live Holding Tanks in Seattle, WA Markets

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ABSTRACT

Oyster *(Crassostrea gigas)* and water samples from Live Holding Tanks at five different Seattle area retail markets were analyzed for microbiological quality indicators and for potential pathogens monthly from March to September, 1987. *Aeromonas hydrophilia* was the most frequently isolated potential pathogen in this study with a higher incidence in oysters (78%) compared to water (53%). *Vibrio cholerae* non 01 and *V. fluvialis* were isolated from oyster samples from two different markets but not from water. *V. alginolyticus* was isolated from 53% of the water samples but was not found in any of the oysters. One oyster sample had a non-pathogenic *Yersinia entercolitica*. *Yersinia* spp. were isolated from oyster samples from one tank at two sampling periods. *Salmonella typhimurium* was isolated from one oyster sample. Samples were examined for *Listeria* spp. during the August sampling period and none were detected. The aerobic plate count was similar for both oyster and water samples and averaged 2000 CFU/gm. Total coliform levels were significantly higher ($P<.05$) for oysters (525MPN/100gm) compared to water (11MPN/100ml). The degree of water turbidity, crowding and species diversity varied between markets and sampling periods.

Live Holding Tanks (LHT's) are increasingly being used for the display of retail seafood products and for wholesale storage. It is imperative that these tanks be set up and maintained correctly to avoid potential microbiological problems since many seafood products likely to be contained in an LHT system are eaten raw or slightly cooked *(2,8)*. It should be noted that these LHT systems are not designed for depuration but only for holding shellfish in an environment that does not result in contamination.

There are several parameters to be considered when operating this type of system. Lappin *(12)* has suggested the following guidelines: the tank must be of sturdy construction and not contain any toxic components that could leach into the water; tanks must be sufficiently large to provide enough water for the product (75 lbs of shellfish/100 gallons of water is recommended); the design of the system should also allow adequate circulation that introduces sufficient oxygen into the water; the filtering and disinfecting system should maintain good water quality; the optimum water temperature (as directed by the season of harvest and the species) should be from 40-60°F (4-16°C), and the salinity held between 25-36 ppt.

This study was conducted to determine the microbiological quality of water and shellfish in LHT's. The Pacific oyster *(Crassostrea gigas)* is the most common species of shellfish held in LHT's in the Seattle study area and was chosen for evaluation.

MATERIALS AND METHODS

Sample collection

Oyster samples consisting of 12-18 animals were purchased from each of five Seattle, WA area markets from March to September 1987. All oysters had been harvested from Puget Sound (total of 27 oyster samples). Water samples were also taken at the same time with the exception of the first sampling period (total of 17 water samples). Samples were transported immediately to the laboratory at ambient temperatures and analyzed within 3 h of collection. Water temperature was measured using a mercury thermometer immediately following collection. The salinity of the water was determined with a refractometer (ATAGO Co. LTD., Japan). The refractometer was standardized before each use with distilled water. Water turbidity, crowding, and comingling of species was noted at each collection time to indicate the general conditions of the systems (Table 1). Turbidity was rated subjectively on a 5 point scale (1=very clear to 5=very turbid). Animal density was rated as a function of approximate water volume versus area occupied by animals on a 3 point scale (1=4 parts water to 3 parts oyster, 2=1 part water to 1 part oyster, 3=<1 part water to 1 part oyster). There was considerable variation in tank design from market to market (Fig. 1).

Sample preparation

Oysters were rinsed with tap water and shucked. The oyster meat and liquid were aseptically transferred to a sterile blender jar, a 1:2 dilution was made with Butterfield's Phosphate Buffered dilution water, followed by blending for 90 sec.*(1)*.

Microbiological analysis

Samples were evaluated for aerobic organisms (APC) and

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total and fecal coliforms *(1)*. *E. coli* were enumerated by the MUG *(11)* test using EC medium supplemented with MUG reagent (4-methyl umbilliferone glucuronide).

Samples were also examined for species of *Vibrio*, *Aeromonas*, *Yersinia*, and *Salmonella*. Analysis for *Listeria* spp. was conducted during only the August sampling period. For each oyster sample, 50 ml of homogenate was added to 225 ml of each of the broths listed below. For water samples, an equal volume of double strength broth was added.

Enrichment procedures for *Vibrio* species was done by incubating Alkaline Peptone Water (APW) at 42°C for 24 h *(3,10)* followed by streaking contents onto Thiosulfate Citrate Bile Salts Sucrose (TCBS) (Oxoid) *(4)*. Typical Vibrio colonies were transferred to Motility Test Medium (MTM)(Difco), Presumptive *Vibrio parahaemolyticus* Agar (PVP) *(9)*, and Tryptone with 3% NaCl and without NaCl. Isolates were identified with API 20E biochemical strips (Analytab Products Plainview, NY). *V. cholerae* isolates were further examined for serotype reaction *(4)* with polyvalent 01, Inaba, and Ogawa antisera (supplied by Harry Smith, Vibrio Reference Laboratory, Thomas Jefferson University, Philadelphia, PA). Isolates were examined for presence of the cholera toxin gene by DNA homology using 32p *(4,13)*. Isolation and identification of *Aeromonas* spp. followed procedures as described by Abeyta and Stelma *(4)* after enrichment in Tryptic Soy Broth with 30 mg/l Ampicillin (TSBA) for 24 h at 35°C. The procedure described by Weagent et. al. *(4)* was used for detection of *Yersinia* spp. with an initial enrichment in Peptone Sorbitol Bile Broth (PSBB) for 10 d at 10°C. The autoagglutination test was used to determine potential pathogenicity of isolates. Samples were analyzed for *Salmonella*

TABLE 1. *Physical and bacteriological quality indicators for water and oysters in live holding tanks.*

Market	Date	Water[1] Quality	Animal[2] Density	Mixed[3] spp.	Temp (°C)	Salinity (ppt)	APC org/ml Water[4]	APC org/ml Oyster	Total Coliform/100g Water	Total Coliform/100g Oyster	*E. coli*/100g Water	*E. coli*/100g Oyster
A	3-31	5	3	M	--	--		170		170		<18
								930		170		<18
	4-28	5	3	M	8.3	--	600	190000	41	220	6.8	20
	6-15	4	2	M	5.0	33	840	660	6.1	1300	2.0	<18
	8-11	5	2		8.9	34	3270	7400	240	170	79	20
	9-8	4	2	M	5.0	34	1400	1420	350	790	<1.8	<18
B	3-24	2	2	M	--	--		340		45		<18
								245		45		<18
	5-5	2	2	M	7.8	--	1965	21750	49	78	<1.8	<18
	6-15	3	2	M	7.8	37	400	380	2	18	<1.8	<18
	8-11	3	2	M	5.6	37.5	2000	480	2	<18	<1.8	<18
	9-8	3	2	M	5.6	37	660	2050	<1.8	93	<1.8	<18
C	4-14	3	2	M	--	--		600		450		<18
								1035		<18		<18
	5-12	3	2	M	6.7	--	1065	1195	540	490	<1.8	68
	6-16	4	2	M	7.8	30	1850	520	1600	5400	<1.8	2200
	8-12	3	2	M	5.6	31	7940	2830	240	78	2	<18
	9-8	2	2	M	6.1	21	2240	2330	540	790	46	230
D	4-8	2	2	M	1.1	--		570		78		<18
								655		78		<18
	5-12	3	2	M	7.8	--	NAFA[5]	170	NAFA	170	NAFA	<18
	6-16	4	3	M	2.8	28	410	730	22	210	<1.8	<18
	8-12	5	2	M	4.4	30	2110	1900	21	5400	1.8	230
E	3-31	1	1	M	--	--		545		18		<18
								46000		78		<18
	5-5	2	1	O	8.9	--	6825	3650	17	490	<1.8	<18
	6-15	2	1	M	11.1	30	8940	5100	2	170	<1.8	<18

[1]Water Quality: 1=Very Clear.
2=Good/Clear.
3=Fair/Slightly Turbid.
4=Cloudly/Turbid.
5=Murky/Quite Turbid.

[2]Animal Density: 1=≥4 part water to 3 parts oyster.
2=1 part water to 1 part oyster.
3=<1 part water to 1 part oyster.

[3]Mixed Species: M=Other Shellfish and/or crustaceans were present in tank with oysters.
O=Oysters only animal present in tank.

[4]Two oyster samples were collected during the first sampling period at each market, after that one oyster and one water sample were collected at each sampling.

[5]NAFA=Not Availble For Analysis.

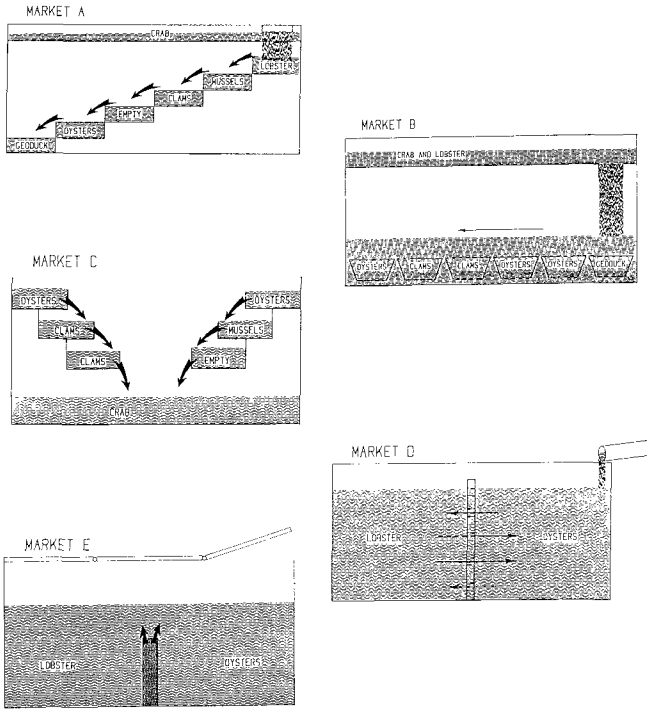

Figure 1. *Design of Live Holding Tanks in five different retail outlets in the Seattle area; arrows indicate direction of water flow.*

using established methods *(4)* with complete serotyping done by Dean Wagner (U.S. FDA, Minneapolis, Center for Microbiological Investigations). Samples were analyzed for *Listeria* species using the method of Lovett *(4)*, which involved an enrichment step for up to 7 d at 30°C in Tryptic Soy Broth with 0.6% Yeast Extract (TSB YE) supplemented with filter sterilized solutions of 15 mg/l Acriflavin HCl (Sigma), 40 mg/l Nalidixic acid (Sigma) and 50 mg/l Cyclohexamide (Sigma).

Bacterial cultures and controls

Positive control cultures were inoculated into portions of homogenates and carried throughout the above procedures at each sampling time. Controls used included the following: *V. cholerae* 154 01 Inaba; *S. typhimurium* ATCC 14028, Serotype B; *Aeromonas hydrophila* isolated from Puget Sound, WA; *Yersinia enterocolitica* WA+ containing the virulence plasmid; *E. coli* 0:157, H:7, hemorrhagic (HEC); *E. coli* BA7, EEC (LT+ST+); and *Listeria monocytogenes* (Scott A). Control cultures were maintained in motility test medium and inoculated directly to enrichment broths with a sterile transfer stick. All control cultures were recovered during each of the 5 sampling periods by the methods used.

RESULTS AND DISCUSSION

Physical Parameters

The degree of water turbidity, crowding and co-mingling of species varied from market to market and sampling period to sampling period (Table 1). Artificial sea water was used at all of the markets and tanks were cleaned at frequencies ranging from once a week to once in three months. Overall, Tank E consistently had the clearest water quality, greatest water to animal ratio and the fewest number of other molluscan and non-molluscan species held in the same tank. The water temperature for all the tanks ranged from 1.11 to 11.1°C. The salinity ranged from 28 to 37.5 ppt. These temperatures (<11.1°C) and salinity ranges (>20.5 ppt) are within those recommended for purification of *C. gigas (14)*.

Bacteriological Quality Parameters

Aerobic plate counts and fecal coliform levels have been used as quality indicators for shellfish. Total coliform and fecal coliform levels have been used to assess classification of growing areas. Wholesale market guidelines for shellfish produced under the sanitary control of the National Shellfish Sanitation Program (NSSP)*(5)* are as follows: APC of 500,000 per g and <230 fecal coliforms per 100 g. If shellfish exceed those levels, a series of procedures recommended by the NSSP would be initiated as a follow-up. No national standard or guideline has been established for water and shellfish in retail LHT systems. Under the 1987 NSSP *(6)*, requirements for commercial wet storage specify supply water to the tanks should have no detectable levels of coliform organisms as measured by a 5 tube MPN test for drinking waters. The standard for direct harvest from shellfish growing waters has been set at a median of <14 fecal coliforms per 100 ml and not more than 10% of the samples to exceed an MPN of 43 for a 5 tube dilution, total coliform levels are not to exceed 230 coliforms/100ml for 10% of the samples or a median of 70 coliforms/100ml *(6)*.

All of the oyster samples met the NSSP recommended market standard for aerobic plate count. The number of Colony Forming Units (CFU'S) detected by the aerobic plate count was similar for both oyster and water samples (Table 1). The average aerobic plate count for oyster and water samples was 2,032 CFU/g and 1,862 CFU/g respectively.

Coliforms. Total Coliform levels were significantly higher (P<.05, using the Student's T test) for oyster (27 samples) compared to water *(16)* samples (Table 1). Oyster samples averaged 525 and 155/100g total and fecal coliforms respectively compared to 54 and 11 total and fecal coliforms respectively for water samples. One oyster sample at 2200 fecal coliforms/100 g exceeded the NSSP market guideline of 230 fecal coliforms/100 g. The water sample that was collected simultaneously had <1.8 MPN/100 ml. Two water samples at 79 and 46 MPN/100 ml exceeded

TABLE 2. *Incidence of potential pathogens in water and oysters from live holding tanks, represents samples from March to September for 5 different tanks.*

	Salmonella	*Vibrio cholerae*	*Vibrio fluvialis*	*Vibrio alginolyticus*	*Aeromonas hydrophilia*	*Yersinia enterocolitica*	*Yersinia* spp.
Oyster (N=27)	3.7%	7.4%	7.4%	0%	78%	3.7%	14.8%
Water (N=17)	0%	0%	0%	53%	53%	0%	0%

the recommended growing water guideline of 14 fecal coliforms/100 ml. All of the water samples except one had detectable levels of coliforms and six of the 16 samples exceeded 230 coliforms/100ml. Each confirmed fecal coliform was also MUG positive, indicating the presence of *E. coli*. Coliform counts may be influenced in part by the length of time the oysters had been in the tank, whether or not they were pumping, the composition of the tank flora and composition and initial level of microorganisms present in the oysters before being placed in the holding system. In two markets (B and E) the fecal coliform levels were consistently below the level of detection for both oyster and water samples.

Potential Pathogens

Salmonella. Salmonella typhimurium was isolated from one oyster sample (Tank B). The organism was isolated during the first sampling period when only oyster samples were collected. Therefore, it is not apparent if the organism was introduced into the tank with the oysters, was introduced into the system by other species and subsequently picked up by the oysters, or was in the tank from some other source. No *Salmonella* spp. were isolated from any of the water samples in this study.

Listeria. Listeria spp. were not isolated from any of the samples during the August sampling period.

Yersinia sp. One non-pathogenic *Yersinia enterocolitica*, determined by the absence of the virulence plasmid *(4)* was isolated from an oyster sample (tank D, June sampling); other *Yersinia* spp. were isolated from two other oyster samples (tank C from the August and Septemtber samplings). No *Yersinia* spp. were isolated from any of the corresponding water samples. *Yersinia* were isolated from tanks ranging in temperature from 2.78 to 6.11°C.

Vibrio spp. The differences between oyster and water sample microbiological flora are readily apparent when considering species of *Vibrios* detected. *Vibrio alginolyticus* was isolated from 53% of the water samples but not from any of the oyster samples. Conversely, *V. fluvialis* (markets A, March and B, September) and non-01 *V. cholerae* were isolated twice from oyster samples. The two *V. cholerae* isolates were from markets C and D during the August sampling period. Presence of *V. cholerae* at this time is most likely due to higher water temperatures prevailing in Puget Sound. Higher temperatures are known to increase survival of *V. cholerae* in the environment *(10)*. None of the *V. cholerae* isolates tested positive for the presence of the cholera toxin gene (CTX A11).

Aeromonas hydrophila. A. hydrophila was the most frequently isolated potential pathogen in this study. Each market consistently had a higher incidence of *A. hydrophila* in oysters compared to water samples; 78% of the oyster samples contained *A. hydrophila* compared to a 53% incidence in water samples. The frequency of isolation also seemed to remain fairly consistent throughout the 6 month sampling time. It is interesting to note that *A. hydrophila* was not isolated from any of the oyster or water samples taken from market E.

It has been suggested that some organisms such as *Aeromonas* and *Vibrio* species are part of the gut flora of shellfish *(13)*. Greenburg and Dubois *(7)* have indicated that it is possible for enteric bacteria indigenous to marine animals to persist during natural or artificial purification processes. Bacteria that are not normally part of marine intestinal habitats on the other hand are readily purged. The results from this study seem to support this. Aeromonas was readily detected in oysters while *E. coli* was generally present in low numbers.

CONCLUSION

These data show that both indicator and pathogenic bacteria can be isolated from oysters and water in LHT's. Samples were collected once a month and the microbiological quality of animals added to the tanks and changes in the microbiological flora during holding were not monitored. Therefore, the sources of the indicator and pathogenic bacteria and their length of survival in the LHT systems is not known. Water quality from these LHT's did not always meet NSSP recommended criteria for wet storage of shellfish and may indicate a need for better water treatment. The potential pathogen *Salmonella typhimurium* was able to survive in shellfish under tank conditions. Although the source of the organism could not be determined, its presence poses a potential health threat and demonstrates a need for further study of tank design, environmental factors and maintenance procedures. All of these parameters can play a role in the survival and pumping action of oysters during their 1-4 week stay in these tanks and can in turn affect their final microbiological quality.

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