한국환경과학회지 제5권(제3호), 329~335, 1996
*J. of the Korean Environmental Sciences Society*

# Persistence of Marine *Vibrio vulnificus* in Oysters within Environmental Parameters

**Bong-Hun LEE* and Heung-Jai PARK**
**Dept. of Chemistry, Pusan National Univ., Pusan 609-735, Korea*
*Dept. of Environmental Science, Inje Univ., Kimhae 621-749, Korea*
(Manuscript received 5 April 1996)

**This project studies marine *Vibrio vulnificus* in oysters in the marine environment and attempts to correlate this bacteria's presence within various environmental parameters; we design this study to determine how different storage temperatures affect the survival of *V. vulnificus* in oysters and whether *V. vulnificus* is able to persist in oysters after exposure to UV light-disinfected seawater. Experimental depuration systems consist of aquaria containing temperature-controlled seawater treated with UV light and 0.2 μm pore size filtration. Results showed that depuration at temperatures higher than 25℃ caused *V. vulnificus* counts to increase in oysters. Throughout the process, depuration water contained high concentrations of *V. vulnificus*, indicating that the disinfection properties of UV radiation and 0.2 μm pore size filtration were less than the release of *V. vulnifcus* into seawater. In contrast, when depuration seawater was maintained at 10℃, the numbers of *V. vulnifcus* were very little and multiplication in oysters was inhibited.**

**Key words** : environmental parameters, temperature, UV light, depuration

## 1. Introduction

Marine microorganisms exist in the marine environment and these cause estuarine environmental pollution as well as bacterial pollution. Marine *Vibrio vulnificus*, a highly virulent pathogen, occurs naturally in estuarine environments of coastal waters (Kaysner et al., 1987, Roberts, et al., 1982, Tacket, et al., 1984, Tison, et al., 1986). This organism can cause gastroenteritis and wound infections (Jenkins, et al., 1986). *V. vulnificus* disease is frequently associated with ingestion of contaminated raw seafood, particularly oysters (*Crassostrea virginica*) and is characterized by an initial gastroenteritis and primary septicema, followed by rapid development of sepsis, secondary skin lesions, and necrosis of surrounding tissues within days. Approximately 60% of infected individuals die, although infections are limited primarily to persons with liver damage or other chronic disease resulting in elevated iron levels in serum (Oliver, 1989).

Only a limited number of reports describe the interaction of *V. vulnificus* with shellfish tissues. A laboratory study of oysters (*C. virginica*) showed that *V. vulnificus* was taken up when present in seawater and was rapidly cleared from the oysters when the bacteria were removed from the water (Kelly et al., 1985).

At all stages of harvest, transport, and storage, oysters may be exposed to temperature extremes. It is conceivable that, especially during elevated temperature, *V. vulnificus* can increase in numbers within the oysters, creating a potentially greater health hazard for high risk individuals who con-

sume raw oysters. Such fact can be responsible for the high incidence of infections associated with the oysters harvest areas.

The literature contains many reports which describe the purification methods of shellfish (e.g., depuration) in which physicochemical parameters of seawater are controlled to reduce bacterial pollutants in shellfish. In the majority of these studies, UV radiation is used to disinfect seawater and lower the number of fecal bacteria. Since *V. vulnificus* and other pathogenic Vibrio species have been recognized as significant seafood pathogens, depuration has been proposed.

This paper presents results from our studies concerning marine *Vibrio vulnificus* and its host, oysters in the environment and attempts to correlate their presence within various environmental parameters; we conduct this study in order to know the effect of storage temperatures on the survival of *V. vulnificus* in oysters and that of UV light on the persistence of *V. vulnificus* in oysters and the release of *V. vulnificus* from oysters.

## 2. Materials and Methods

### 2.1 Oysters

Oysters (*C. virginica*) were obtained from local growers and brought to our laboratory within 24 h of harvest. They were washed with a scrub brush under flowing fresh water, put in pans to dry, and stored at 10℃ for 15 h. They were then warmed for 5 h at 22℃ to avoid cold shock to *V. vulnificus* during inoculation. Seawater samples were taken from just below the water surface by using sterile glass jars.

### 2.2 *Vibrio vulnificus* culture

A virulent *Vibrio vulnificus* was used in this study and cultured in heart infusion broth at 35℃ for 24 h. Cultured cells were centrifuged and washed three times with 0.15 M phosphate-buffered saline (PBS, pH 7.2). Dilutions were made in PBS and colony-forming unit (CFU) was determined by using heart infusion agar-2% NaCl pour plates.

### 2.3 Inoculation of *V. vulnificus* to oysters

An electric drill was used to drill a hole through the shell of each oyster at a center point. 0.1 ml of a suspension of *V. vulnificus* in sterile 1% saline was injected by using a 1 ml of syringe. Control oyster was injected only with 0.1 ml of sterile 1% saline.

### 2.4 Enumeration of microorganism

At each sampling, two live oysters were removed, weighed in a sterile jar, and analyzed separately. One control oyster was examined at each time interval. The internal surface of each oyster shell was rinsed with 1% sterile saline. Smaller oysters (<20 g) were initially diluted in 9 parts of saline and blended. The mixture was blended for 30 sec and diluted serially in 10 fold increments with sterile saline. A 0.1 ml portion of each serial dilution was spread onto the surface of duplicate plates of agars. The plates were incubated at 37℃ for 24 h and typical colonies were enumerated.

### 2.5 Effect of UV light on depuration of *V. vulnificus* in oysters

A depuration system consisted of 80 l aquaria containing 40 oysters and filled with 50 l of autoclaved natural seawater (salinity, 1.5%) which was ci-

rculated through 60 W UV light chambers. Suspended particles were filtered from seawater by using a pump and a diatomaceous earth filter. Water temperature was maintained at 25℃. For control, seawater was circulated with a pump and filtered but not treated with UV light. Whole oysters and seawater were tested at selected time intervals.

### 2.6 Effect of temperature on depuration of *V. vulnificus* in oysters

The effect of temperature on depuration of *V. vulnificus* was tested by using aquaria containing oysters and equipped with 60 W UV light bulb, diatomaceous earth filters. Refrigerated manifolds were placed to maintain seawater temperature at 10 or 25℃. Pooled 10 oysters and 10 ml of seawater were sampled at each time interval.

### 2.7 Measurement of *V. vulnificus* released from oysters

The numbers of *V. vulnificus* organisms and their release from oysters were determined by using four cylindrical chambers. UV light- disinfected artificial seawater was pumped through each chamber by using a peristaltic pump. At each time interval, *V. vulnificus* organisms were enumerated in the effluent seawater of all chambers and in representative samples of oysters before and after experimental protocol.

In a separate experiment, the numbers of *V. vulnificus* released from filter-feeding and non filter-feeding oysters were determined. One chamber contained three unbound and filter-feeding oysters. A second flowthrough chamber contained three secured oysters to measure the number of *V. vulnificus* released from shell surfaces. *V. vulnificus* organisms were enumerated in effluent seawater from each chamber at selected time intervals.

## 3. Results and Discussion

### 3.1 Effect of UV light on depuration of *V. vulnificus* in oysters

When oysters containing *V. vulnificus* were present in depuration aquaria, *V. vulnificus* counts remained elevated in seawater for 3 days, i. e., 103

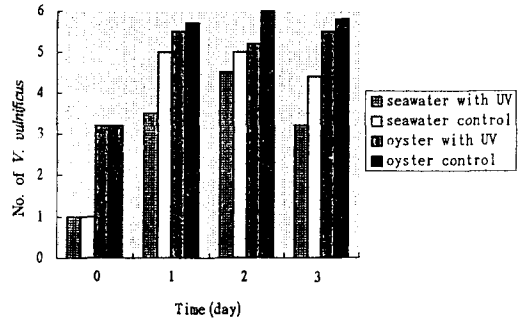


**Fig. 1. Survival of *V. vulnificus* in seawater and oysters by UV light. The plots represent logarithmic analysis of datum points.**

**Table 1. Survival of *V. vulnificus* in seawater and oysters under depuration conditions**

| time(day) | No. of *V. vulnificus* in seawater with UV | No. of *V. vulnificus* in seawater control | No. of *V. vulnificus* in oyster with UV | No. of *V. vulnificus* in oyster control |
|---|---|---|---|---|
| 0 | $10^1$ | $10^1$ | $3\times10^3$ | $3\times10^3$ |
| 1 | $4\times10^3$ | $10^5$ | $4\times10^5$ | $6\times10^5$ |
| 2 | $5\times10^4$ | $10^5$ | $3\times10^5$ | $10^6$ |
| 3 | $3\times10^3$ | $5\times10^4$ | $4\times10^5$ | $6\times10^5$ |

to 105 organisms per ml. *V. vulnificus* counts were similar in oysters exposed to UV light and those exposed to untreated seawater (Table 1, Fig. 1). For both test and control specimens, the numbers of *V. vulnificus* were increased more than 100 fold in oysters within 24 h and remained elevated for 3 days of treatment. In a separate experiment, 0.2 μm filters were added to depuration systems to reduce residual *V. vulnificus* organisms in seawater, limit feedback to oysters, and potentially increase depuration efficiency. This procedure reduced *V. vulnificus* counts in seawater compared to those in depuration systems without filtration, but *V. vulnificus* counts in oysters and seawater remained elevated throughout 7 days of treatment.

Above results suggested that *V. vulnificus* did not depurate from oysters when UV light and/or 0.2 μm filtration treatments of seawater were used. Importantly, at elevated seawater temperatures (e.g., >20℃), *V. vulnificus* multiplied markedly in oysters and large numbers of *V. vulnificus* were released into surrounding seawater at rates exceeding the bactericidal activities of UV light.

### 3.2 Effect of temperature on depuration of *V. vulnificus* in oysters

The temperature of depuration seawater markedly affected the growth of *V. vulnificus* in oysters and seawater (Table 2, Fig. 2). At 10℃, the counts of *V. vulnificus* in oysters remained low after 5 days of treatment, similar to pretreatment counts. In contrast, oysters maintained in 25℃ seawater contained $3\times10^4$ to $6\div10^5$ *V. vulnificus* organisms, concentrations $10^4$ greater than pretreatment concentrations. Likewise, *V. vulnificus* concentrations in 25℃ seawater were markedly elevated (i. e., > $10^5$/ml).

### 3.3 *V. vulnificus* released from oysters

Large numbers of *V. vulnificus* organisms were released from oysters into surrounding seawater within 1 day (Table 3, Fig. 3). The rate of release

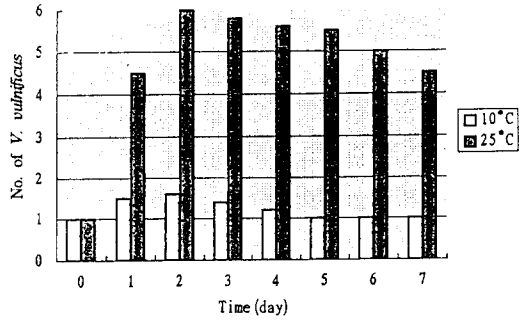


**Fig. 2. Effect of seawater temperature at 10 and 25℃ on survival of *V. vulnificus* in oysters. The plots represent logarithmic analysis of datum points.**

**Table 2. Effect of seawater temperature at 10 and 25℃ on the survival of *V. vulnificus* in oysters**

| time (day) | No. of *V. vulnificus* at 10℃ | No. of *V. vulnificus* at 25℃ |
|---|---|---|
| 0 | $10^1$ | $10^1$ |
| 1 | $3\times10^1$ | $4\times10^4$ |
| 2 | $4\times10^1$ | $10^6$ |
| 3 | $3\times10^1$ | $6\times10^5$ |
| 4 | $2\times10^1$ | $4\times10^5$ |
| 5 | $10^1$ | $4\times10^5$ |
| 6 | $10^1$ | $10^5$ |
| 7 | $10^1$ | $3\times10^4$ |

**Table 3. Release of *V. vulnificus* from filter feeding oysters into seawater**

| time (day) | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|---|
| No. of *V. vulnificus* | $6\times10^6$ | $6\times10^5$ | $4\times10^5$ | $2+10^5$ | $10^5$ | $7\times10^4$ | $3\times10^4$ | $2\times10^4$ |

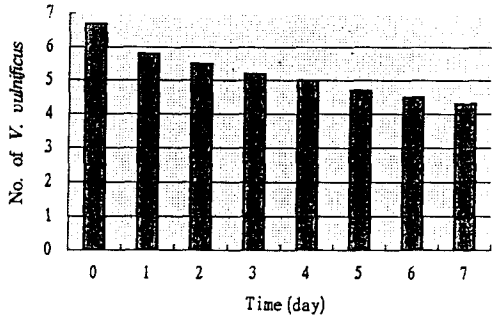


**Fig. 3. Release of *V. vulnificus* from filter feeding oysters into seawater. The plots represent logarithmic analysis of datum points.**

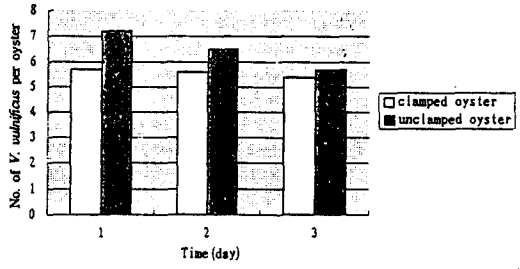


**Fig. 4. Release of *V. vulnificus* from clamped and unclamped oysters surfaces. The plots represent logarithmic analysis of datum points.**

was declined over 5 days to approximately $10^5$ *V. vulnificus* organisms per oyster. After 7 days of exposure to one-pass disinfected seawater, a total of more than $10^6$ *V. vulnificus* organisms were released per oyster.

The numbers of *V. vulnificus* organisms released from clamped and unclamped oysters surfaces were also determined (Table 4, Fig. 4). From 1 to 3 days, *V. vulnificus* counts released from unclamped, filter-feeding specimens were declined from $2\times10^7$ to $6\times10^5$. *V. vulnificus* organisms released from the shell surfaces of the clamped oysters were decreased from approximately $6\times10^5$ to $3\times10^5$. We speculated that internal anaerobic conditions of clamped oysters reduced specimen viability.

As has been shown in other environmental and laboratory studies, low seawater temperature markedly inhibited *V. vulnificus* growth. Under depuration conditions at 10℃, the numbers of *V. vulnificus* in oysters remained low, similar to pretreatment concentrations (Fig. 2). In contrast, at 25℃, *V. vulnificus* counts in oysters can increase 10,000 fold (Tamplin et al., 1989). Further experiments are needed to identify the specific low temperatures which initiates and inhibits *V. vulnificus* growth in detail.

When seafoods are eaten raw, prevention of Vibrio infection is difficult, and it may be impossible to guarantee that any raw seafood is totally safe. Perhaps even healthy persons anxious to minimize the risks of food-borne Vibrio infections should not eat raw seafoods during the warm summer and

**Table 4. Release of *V. vulnificus* from clamped and unclamped oysters surfaces**

| time (day) | No. of *V. vulnificus* in clamped oysters | No. of *V. vulnificus* in unclamped oysters |
|---|---|---|
| 1 | $6\times10^5$ | $2\times10^7$ |
| 2 | $4\times10^5$ | $4\times10^6$ |
| 3 | $3\times10^5$ | $6\times10^5$ |

fall months.

It does appear that *V. vulnifcus* is normal flora in coastal waters and therefore can be concentrated in oysters. Such a conclusion would receive support by the observation that most human infections show a history of seawater contact or consumption of raw seafood. The survival of *V. vulnificus* in oysters has been examined by Oliver, who concluded that properly chilled oysters should not pose a health hazard unless consumed by a person with elevated serum iron levels (Wright, et al., 1981). The survival of *V. vulnificus* for up to 2 weeks in commercial shellstock and at least 6 days in shucked oysters at refrigeration temperatures is potentially significant to public health. Although most reports of *V. vulnificus* disease concern the consumption of raw oysters, little information exists regarding disease from properly stored oysters. The numbers of *V. vulnificus* did not increase during storage; the organism did survive, however. This implies that consumption of raw oysters, even those that are properly handled during storage, may result in ingestion of *V. vulnificus*. In addition, oyster drip may contain *V. vulnificus*, which may cross contaminate other oysters and seafood.

## References

Jenkins, R. D. and J. M. Johnson, 1986, Inland presentation of *Vibrio vulnificus*; primary septicemia and necrotizing fasciitis, West. J. Med., 144, 78~80.

Kaysner, C. A., C. Abeyta, Jr., M. M. Wekell, A. Depaola, R. F. Stott, and J. M. Leitch, 1987, Virulent strains of *Vibrio vulnificus* isolated from estuaries of the U.S. West Coast, Appl. Environ. Microbiol., 53, 1349~1351.

Kelly, M. T. and A. Dinuzzo, 1985, Uptake and clearance of *Vibrio vulnificus* from Gulf Coast oysters (Crassostrea virginica), Appl. Environ. Microbiol., 50, 1548~1549.

Oliver, J. D, 1989, *Vibrio vulnificus*, In M. Doyle (ed.), Foodborne bacterial pathogens, Marcel Dekker, Inc., New York. 569~599.

Roberts, N. C., R. J. Seibeling, J. B. Kaper, and H. B. Bradford, Jr, 1982, Vibrios in the Louisiana Gulf Coast environment, Microb. Ecol., 8, 299~312.

Tacket, C. O., F. Brenner, and P. A. Blake, 1984, Clinical features and an epidemiological study of *Vibrio vulnificus* infections, J. Infect. Dis., 149, 558~561.

Tamplin, M. L, 1989, Ecology of *Vibrio vulnificus* in Gulf Coast estuaries, Shellfish Institute of North America, Washington, D.C.

Tison, D. L., and M. T. Kelly, 1986, Virulence of *Vibrio vulnificus* strains from marine environments. Appl. Environ. Microbiol., 51, 1004~1006.

Wright, A. C., L. M. Simpson, and J. D. Oliver, 1981, Role of iron in the pathogenesis of *Vibrio vulnificus* infections, Infect. Immun., 34, 503~507.

Persistence of Marine *Vibrio vulnificus* in Oysters within Environmental Parameters

# 환경 변수에 대한 생굴에서의 해양 Vibrio vulnificus의 인내성

이봉헌* · 박흥재
부산대학교 자연과학대학 화학과 • 인제대학교 자연과학대학 환경학과
(1996년 4월 5일 접수)

본 연구는 해양 환경에서 생굴과 해양 *Vibrio vulnificus*에 관한 연구로서 이들의 존재와 여러 가지 환경 변수와의 상호 관계를 알아보고자 수행하였다. 즉, 저장 온도가 생굴에서의 *V. vulnificus*의 생존에 어떻게 영향을 미치는지 그리고 자외선을 처리한 해수에 노출된 후 생굴에서 *V. vulnificus*가 어느 정도 견뎌내는지를 알아보기 위해서이다. 실험적 정화계는 자외선으로 처리한 온도 조절 가능한 해수와 0.2 μm pore size의 여과 장치로 구성되어 있다. 25℃ 보다 높은 온도에서 수행한 정화계의 실험 결과 생굴에서 *V. vulnificus*수가 증가하였다. 전 과정에서 정화수에는 *V. vulnificus*가 고농도로 존재하였는데 이는 자외선의 멸균 특성과 0.2 μm pore size의 여과가 *V. vulnificus*가 해수로 유출되는 것보다 적다는 것을 나타내 준다. 대조적으로 정화수를 10℃로 유지하였을 때 *V. vulnificus*수는 적었고 생굴에서의 증식도 억제되었다.