Effects of temperature abuse on survival of *Vibrio vulnificus* in oysters

Effets d'une température excessive sur la survie de Vibrio vulnificus *dans les huîtres*

SUSAN MURPHY, JAMES D. OLIVER
Department of Biology, University of North Carolina at Charlotte, Charlotte, NC, 28223, USA

Abstract

Of the several bacterial diseases which may result from consumption of shellfish, those caused by marine bacteria of the genus *Vibrio* are the most abundant. In the United States, up to 10,000 non-fatal cases per year are estimated to be caused by the various members of this genus. More than 95% of all deaths in the United States which result from seafood consumption are caused by a single bacterium, *Vibrio vulnificus*. The bacterium is a normal inhabitant of estuarine waters, and occurs naturally in especially high numbers in molluscan shellfish. Infections following consuption of raw or undercooked shellfish, especially oysters, result in fatality rates of over 60%.

Because most oysters in the United States are transported large distances before marketing, the possibility of temperature abuse of oysters as a factor in the epidemiology of this disease was investigated. Oysters (*Crassostrea virginica*) were allowed to take up cells of *V. vulnificus* (CVD713) which had been added with algal cells. Following uptake, the live, shellstock oysters were placed at temperatures of 0.5, 5, 10, 17 and 22°C and maintained at those temperature for up to 10 days. At intervals, groups of five oysters were removed and assayed for the number of *V. vulnificus* cells present. Strain CVD713 contains the transposon Tn*pho*A, which carries genes for kanamycin resistance and alkaline phosphatase production. These markers permit significant selectivity of this strain from other normal flora bacteria, as well as differentiation from other kanamycin resistant cells that may be present. Temperature studies demonstrated that the transposon was stable at all investigation temperatures (figure 1).

Results indicated that, regardless of storage temperature, the number of *V. vulnificus* cells present in shellstock oysters decreased gradually with time, with the greatest decreases occurring at the higher temperatures (figure 2). A similar result was seen when a 1000-fold lower number of cells were initially present in the oysters (figure 4). No significant differences in cell survival were noted between opaque (encapsulated) cells (figure 2) and translucent (non encapsulated) cells (figure 6) of *V. vulnificus*. Similar results were obtained when an identical study was carried out employing a strain of *V. cholerae* harboring this transposon (data not shown).

In contrast to the results observed when the storage studies were conducted on shellstock oysters, a rapid and dramatic decrease in *V. vulnificus* cell survival was observed at all temperatures when oysters were shucked prior to storage (figure 7).

Our results suggest that temperature abuse may not be a major factor in the epidemiology of infections by *V. vulnificus*. However, storage of *V. vulnificus* at low temperatures does not appear to significantly reduce the populations of this human pathogen.

Résumé

Parmi les maladies bactériennes dues à la consommation de coquillages, celles provoquées par les bactéries marines du genre *Vibrio* sont les plus courantes. Aux États-Unis, on

estime à environ 10 000 le nombre de cas non mortels de maladie provoquée par divers germes de ce genre. Plus de 95 % des décès dus à la consommation de coquillages aux États-Unis sont provoqués par une seule bactérie, *Vibrio vulnificus*. Cette bactérie est un résident habituel des eaux estuariennes et apparaît naturellement en très grand nombre chez les mollusques. Les infections consécutives à la consommation de coquillages crus ou peu cuits, donnent lieu à des taux de décès supérieurs à 60 %.

La majorité des huîtres aux États-Unis subissant un transport sur de longues distances avant leur consommation, la possibilité d'une température excessive en tant que facteur dans l'épidémiologie de ces infections a été étudiée. On a laissé des huîtres (*Crassostrea virginica*) accumuler des cellules de *V. vulnificus* (CVD713) ajoutées à des cellules algales. Après absorption, les huîtres vivantes ont été placées à des températures de 0,5, 5, 10, 17 et 22 °C, et maintenues à ces températures pendant une période allant jusqu'à 10 jours. A intervalle régulier, des groupes de cinq huîtres ont été prélevés et analysés pour dénombrer les cellules de *V. vulnificus*. La souche CVD713 contient le transposon Tn*phoA* qui est porteur de gènes de résistance à la kanamycine et de production de phosphatase alcaline. Ces marqueurs permettent une sélectivité importante de la souche par rapport aux autres bactéries normale de la flore, ainsi qu'une bonne différenciation entre les autres cellules résistantes à la kanamycine éventuellement présentes. Les études de température montrent que le transposon reste stable à toutes les températures d'essai (figure 1).

Les résultats indiquent que, quelle que soit la température de stockage, le nombre de cellules de *V. vulnificus* présentes dans les huîtres vivantes diminue progressivement en fonction du temps, les diminutions les plus importantes se produisant à haute température (figure 2). Un résultat semblable a été observé avec un nombre 100 fois inférieur de cellules présentes initialement dans les huîtres (figure 4). Aucune différence significative dans la survie cellulaire n'a été constaté entre les cellules opaques (avec enveloppe) (figure 2) et translucides (dépourvues d'enveloppe) (figure 6) de *V. vulnificus*. Des résultats similaires ont été obtenus lors d'une deuxième étude identique réalisée sur une souche de *V. cholerae* hébergeant ce transposon (données non présentées).

Par opposition avec les résultats observés lors des études de stockage sur les huîtres vivantes, on observe une diminution rapide et spectaculaire du temps de survie des cellules de *V. vulnificus* à toutes les températures lorsque les huîtres sont écaillées avant stockage (figure 7).

Nos résultats suggèrent que les températures excessives ne semblent pas constituer un facteur important dans l'épidémiologie des infections dues à *V. vulnificus*. Cependant, le stockage de *V. vulnificus* à basse température ne semble pas réduire de façon significative les populations de ce pathogène humain.

Vibrio vulnificus is a pathogenic marine bacterium capable of producing severe infections in individuals with certain underlying chronic diseases. Infections are generally associated with the consumption of raw seafood, especially oysters, where the bacterium becomes concentrated through filter feeding. An unusual consequence of septicaemia is the production of secondary skin lesions, particularly of the extremities, characterized by rapid swelling, reddening, fluid accumulation, and necrosis of surrounding tissues within days. Death results in approximately 60% of the infected individuals, although infections are limited primarily to persons with liver damage or other chronic diseases resulting in elevated serum iron levels (Oliver, 1989).

In 1985, Yoshida *et al.*, reported the existence of two colony morphotypes for *V. vulnificus*, dependent on colony opacity. Our laboratory subsequently found that only the opaque colony types were virulent, with the translucent isotypes being avirulent (Simpson *et al.*, 1987).

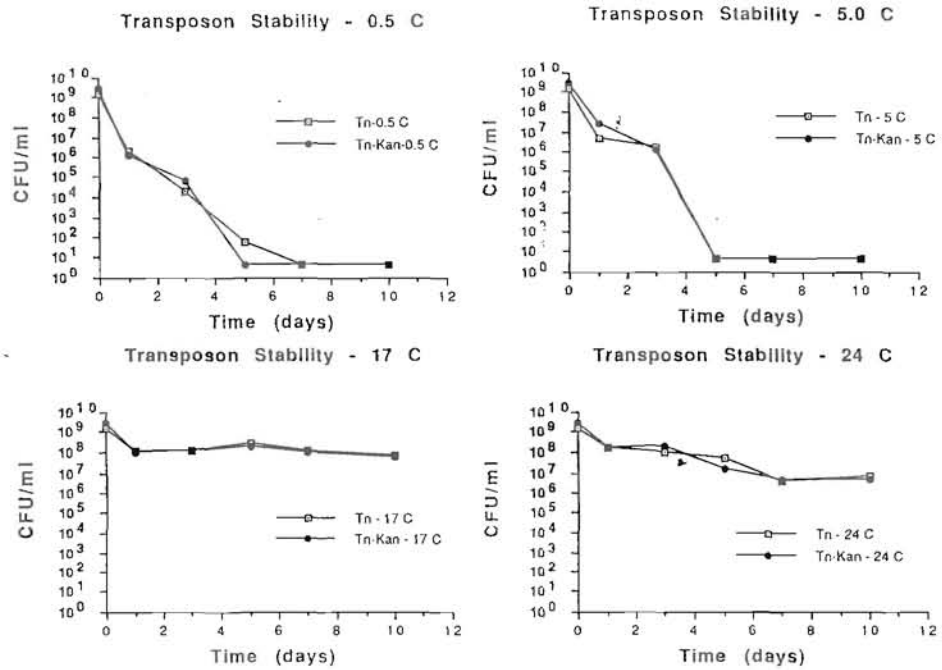

Figure 1: Stability of the transposon Tn*pho*A in *V. vulnificus* CVD713 (opaque) incubated in ASW for 10 days at temperatures from 0.5 to 24°C. Bacteria were plated onto L-agar with or without kanamycin, to determine viability of the population and retention of the transposon. Data for the 10°C sample are not shown, but revealed no difference on the two media

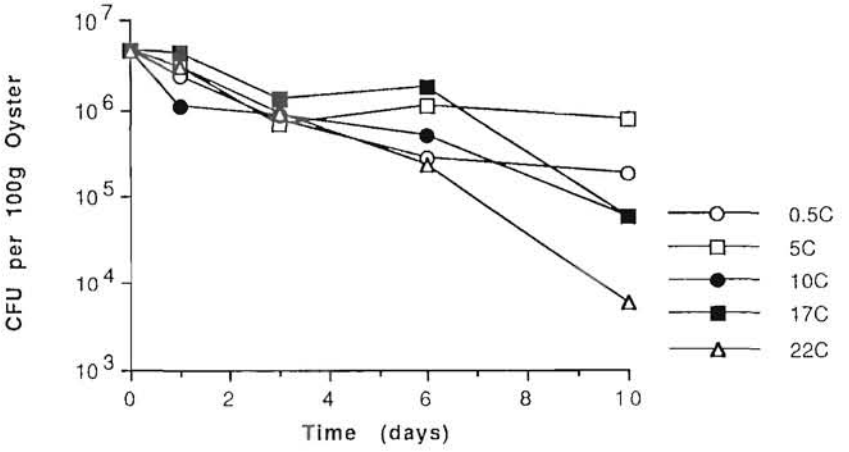

Figure 2: Effect of temperature on survival of *V. vulnificus* CVD713 (opaque) in shellstock oysters

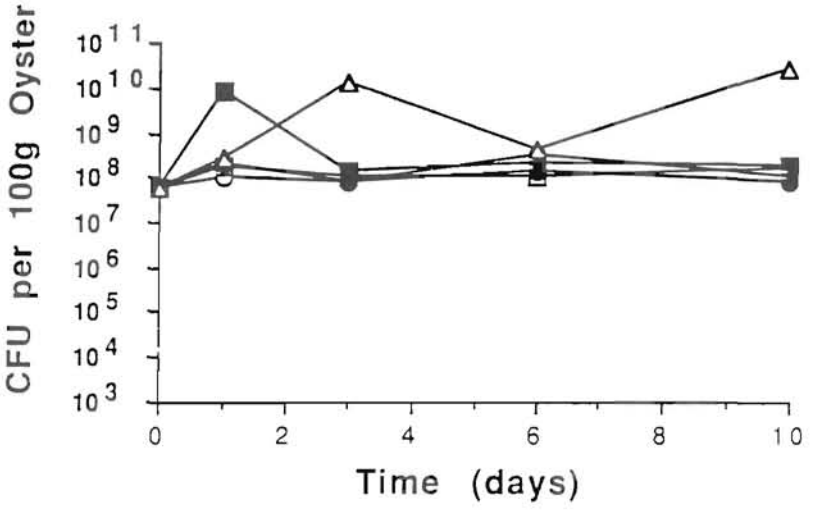

Figure 3: Effect of temperature on survival of total bacteria in shellstock oysters. Oyster holding temperatures are as shown in figure 2

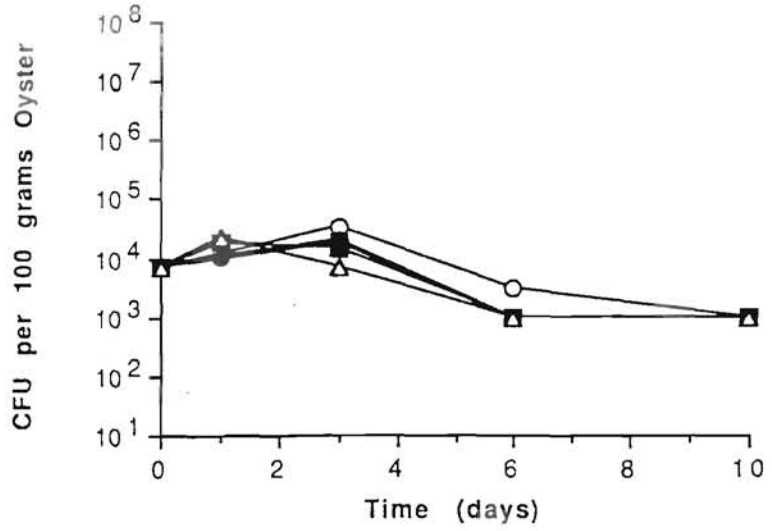

Figure 4: Survival of low inocula of *V. vulnificus* CVD713 (opaque) in shellstock oysters. Oyster holding temperatures are as show in figure 2

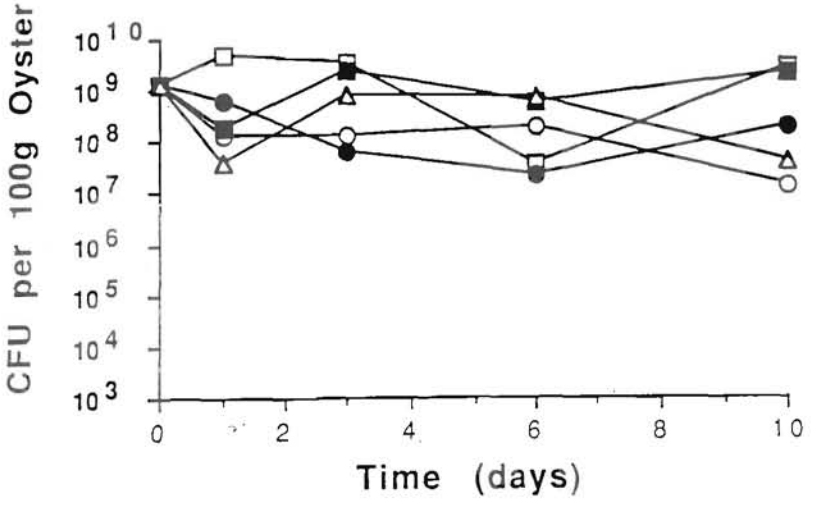

Figure 5: Effect of temperature on survival of total bacteria in shellstock oysters receiving low inocula of *V. vulnificus*. Temperatures are as show in figure 2

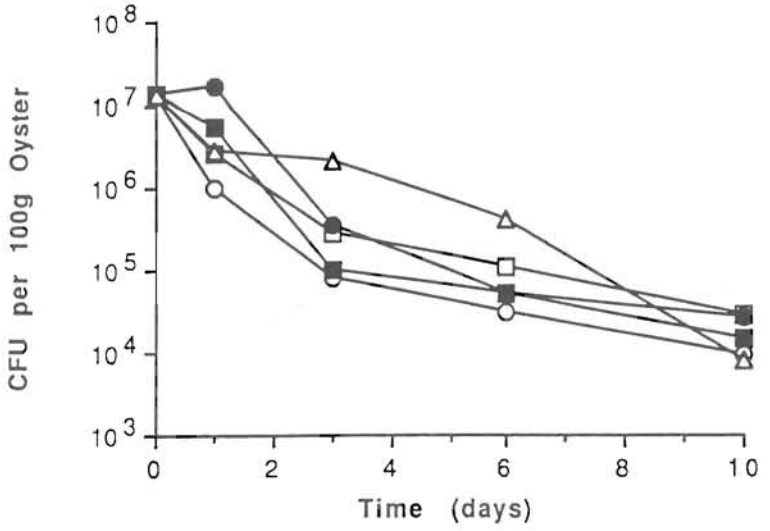

Figure 6: Survival of *V. vulnificus* CVD713 (translucent strain) in shellstock oysters. See figure 2 for details

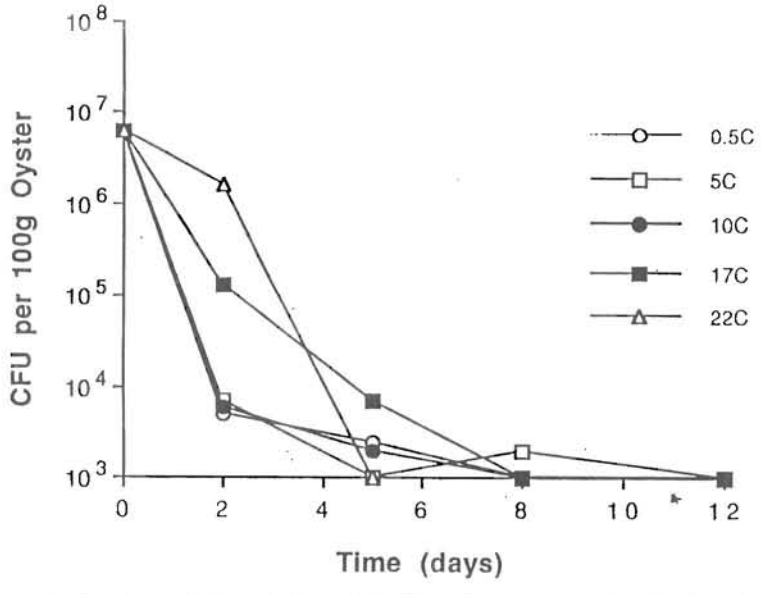

Figure 7: Survival of *V. vulnificus* CVD713 (opaque) in shucked oysters. See figure 2 for details

At all stages of harvest, transport, and storage, oysters may be exposed to temperature extremes. It is conceivable that, especially during elevated temperature abuse, *V. vulnificus* could increase in numbers within the oysters, creating a potentially greater health hazard for high risk individuals who consume raw oysters. Such abuse could be responsible for the high incidence of infections associated with the oyster harvesting areas along the Gulf Coast of the United States.

The intent of this study was to simulate elevated temperature abuse that may occur in oysters, and compare the levels of *V. vulnificus* and total bacteria in these oysters with those stored properly at refrigeration temperatures. Previous studies of this nature have required the use of enrichment cultures and MPN enumeration procedures, in all cases followed by taxonomic studies. In the present study, a transposon-containing strain of *V. vulnificus* was used which allowed direct plating and rapid (within 24 hours) confirmation of the presence of *V. vulnificus* from oyster samples. This eliminated the tedious, time-consuming, and inherently inaccurate methods employed in previous studies.

Tests were initially performed to determine the stability of the Tn*phoA* transposon insertion. Cells of CVD713 (opaque strain) were suspended in ASW and placed at the various experimental temperatures (figure 1). At intervals up to 12 days, aliquots of each suspension were plated to L-agar with or without kanamycin. At 0.5 and 5°C, the plate counts of *V. vulnificus* underwent a rapid decline, which is known to be a result of this bacterium entering into the viable but nonculturable state (Oliver *et al.*, 1991). The transposon, however, was found to be stable at all temperatures over the 10 day sample period. Further, we have found that this insertion does not affect putative virulence factors of *V. vulnificus*, and that the bacterium enters both the starvation state and the viable but nonculturable state similar to the parent strain.

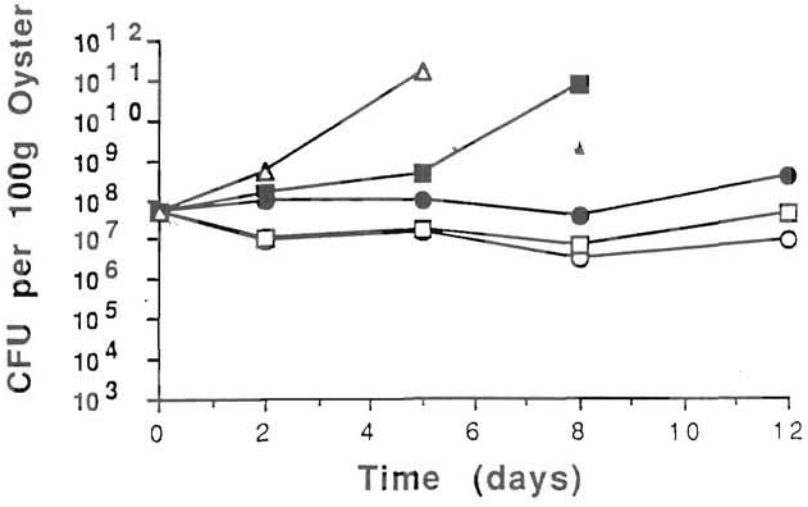

Figure 8: Effect of temperature on survival of total bacteria in shucked oysters. See figure 2 for details

At all temperatures, levels of the opaque morphotype of *V. vulnificus* declined over the 10 day study period (figure 2). At 5°C, there was roughly a half-log decrease while at 0.5, 10, and 17°C there was a one to two log decrease. The most dramatic decrease in *V. vulnificus* numbers was at 22°C, which fell from 4.7×10^6 CFU per 100 grams to 6.0×10^3 CFU per 100 grams, a decrease of almost three orders of magnitude.

Total counts of bacteria present in the oysters, as monitored on heart infusion agar, showed little change over the sampling period at all temperatures (figure 3). The time zero sample contained 4.0×10^7 CFU per 100 grams, with a slight increase in number observed only for the 17°C sample, to 1.8×10^8 CFU per 100 grams.

To eliminate the possibility that the high cell density of *V. vulnificus* employed in our experiments was at a level which would prevent an increase in number following temperature abuse, we repeated the study using an initial inoculum of almost 1000 times lower than this study. The time zero level of *V. vulnificus* in this low inoculum study was 7.10^3 CFU per 100 grams (figure 4). By day three, *V. vulnificus* had increased slightly at all temperatures to roughly 10^4 CFU per 100 grams. By day 10, however, culturable *V. vulnificus* had fallen to at or below the limits of detection of our plating technique ($< 10^3$ CFU per 100 grams). Thus, altering the initial inoculum size did not significantly affect our results, as the shellstock oysters also showed a decrease in *V. vulnificus* over the 10 day storage period. In this low inoculum *V. vulnificus* study, total bacterial counts remained relatively stable at all but the highest and lowest temperatures over the 10 day study period (figure 5). Only at 0.5 and 22°C were declines in numbers observed.

Because *V. vulnificus* produces two colony types, and due to the significance of these colony types in the pathogenicity of this bacterium, the translucent morphotype was also included in our studies. Beginning with a time zero density of 1.3×10^7 CFU per 100 grams, a gradual decline (ca. three logs) of the translucent strain of *V. vulnificus* occurred over the 10 days at all temperatures (figure 6). Thus, the translucent morphotype of *V. vulnificus* appears to respond similarly to the opaque strain, with decreasing cell numbers over the course of the study. While it is not known to what degree the translucent variety of this bacterium exists in nature, it is known that conversions between the opaque and translucent isotypes can occur (Simpson *et al.*, 1987). No such conversions were observed with strain CVD713 in our experiments, however.

We also carried out similar studies using shucked instead of shellstock oysters. In these studies, after feeding on the opaque strain of *V. vulnificus*, oysters were immediately shucked and placed into beakers at the five designated temperatures. At intervals, including time zero, 10 oysters were removed from each beaker along with their liquor, homogenized, and plated to determine the levels of *V. vulnificus* and total bacteria present.

By day two of this study, there was a three log decrease in *V. vulnificus* numbers at 0.5, 5, and 10°C (figure 7). By day five, the 17 and 22°C samples also had dropped over three log units. Oyster meats at 22 and 17°C were discarded at days 8 and 12, respectively, because of putrification. Samples taken from

these two temperatures prior to discard showed *V. vulnificus* to be below the levels of detection. By day twelve, the 0.5, 5, and 10°C samples were also at or below detectable levels for *V. vulnificus*.

Total aerobic counts of these oyster meats showed an opposite trend (figure 8). Beginning at $5.4x10^7$ CFU per 100 grams at time zero, large increases were evident by day five in the 22°C sample, to over 10^{11} CFU per 100 grams. The 17°C oysters also increased, to $6.7x10^{10}$ by day height. Such increases account for the putrification observed in the oyster held at these elevated temperatures. The 0.5, 5, and 10°C counts remained relatively stable throughout the 10 day study.

Throughout these studies, one surprising but obvious consistency was the observation that elevated temperature abuse of oysters, both shellstock and shucked meats, did not contribute to an increase in *V. vulnificus* levels. These findings are in agreement with an earlier study by Kaysner *et al.* (1989) which described decreases in *V. vulnificus* cell numbers in shellstock and shucked oysters at temperatures up to 22°C. Similarly, their study found *V. vulnificus* to be retained in shellstock oysters for up to 14 days at 2°C. In our study, *V. vulnificus* remained at culturable levels (in the high inoculum study) at all temperatures over the 10 day study. In our studies, both the opaque and translucent variants of *V. vulnificus* responded similarly to storage at the various temperatures.

It is interesting to note the rapid decrease in plate counts exhibited by *V. vulnificus* when incubated in ASW at 0.5 or 5°C (figure 1) compared to the relative stability of the population at these temperatures when present in oysters (figures 2, 4 and 6). Apparently *V. vulnificus* does not enter into the viable but nonculturable state when present in oysters, or requires more than the ten day period employed in these studies. These possibilities are currently under investigation in our laboratory.

Studies by Hood *et al.* (1983) reported increases in *V. vulnificus* numbers in shellstock oysters at 8 and 20°C after seven days, with decreases evident only at 14 and 21 days. Similarly, Cook and Ruple (1989) concluded that transport temperatures were a controlling factor in determining levels of *V. vulnificus* in oysters. In the present study, decreases were evident in *V. vulnificus* levels at all temperatures after 10 days of storage. The studies of Hood et *al.* (1983) and Cook and Ruple (1989) may differ from ours due to the methods employed in their studies (MPN, alkaline peptone enrichment culture) for isolation and enumeration, and the taxonomic studies required to identify *V. vulnificus*. These steps may have led to an inaccurate portrayal of the presence of this bacterium in oysters. Indeed, the proper identification of this bacterium from natural sources is extremely difficult (Oliver, 1989 ; Oliver *et al.*, 1992). The use of the Tn*phoA* strain in the present study eliminated these tedious, time-consuming, and often inaccurate methods, and allowed direct and specific results within one day.

While it is clear in our study that levels of *V. vulnificus* did not increase during storage at elevated temperatures, the same was not always true for other indigenous species of aerobic, heterotrophic bacteria in the oysters. Whereas at most temperatures shellstock bacteria showed little change over the 10 day incubation period, increases in total aerobic counts of bacteria were evident at

the highest storage temperatures, indicating that it is possible for microflora in oysters to multiply during temperature abuse. Such a temperature response was especially evident in the shucked oyster study, where the aerobic counts showed great increases at both the 17 and 22°C temperatures. This response is probably due to the lack of any host defences in the dead oysters.

We conclude that, while a higher storage temperature does not, in itself, cause *V. vulnificus* to multiply within oysters and thus increase the possibility of disease outbreak, proper storage temperatures are by no means a safeguard to insure that consumption of *V. vulnificus* is not without some potential public health hazard.

REFERENCES

Hood M.A., G. Ness, G. Rodrick, N. Blake, 1983. Effects of storage on microbial loads of two commercially important shellfish species, *Crassostrea virginica* and *Mercenaria campechiensis*. *Appl. Environ. Microbiol.*, **45**, 1221-1228.

Kaysner C.A., M. Tampling, M. Wekell, R. Stott, K. Colburn, 1989. Survival of *Vibrio vulnificus* in shellstock and shucked oysters (*Crassostrea gigas* and *Crassostrea virginica*) and effects of isolation medium on recovery. *Appl. Environ. Microbiol.*, **55**, 3072-3079.

Oliver J.D., 1989. *Vibrio vulnificus*. In: Foodborn Bacterial Pathogens, M. Doyle ed., Marcel Dekker, Inc., N.Y., 569-599.

Oliver J.D., L. Nilsson, S. Kjelleberg, 1991. Formation of nonculturable *Vibrio vulnificus* cells and its relationship to the starvation state. *Appl. Environ. Microbiol.*, **57**, 2640-2644.

Oliver J.D., K. Guthrie, J. Preyer, A. Wright, L.M. Simpson, R. Siebeling, J.G. Morris Jr., 1992. Use of colistin-polymyxin B-cellobiose agar for isolation of *Vibrio vulnificus* from the environment. *Appl. Environ. Microbiol.*, **58**, 737-739.

Simpson L.M., V. White, S. Zane, J.D. Oliver, 1987. Correlation between virulence and colony morphology in *Vibrio vulnificus*. *Infect. Immun.*, **55**, 269-272.

Wright A.C., L.M. Simpson, J.D. Oliver, J.G. Morris Jr., 1990. Phenotypic evaluation of acapsular transposon mutants of *Vibrio vulnificus*. *Infect. Immun.*, **50**, 1769-1773.

Yoshida S.I., M. Ogawa, Y. Mizuguchi, 1985. Relation of capsular materials and colony opacity to virulence of *Vibrio vulnificus*. *Infect. Immun.*, **47**, 446-451.