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Effect of *Vibrio alginolyticus* on larval survival of the blue mussel *Mytilus galloprovincialis*

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ABSTRACT: The effect of increasing concentrations of *Vibrio alginolyticus* on survival of *Mytilus galloprovincialis* larvae was studied in a 48 h static bioassay in 1 l glass bottles. Five bacterial densities were tested ranging from 10^2 to 10^6 bacteria ml^{-1}. Larval survival and normality (veliger larvae with the typical D-shape) were evaluated after 48 h. An inverse relationship between bacterial concentration and larval survival and normality was observed. In spite of high larval survival (79%) under conditions of high bacterial density (10^5 bacteria ml^{-1}), the percent of normal larvae was 11%. Besides an irregular shape, abnormal larvae also presented velum reduction. Results from this study suggest that concentrations of *V. alginolyticus* lower than 10^3 bacteria ml^{-1} should be maintained during *M. galloprovincialis* larval culture.

KEY WORDS: Mussels · *Mytilus* · Pathogenicity · Vibriosis · Mollusks · *Vibrio alginolyticus*

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INTRODUCTION

Blue mussel farming has become an important activity in Ensenada, Baja California, Mexico. Mussel production increased from 205 to 343 metric tons during the period 1996 to 2001 (H. Valles, SAGARPA, Ensanada BC, Mexico, pers. comm.). Since 1989, blue mussel spat have been produced in aquacultural laboratories of Ensenada, where epizootic events have occurred during the larval stage (García-Pámanes 1990); however, no attempts have been made to identify the cause of such mortalities.

Studies in Mexican invertebrate hatcheries have documented *Vibrio*-like bacteria (VLB) at concentrations between 10^2 and 10^3 bacteria ml^{-1} (Lizárraga-Partida et al. 1998, López-Torres & Lizárraga-Partida 2001, López-Torres et al. 2001). Similar concentrations have been reported in samples from highly sewage-polluted areas (Lizárraga-Partida & Vargas-Cárdenas 1996, Portillo-López & Lizárraga-Partida 1997). However, it has been observed in aquaculture facilities that high concentrations of VLB do not always result in significant larval mortality (Lizárraga-Partida et al. 1997, López-Torres & Lizárraga-Partida 2001, López-Torres et al. 2001).

Different species of *Vibrio* have been identified as major pathogens in bivalve larval cultures, especially oysters and clams (Guillard 1959, Brown 1983, Douillet & Langdon 1993, Riquelme et al. 1995, Sainz et al. 1998, Sugumar et al. 1998). The most common problem reported is bacillary necrosis (Tubiash et al. 1965, Lodeiros et al. 1987); however, the inhibition of filtration (McHenery & Birkbeck 1986, Birkbeck et al. 1987) and the loss of swimming ability have also been observed (Nottage & Birkbeck 1987, Nottage et al. 1989). *Vibrio alginolyticus* has been reported as one of the most pathogenic bacteria for molluscan larvae (Tubiash & Otto 1986, Anguiano-Beltrán et al. 1998, Luna-González et al. 2002). This study investigated the effects of increasing concentrations of *V. alginolyticus* ATCC 17749 on survival and normality of blue mussel *Mytilus galloprovincialis* larvae.

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MATERIALS AND METHODS

A completely randomized experimental design was followed to evaluate the effects of bacteria on *Mytilus galloprovincialis* larvae. Mussel eggs were obtained by spawning ripe adults according to standard procedures (Loosanoff & Davis 1963). To remove bacterial flora, fertilized eggs were washed with 0.45 µm-filtered, autoclaved seawater and placed in 1 l experimental units (EUs) filled with 600 ml of 0.45 µm filtered and autoclaved seawater at a density of 67 ± 6.8 eggs ml^{-1} and maintained at constant temperature (20 ± 1°C). Air was bubbled into each EU after passing through Gelman (bacterial air vent) 0.45 µm-filters. Previous experiments to test the sterility of the EUs indicated that after 72 h of bubbling air into peptone broth, no bacteria were introduced by the air system. After the introduction of fertilized eggs into each EU, a 0.1 ml water sample was seeded into TCBS agar to evaluate the possible introduction of VLB.

A reference strain (Gherna & Pienta 1992) of *Vibrio alginolyticus* (ATCC 17749) was cultured on Zobell agar media (Difco 1984) and incubated at 26 ± 1°C for 24 h. Bacteria were harvested, diluted and inoculated into EUs to obtain 5 treatment concentrations (10^2, 10^3, 10^4, 10^5 and 10^6 bacteria ml^{-1}). Cell concentrations were derived from a previously obtained equation relating spectrophotometric absorbance (600 nm) and bacterial concentration after staining with DAPI (Porter & Feig 1980, Anguiano-Beltrán 1996). As a control condition, EUs without bacterial inoculums were used. Actual inoculum concentrations were evaluated on TCBS agar after inoculation of *V. alginolyticus* into EUs. Five replicates of each bacterial treatment were used.

Samples of larvae (1 ml) were taken from each EU at the beginning of the experiment and after 48 h. Samples were examined under a light microscope using a Sedgwick-Rafter chamber. Survival of larvae was calculated as the number of veliger larvae developed from the initial number of fertilized eggs. This number included normal and abnormal veliger larvae. Normal larvae were considered as those that developed the standard 'D'-shape shell and a typical velum, while abnormal larvae did not develop this feature and presented a reduced velum. One-way ANOVA on transformed data (arcsine $\sqrt{p}$) and linear regression analyses were used to test the effect of treatments on the percentage of larvae survival and normality (Sokal & Rohlf 1995).

Table 1. One-way ANOVA for percent survival of *Mytilus galloprovincialis* larvae cultured in different *Vibrio alginolyticus* concentrations

Source	SS	df	MS	*F*	p
Between concentrations	1.10966	5	0.221932	11.49	9.62×10^{-6}
Within concentrations	0.463374	24	0.019307		
Total	1.573034	29			

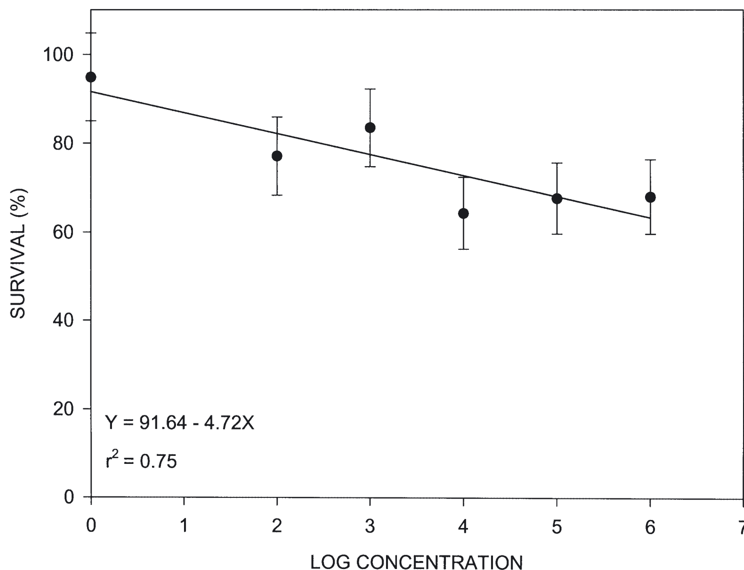

Fig. 1. *Mytilus galloprovincialis*. Survival of larvae in different *Vibrio alginolyticus* concentrations after 48 h. Solid line is the fitted regression, vertical bars are SEs (n = 5)

RESULTS

Negative results of VLB were detected in all samples collected after the introduction of fertilized eggs into the EUs, suggesting that VLBs were not present on the mussel eggs. The actual initial concentrations of *Vibrio algynolyticus* for each treatment were 3×10^2, 2×10^3, 3×10^4, 4×10^5 and 3×10^6 bacteria ml^{-1}.

Larval survival (Fig. 1) was between 64 and 95% for the different bacterial concentrations. Minimum survival (64%) was found in 3×10^4 bacteria ml^{-1}, and the maximum in the control treatment (95%). ANOVA of mussel larvae survival indicated that significant differences were found among treatments ($p < 0.001$, Table 1), and linear regression of survival versus bacterial concentration was significant ($p < 0.05$).

Larval normality showed highly significant differences among treatments ($p < 0.001$; Table 2). In the

Table 2. One-way ANOVA for normal percent of *Mytilus galloprovincialis* larvae cultured in different *Vibrio alginolyticus* concentrations

Source	SS	df	MS	*F*	p
Between concentrations	3.967642	5	0.793528	98.97	3.26×10^{-15}
Within concentrations	0.192414	24	0.008017		
Total	4.160056	29			

control, a mean percentage of 91% normal larvae was found versus 12% in 3×10^6 bacteria ml^{-1} of *Vibrio algnolyticus* (Fig. 2). Normality decreased as the bacterial concentration increased, and linear regression of larval normality versus bacterial concentration was significant ($p < 0.01$).

DISCUSSION

Large differences were observed among the percentages of larval normality in increasing concentrations of *Vibrio alginolyticus*, but larval survival did not show such dramatic differences. The difference between maximum (95%) and minimum (64%) survival was only 31% (Fig. 1).

In contrast, evaluation of larval normality clearly shows sub-lethal effects of the bacteria on veliger mussel larvae. In the control treatment (Fig. 2) 91% of the larvae developed the standard D-shaped shell, but at 3×10^2 bacteria ml^{-1}, this number decreased to 57%. A critical concentration threshold seems to be located between 2×10^3 and 3×10^4 bacteria ml^{-1}, where normal D-veliger larvae change from a mean percentage of 50 to 20%. These results are in agreement with Brown & Losee (1978), who reported that an inoculum of 1.6×10^3 bacteria ml^{-1} of *Vibrio anguillarum* in *Crassostrea virginica* larvae cultures (fertilized eggs to D-veliger phase) resulted in 22% survival, whereas 2.4×10^4 bacteria ml^{-1} resulted in only 2.4% survival. These authors suggested that some type of toxic substance could act as a teratogen, promoting abnormal development of fertilized oyster eggs, and also mentioned that *Vibrio* concentrations of 1×10^6 bacteria ml^{-1} allowed the potentially teratogenic metabolite to accumulate to its effective level before the fertilized eggs reached the straight-hinge stage; however, the results in this work show that a concentration of 3×10^4 decreased the normal development of *Mytilus galloprovinciallis* larvae drastically (by 80%).

Table 3 summarizes previous reports regarding the effects of *Vibrio* spp. on several mollusk larvae. Pathogenic effects of the same bacteria may not be similar for different species of larvae. In this study, a 32% mortality of *Mytilus galloprovincialis* larvae was observed in 3×10^6 bacteria ml^{-1} of *V. alginolyticus* whereas in a previous study, Anguiano-Beltrán et al. (1998) reported mortalities of 100% in 1×10^6 bacteria ml^{-1} with *Haliotis rufescens* larvae and post-larvae after a period of 24 h of exposure to the same bacterial species. Luna-González et al. (2002) reported that *Argopecten ventricosus*, *Atrina maura* and *Nodipecten subnudosus* were more susceptible to *V. alginolyticus* than was *Crassostrea gigas* (Table 3). Also Nicolas et al. (1996) reported that a *Vibrio* strain related to *V. splendidus* was virulent to *Pecten maximus* larvae, but was not pathogenic for oyster larvae. However, Sugumar et al. (1998) found that some isolates of *V. splendidus* were pathogenic for oyster larvae (*C. gigas*) at 10^5 bacteria ml^{-1}, producing 100% of mortality in 24 h.

Likewise, differences in susceptibility of the same molluscan larvae to different bacterial species are also reported. Elston & Leibovitz (1980) found that *Crassostrea virginica* larvae were more resistent to *Vibrio* sp. Isolate 981 (50% of mortality in 10^6 bacteria ml^{-1}) than to *Vibrio* sp. Isolate 1031 (50% of mortality in 5×10^3 bacteria ml^{-1}) in a period of 6 and 5 d, respectively, whereas Brown (1981) reported that *Vibrio anguillarum* Strains S1 and S2 produced high mortal-

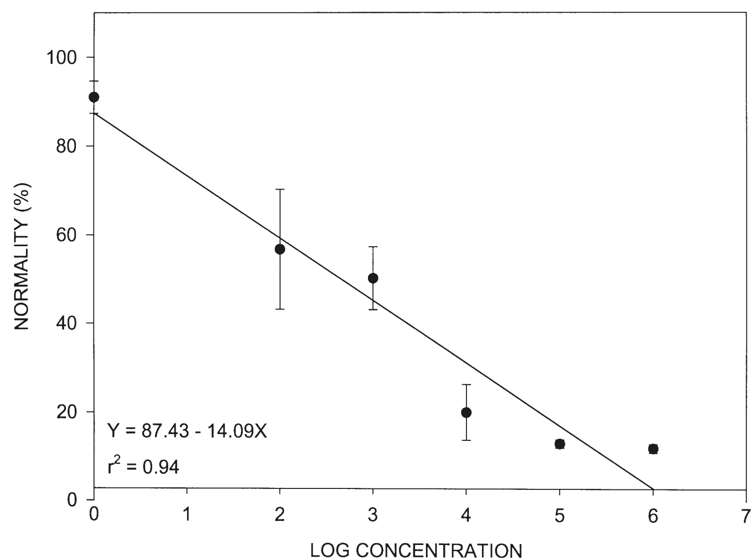

Fig. 2. *Mytilus galloprovincialis*. Normality of larvae in different *Vibrio alginolyticus* concentrations after 48 h. Solid line is the fitted regression, vertical bars are SEs (n = 5)

Table 3. Susceptibility of several molluscan larvae to different *Vibrio* species

Mollusk	Bacteria	Concentration (bacteria ml^{-1})	Mortality (%)	Time of exposure (h)	Source
Crassostrea virginica	*Vibrio* spp.	1.2×10^5	12.9	48	Brown (1973)
Crassostrea virginica	*Vibrio* spp.	3.9×10^8	99.9	48	Brown (1973)
Crassostrea virginica	*Vibrio anguillarum*	2.4×10^4	98	24	Brown & Losee (1978)
Crassostrea virginica	*Vibrio* sp. Isolate 98	1×10^6	50	144	Elston & Leibovitz (1980)
Crassostrea virginica	*Vibrio* sp. Isolate 1031	5×10^3	50	120	Elston & Leibovitz (1980)
Crassostrea virginica	*Vibrio anguillarum* S1	3×10^5	100	24	Brown (1981)
Crassostrea virginica	*Vibrio anguillarum* S2	1×10^5	100	24	Brown (1981)
Crassostrea gigas	*Vibrio* spp.	2.1×10^5	>90	48	Jeffries (1982)
Ostrea edulis	*Vibrio* spp.	2.2×10^6	>90	24	Jeffries (1982)
Ostrea edulis	*Vibrio tubiashii* EX1	1.7×10^2	70	36	Lodeiros et al. (1987)
Argopecten ventricosus	*Vibrio alginolyticus*	90×10^5	100	48	Sainz et al. (1998)
Crassostrea gigas	*Vibrio splendidus*	1×10^5	100	24	Sugumar et al. (1998)
Haliotis rufescens (larvae)	*Vibrio alginolyticus*	1×10^6	100	24	Anguiano-Beltrán et al. (1998)
Haliotis rufescens (postlarvae)	*Vibrio alginolyticus*	1×10^6	100	24	Anguiano-Beltrán et al. (1998)
Atrina maura[a]	*Vibrio alginolyticus*	5×10^5	>95	120	Luna-González et al. (2002)
Atrina ventricosus[a]	*Vibrio alginolyticus*	5×10^5	>95	120	Luna-González et al. (2002)
Nodipecten subnudosus[a]	*Vibrio alginolyticus*	5×10^5	>95	120	Luna-González et al. (2002)
Crassostrea gigas[a]	*Vibrio alginolyticus*	5×10^5	60	120	Luna-González et al. (2002)
Mytilus galloprovincialis	*Vibrio alginolyticus*	3×10^6	32	24	This study

[a]Data from figures in the literature source

ity (100% in 10^5 bacteria ml^{-1}) on *C. virginica* larvae in only 1 d. These results indicate differences of pathogenic effects according to the bacterial pathogen and differences in susceptibility among mollusk species.

Our data show that *Vibrio alginolyticus* do not cause massive mortality of mussel larvae, but that normal development can be severely affected. Nevertheless, in commercial farming operations these abnormal larvae will be discharged or they will die in the next phase of development. Therefore as a practical consideration, maintenance of VLB concentrations lower than 10^3 bacteria ml^{-1} would be recommended to avoid the abnormal development of mussel larvae.

Specific detection of pathogenic *Vibrio* by polymerase chain reaction (PCR), genetic probes or monoclonal antibodies would help in the development of control measures in bivalve larval culture before bacterial problems appear; however, commercial laboratories do not have trained personnel and routine monitoring of *Vibrio* is not practiced.

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LITERATURE CITED

Anguiano-Beltrán C (1996) Patogenicidad de *Vibrio alginolitycus* sobre larvas del mejillón europeo *Mytilus galloprovincialis* y larvas y poslarvas del abulón rojo *Haliotis fulgens*. MSC thesis, CICESE, Ensenada

Anguiano-Beltrán C, Searcy-Bernal R, Lizárraga-Partida ML (1998) Pathogenic effects of *Vibrio alginolyticus* on larvae and poslarvae of the red abalone *Haliotis rufescens.* Dis Aquat Org 33:119–122

Birkbeck TH, McHenery JG, Nottage AS (1987) Inhibition of filtration in bivalves by marine vibrios. Aquaculture 67(1–2):247–248

Brown C (1973) The effects of some selected bacteria on embryos and larvae of the American oyster, *Crassostrea virginica*. J Invertebr Pathol 21:215–223

Brown C (1981) A study of two shelfish pathogenic *Vibrio* strains isolated from Long Island during a recent outbreak of disease. J Shellfish Res 1(1):83:87

Brown C (1983) Bacterial diseases in bivalve larval cultures and their control. P 230–242. In: Berg CJ Jr (ed) Culture of marine invertebrates selected readings. Hutchinson Publishing Company, Stroudsburg

Brown C, Losee C (1978) Observations on natural and induced epizootics of vibriosis in *Crassostrea virginica* larvae. J Invertebr Pathol 31:41–47

Difco (1984) Dehydrated culture media and reagents for microbiology, 10th edn. Difco laboratories, Detroit

Douillet PA, Langdon CJ (1993) Effects of marine bacteria on the culture of axenic oyster *Crassostrea gigas* (Thunberg) larvae. Biol Bull 184:36–51

Elston R, Leibovitz L (1980) Pathogenesis of experimental vibriosis in larval american oysters, *Crassostrea virginica.* Can J Fish Aquat Sci 37:964–978

García-Pámanes LE (1990) El cultivo de mejillón en México y la problemática asociada a la actividad. Serie Científica UABCS, México 1:19–24

Gherna R, Pienta P (eds) (1992) Catalogue of bacteria and bacteriofagues, 18th edn. ATCC, Rockville

Guillard RRL (1959) Further evidence of the destruction of bivalve larvae by bacteria. Biol Bull 117:258–266

Jeffries VE (1982) Three *Vibrio* strains pathogenic to larvae of *Crassostrea gigas* and *Ostrea edulis*. Aquaculture 29: 201–226

Lizárraga-Partida ML, Vargas-Cárdenas G (1996) Influence of water circulation on marine and faecal bacteria in a mussel-growing area. Mar Pollut Bull 32:196–201

Lizárraga-Partida ML Montoya-Rodriguez L, Gendrop-Funes V (1997) The use of bacterial count in two mexican shrimp hatcheries. Cienc Mar 23(1):129–140

Lizárraga-Partida ML, Anguiano-Beltrán C, Searcy-Bernal R, Vazquez-Moreno E (1998) Bacterial water quality in abalone farms of Baja California. J Shellfish Res 17(3): 689–692

Lodeiros C, Bolinches J, Dopazo CP, Toranzo AE (1987) Bacillary necrosis in hatcheries of *Ostrea edulis* in Spain. Aquaculture 65:15–29

Loosanoff VL, Davis HC (1963) Rearing of bivalve molluscs. In: FS Russell (ed) Adv Mar Biol 1:1–136

López-Torres MC, Lizárraga-Partida ML (2001) Bacteria isolated on TCBS media associated with hatched *Artemia* cysts of commercial brands. Aquaculture 194:11–20

López-Torres MC, Macías Montes de Oca P, Lizárraga-Partida ML (2001) Evaluación de la calidad bacteriológia de dispositivos para limpieza de agua utilizados en acuacultura. Biotecnia 3(2):59–63

Luna-González A, Maeda-Martínez AN, Sainz JC, Ascencio-Valle F (2002) Comparative susceptibility of veliger larvae of four bivalve mollusks to a *Vibrio alginolyticus* strain. Dis Aquat Org 49:221–226

McHenery JG, Birkbeck TH (1986) Inhibition of filtration in *Mytilus edulis* L. by marine vibrios. J Fish Dis 9:257–261

Nicolas JL, Corre S Gauthier G, Robert R, Ansquer D (1996) Bacterial problems associated with scallop *Pecten maximus* larval culture. Dis Aquat Org 27:67–76

Nottage AS, Birkbeck TH (1987) The role of toxins in *Vibrio* infections of bivalve mollusc. Aquaculture 67:244–246

Nottage AS, Sinclair PD, Birkbeck TH (1989) The role of low-molecular ciliostatic toxins in Vibriosis of bivalve mollusks. J Aquat Anim Health 1:180–186

Porter KG, Feig YS (1980) The use of DAPI for identifying and counting aquatic microflora. Limnol Oceanogr 25:943–948

Portillo-López A, Lizárraga-Partida ML (1997) Detection of *Vibrio cholerae* O1 in diferent habitats of Todos Santos Bay, Baja California. Cienc Mar 23(4):435–447

Riquelme C, Hayashida G, Toranzo AE, Vilches J, Chavez P (1995) Pathogenicity studies on a *Vibrio anguillarum* related (VAR) strain causing an epizootic in *Argopecten purpuratus* larvae cultured in Chile. Dis Aquat Org 22:135–141

Sainz JC, Maeda-Martinez AN, Ascencio F (1998) experimental vibriosis induction with *Vibrio alginolyticus* of larvae of the catarina scallop (*Argopecten ventricosus* = *circularis*) (Serby II, 1842). Microb Ecol 35:188–192

Sokal RR, Rohlf FS (1995) Biometry: the principles and practice of statistics in biological research, 3rd edn. Freeman, New York

Sugumar G, Nakai T, Hirata H, Matsubara D, Muroga K (1998) *Vibrio splendidus* Biovar II as the causative agent of bacilary necrosis of Japanese oyster *Crassostrea gigas* larvae. Dis Aquat Org 33:111–118

Tubiash HS, Otto SV (1986) Bacterial problems in oysters. A review. In: Vivarès CP, Bonami JR, Jasper E (eds) Pathology in marine aquaculture. European Aquaculture Society, Spec Publ 8, p 233–242

Tubiash HS, Chanley PE, Leifson E (1965) Bacillary necrosis, a disease of larval and juvenile bivalve mollusks. J Bacteriol 90(1):1036–1044

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