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Vibrios in hatchery cultures of the razor clam, *Solen marginatus* (Pulteney)

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Abstract

Hatchery culture of the razor clam, *Solen marginatus* (Pulteney), has recently been developed in Galicia (NW Spain). However, recurrent episodes of mortalities of larval and post-larval cultures have been recorded during the course of various studies. The disease signs were similar to those described for other bivalve species in outbreaks caused by bacteria of the genus *Vibrio*. In this article, we present the results of microbiological monitoring of two batches of razor clams with different survival rates. All fermentative isolates were identified as members of the Splendidus clade within the genus *Vibrio*. Some of these isolates, identified as *Vibrio splendidus*-like, were clearly associated with the batch suffering mortalities, indicating their possible role as pathogens. Similar strains were found in the broodstock, suggesting vertical transmission of these bacteria. This is the first study of the microbiota associated with hatchery culture of *S. marginatus*, and the results will provide useful information for the optimization of a protocol for hatchery culture of this bivalve species.

Keywords: razor clam, shellfish hatchery, *Vibrio*.

Introduction

The razor clam, *Solen marginatus* (Pulteney), is the most abundant species of solenid (Bivalvia: Solenidae) in Spain. The commercial value of the species, together with that of other razor clams (*Ensis* spp. and *Solen* spp.), has recently increased in Europe and in new markets like Japan. Shellfish gatherers have focused on *S. marginatus* as a substitute for other overexploited razor clam resources. A supply of spat is therefore needed to counteract fishing pressure and the decline of natural beds.

Hatchery production of *S. marginatus* has been developed in the Centro de Investigacións Mariñas, CIMA-Ribadeo (Galicia, NW Spain), where the life cycle was completed by obtaining larvae from individuals previously reared in hatchery facilities (da Costa & Mart´ınez-Patiño 2009). This confirmed the potential suitability of this razor clam for aquaculture production. However, recurrent episodes of mortality have been observed in larvae and early post-larvae during experimental cultures, representing a bottleneck for production. Microbiota occur in large numbers in bivalve hatcheries, where the optimal conditions enhance the growth of bacteria, including opportunistic pathogens common in the marine environment. These proliferate under favourable conditions caused by the high stocking densities in larval cultures, the food supply and metabolite accumulation between water changes (Elston 1984; Prado *et al.* 2005).

Vibriosis is the main disease currently hindering the success of the hatchery culture of bivalves. Different species of *Vibrio* have been described as opportunistic pathogens associated with mortalities during larval development: *V. alginolyticus*, *V. anguillarum*, *V. neptunius*, *V. pectenicida*, *V. splendidus* and *V. tubiashii* (Tubiash, Chanley & Leifson

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1965; Brown 1981; Jeffries 1982; Riquelme *et al.* 1995, 1996; Nicolas *et al.* 1996; Lambert *et al.* 1998; Sugumar *et al.* 1998; G´omez-Le´on *et al.* 2005; Prado *et al.* 2005; Elston *et al.* 2008; Kesarcodi-Watson *et al.* 2009). These studies reported the disease in a wide range of bivalve species, including oysters (*Ostrea*, *Crassostrea*), clams (*Mercenaria*, *Venerupis*, *Panope*), scallops (*Pecten*, *Argopecten*) and mussels (*Perna*).

This is the first study that focuses on the microbiota associated with hatchery culture of *Solen marginatus* during the development from larvae to spat. We report the results of the microbiological monitoring of two batches of razor clams with different survival rates. The presence of vibrios and the effect of the use of antibiotics on the microbial populations were studied. The microbiological data obtained in the present study will help to improve the protocols for hatchery culture of this bivalve species.

Materials and methods

Larval cultures

Two batches (*A* and *B*) of larval razor clams were obtained by spontaneous spawning in the hatchery (CIMA-Ribadeo) and were cultured following the methodology described by da Costa & Mart´ınez-Patiño (2009). Briefly, the gametes released from the conditioned broodstock were transferred to 150-L culture tanks at an initial density of 5 eggs mL^{-1}. The temperature was set at 19 1 °C and the sea water (1-lm sand-filtered, UV-sterilized sea water) was changed every 2 days. No food was provided until full development of D-shaped veligers (day 1 post-fertilization). A mixed diet, consisting of *Isochrysis galbana, Diacronema lutheri*, *Chaetoceros calcitrans* and *Tetraselmis suecica*, was provided on a daily basis at a rate of 40 cells lL^{-1}. Growth and survival rate were determined.

Failure of hatchery cultures due to recurrent outbreaks of disease led to testing the experimental prophylactic use of antibiotic, in an attempt to elucidate the role of bacteria in the disease outbreaks. With this aim, chloramphenicol was added with every change of sea water (2.6 mg L^{-1}) during larval development until settlement.

Microbiology

Larvae and sea water were sampled from culture tanks every week, at change of water, and immediately processed *in situ*. The culture media used were marine agar (MA, Pronadisa), for marine heterotrophic bacteria, and thiosulphate–citrate–bile–sucrose (TCBS, Oxoid) for vibrios. The larvae were streaked on to agar plates, with an inoculat- ing loop (1 lL), to obtain comparable estimates of bacterial numbers in the different samples. The sea water from culture tanks was collected in sterile containers. In addition, adult clams used as broodstock were sampled for microbiological analysis. Fragments of gonadal tissues were aseptically excised, weighed and homogenized in sterile sea water (SSW). Serial dilutions of sea water or gonad were made in SSW and spread on agar plates.

The plates were incubated at 23 1 °C, 24–48 h (TCBS) or 7 days (MA). The different types of colonies were isolated and preserved as described by Prado *et al.* (2005). The basic characteristics of the bacterial isolates were determined: cell morphology and motility, oxidase and catalase activity, Gram reaction, growth in TCBS, glucose oxidation–fermentation in ZOF and aminoacid decarboxylation in Moeller's medium. Further studies were carried out with the fermentative isolates (Prado *et al.* 2005), including nitrate reduction, indol production and methyl-red tests. The extracellular enzyme activities were examined using starch, esculin, gelatine and Tween 80. The NaCl requirements (0, 3, 6, 8 and 10% NaCl) and the temperature range for growth (6, 25 and 37 °C) were determined. The sensitivity to the vibriostatic agent pteridine (O/129 150 lg) was also evaluated.

The sensitivity of the *Vibrio* isolates to chloramphenicol was evaluated using the disc diffusion method. The inhibition zone was determined on Mueller–Hinton agar (Oxoid) supplemented with 1% NaCl (MHA-1), after 24–48 h at 25 °C, using 30-lg discs (Oxoid).

Genetic characterization of fermentative isolates was performed by sequening of the 16S rRNA gene. Genomic DNA from pure cultures was extracted using the Instagene matrix (Bio-Rad), following the manufacturer's reommendations. The 16S rRNA genes were ampliied by PCR using standard primers 27F, 926F, 100R and 1510R (Lane 1991). The PCR productswere purified using UltraClean PCR Clean-up(Mo Bio Laboratories). The GenomeLab DTCS Quick Start Kit (Coulter) was use for the sequencing reactions. The resulting PCR products were processed in an

Automatic DNA Sequencer (CEQ 8000, Beckman Coulter). Sequence data sets were assembled using the Seqman program of the Lasergene software package (Dnastar) (Burland 2000). Comparative analyses were carried out in EzTaxon-e server (Kim *et al.* 2012). MEGA4 software (Tamura *et al.* 2007) was used to align the sequences and to construct a phylogenetic tree by the neighbour-joining method.

Results

Substantial differences were observed in the development of the two larval cultures and in the type of microbiota associated with the cultures.

Batch *A* settled by day 9 with a good survival rate (46.2%). A total of 9 bacterial isolates were obtained from larval samples, corresponding to the different types of colonies grown on the MA plates. Only two isolates from the initial samples showed fermentative metabolism, and strain 71 predominated. Isolate 298 was in a minority relative to the oxidative bacteria (Table 1).

In the sea water from culture tanks, the total heterotrophic bacteria counts were in the range 3.4×10^4–2.1×10^5 colony-forming units per mL (cfu mL^{-1}); this included the sample from post-larval stage (day 19) after the cessation of antibiotic treatment at the beginning of settlement. All isolates from sea water were oxidative bacteria obtained from MA plates, and no growth was recorded on the TCBS plates.

Batch *B* showed problems in larval development from the first days, and the survival rate was low at the end of the larval stage (5.9%, day 13). Complete mortality occurred at the post-larval stage, by day 27, despite the antibiotic treatment. A total of 17 representative isolates were obtained. The 13 fermentative strains corresponded to the razor clams (8) and sea water (4) samples in TCBS, and only one isolate was from the sample of dead post-larvae in MA (Table 1).

Table 1 Fermentative isolates obtained from the razor clam larval cultures in hatchery

Isolate	Batch	Sample	Day of culture	Culture medium
71	A	Larvae	2	MA
298	A	Larvae	5	MA
74	B	Larvae	0	TCBS
75	B	Larvae	0	TCBS
76	B	Larvae	3	TCBS
24	B	Sea water	3	TCBS
25	B	Sea water	3	TCBS
77	B	Larvae	6	TCBS
78	B	Larvae	6	TCBS
26	B	Sea water	6	TCBS
299	B	Larvae	13	TCBS
317	B	Sea water	20	TCBS
402	B	Dead post-larvae	27	TCBS
403	B	Dead post-larvae	27	TCBS
445	B	Dead post-larvae	27	MA

The total heterotrophic bacterial counts in the sea water from culture tanks were similar to those recorded for the other batch, ranging from 1.0×10^4 –1.3×10^5 cfu mL^{-1}. The main difference between the two batches was the presence of presumptive vibrios in TCBS at levels of $\approx 10^2$ cfu mL^{-1} in batch *B*, in contrast to the absence of these in batch *A*.

Characterization of the 15 fermentative isolates showed that they were Gram-negative, oxidase- and catalase-positive motile rods. All strains tested positive for glucose fermentation, nitrate reduction, gelatinase and lipase activities, as well as for growth at 4 °C and 6% NaCl. Negative results were obtained in the tests for lysine and ornithine decarboxylases and growth without NaCl.

The 16S rDNA sequences confirmed that the fermentative isolates were *Vibrio s*pp. The analytical results enabled division of the isolates into two groups within the Splendidus clade, clearly separated in the phylogenetic tree constructed with the closest relatives (Fig. 1).

Group 1 was formed by 7 isolates from TCBS samples of batch *B,* including larvae, post-larvae and sea water. In all cases, this was the dominant colony type in the corresponding plates.

The closest relative of all these strains, on the basis of the 16S rRNA gene, was *V. splendidus*, with similarity percentages in the range 99.70–99.71%. The group shared a separate branch with the type strain of this species in the phylogenetic tree (Fig. 1).

Besides the characteristics common to all the fermentative isolates, all 7 strains grew in TCBS, forming green colonies, and tested positive for indol production and amylase activity. The differential characteristics are listed in Table 2.

Group 2 included 8 strains from both batches, obtained from samples in MA and TCBS media. These were isolated mainly from larvae and post-larvae (only one was isolated from sea water) and were not present as the dominant type of colony.

The closest relatives of all the isolates in Group 2, as reported in EzTaxon-e, were *V. tasmaniensis*

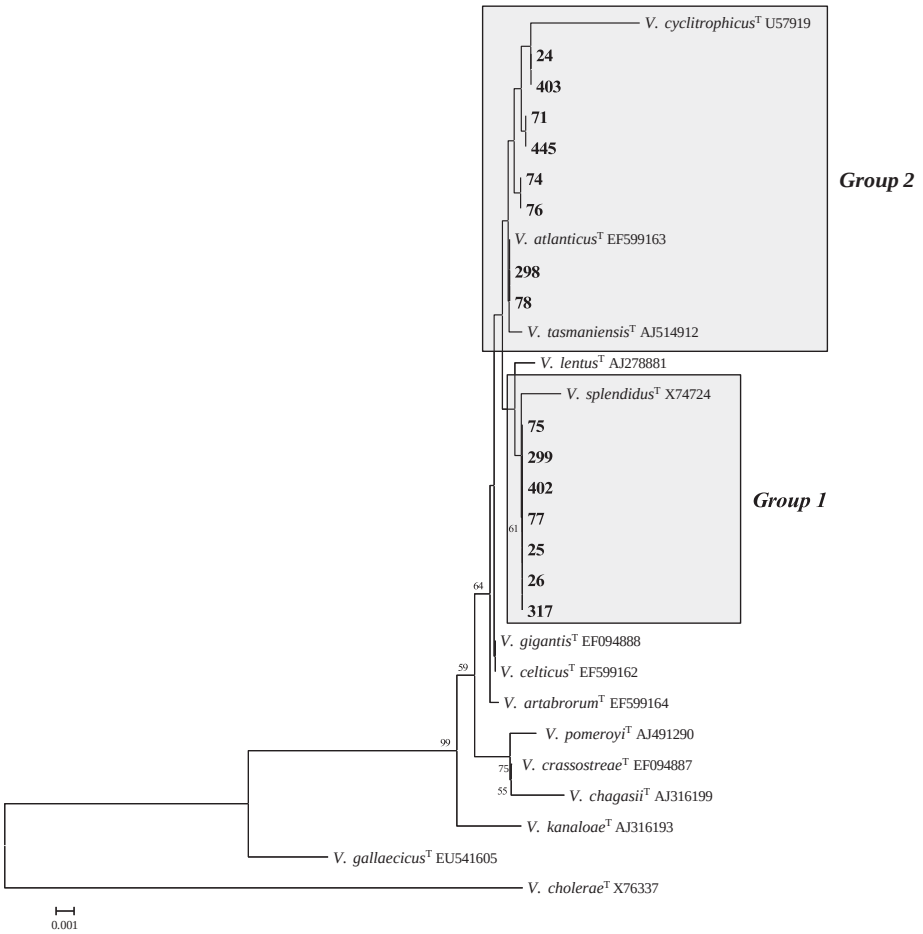

Figure 1 Phylogenetic tree of the sequences of the 16S rRNA genes (> 1400 bp) for the isolates from razor clam cultures included in this study and the type strains of their closest relatives, the members of Splendidus clade, using the neighbour-joining method. *Vibrio cholerae* was employed as outgroup. Horizontal branch lenghts are proportional to evolutionary divergence. Significant bootstrap values of 1000 replicates appear next to the corresponding branch.

Table 2 Differential phenotypic characteristics of the Group 1 isolates

Isolate	75	25	26	77	299	317	402
Source	L	SW	SW	L	L	SW	PL
Day of culture	0	3	6	6	13	20	27
Culture medium	TCBS	TCBS	TCBS	TCBS	TCBS	TCBS	TCBS
ADH	—	—	—	+	—	—	—
Growth							
37 °C	—	+	—	+	—	—	—
8% NaCl	—	+	—	—	—	—	—
O/129	R	S	R	R	R	R	R
Chloramphenicol (C30)	S	S	R	R	R	S	R

L = larvae; SW = sea water from culture tanks; PL = post-larvae; ADH = arginine dihydrolase; S = sensitive; R = resistant.

and *V. atlanticus*, with percentages of similarity between 99.66 and 100%. In the phylogenetic tree, they were located near the type strains of these species (Fig. 1). This was a phenotypically heterogeneous group, sharing only the characteristics included in the general description of all the fermentative isolates. The differences within the group are summarized in Table 3.

The assays for sensitivity to chloramphenicol revealed resistant strains in Group 1, the dominant

Table 3 Differential phenotypic characteristics of Group 2 isolates. See abbreviations in Table 2

Isolate	74	71	76	24	298	78	403	445
Source	L	L	L	SW	L	L	PL	PL
Day of culture	0	2[a]	3	3	5[a]	6	27	27
Culture medium	TCBS	MA	TCBS	TCBS	MA	TCBS	TCBS	MA
TCBS	y	y	y	y	g	y	y	y
ADH	—	+	—	—	+	+	+	+
Indol	+	+	+	+	+	+	—	—
Amylase	+	+	+	+	+	+	+	—
Growth								
37 °C	+	+	+	+	—	+	+	+
8% NaCl	—	+	—	+	—	—	+	+
O/129	S	S	S	S	S	S	R	S
Chloramphenicol (C30)	S	S	S	S	S	S	S	S

[a]batch A; y = yellow colonies; g = green colonies.

types of colony, after only 6 days of larval culture and antibiotic treatment (Table 2). Group 2 was sensitive to chloramphenicol (Table 3).

To find the source of potential pathogens in batch *B*, vertical transmission of bacteria from adults to larval cultures was considered. The 7 different types of colony isolated from the samples of broodstock gonad in TCBS (RSm-T1 to RSm-T7) were identified on the basis of their 16S rDNA sequences. They all belonged to the Splendidus clade of the genus *Vibrio* (Fig. 2). Strains similar to Groups 1 and 2 were found together with isolates related to the species *V. gigantis–V. celticus*, which were not detected in any batch. These results strongly indicate the importance of the microbiota present in the gonad of broodstock and the vertical transmission of bacteria, including potential pathogens, as demonstrated by the clustering of strains from adults and larvae.

Discussion

Bivalve aquaculture is highly diversified and new species are cultured in order to replenish natural beds and expand commercial exploitation. Among razor clam species, *S. marginatus* displays the best yields for hatchery culture. Hatchery culture of *S. marginatus* throughout the complete life cycle, including the development of larval and post-larval stages, has been accomplished by da Costa & Mart´ınez-Patiño (2009). However, episodes of mortalities indicated the existence of problems related to the presence of bacteria. As most studies of mortalities in bivalve hatcheries have identified the aetiological agents as members of the genus *Vibrio*, we focused our attention on these populations in two cultures with markedly different survival rates.

The duration of larval development and the survival rate of batch *A* were as expected for successful culture of *S. marginatus*, while in batch *B* development took longer and mortality was higher (da Costa & Mart´ınez-Patiño 2009).

The total counts of heterotrophic bacteria in the sea water from the culture tanks were similar for both batches. Moreover, only 3 of 27 isolates from MA corresponded to fermentative strains. These results ruled out this value as a practical indicator of health problems.

The main difference between batches was in the TCBS counts, indicating the presence of vibrios in larvae, post-larvae and sea water from batch *B*, but not from batch *A*. The sea water and microalgae were ruled out as sources of bacterial contamination on the basis of the results of routine bacteriological controls carried out in the hatchery.

TCBS medium is a useful tool in hatchery culture. It has practical advantages such as the rapid and easy interpretation of the results by personnel with only basic knowledge of microbiology. Growth in this medium is a good indicator of the health status of cultures, not only of the presence of pathogens. In the present study, bacterial counts $\approx 10^2$ cfu mL^{-1} in the sea water from culture tanks indicated a health risk, that is, lower values than those proposed by other authors: 10^3 cfu mL^{-1} (Lodeiros *et al.* 1987) and 10^4 cfu mL^{-1} (Widman *et al.* 2001). However, the risk of the presence of vibrios must be determined in each hatchery.

Special attention should be focused on vibrios associated with larvae. Larvae can act as a reservoir

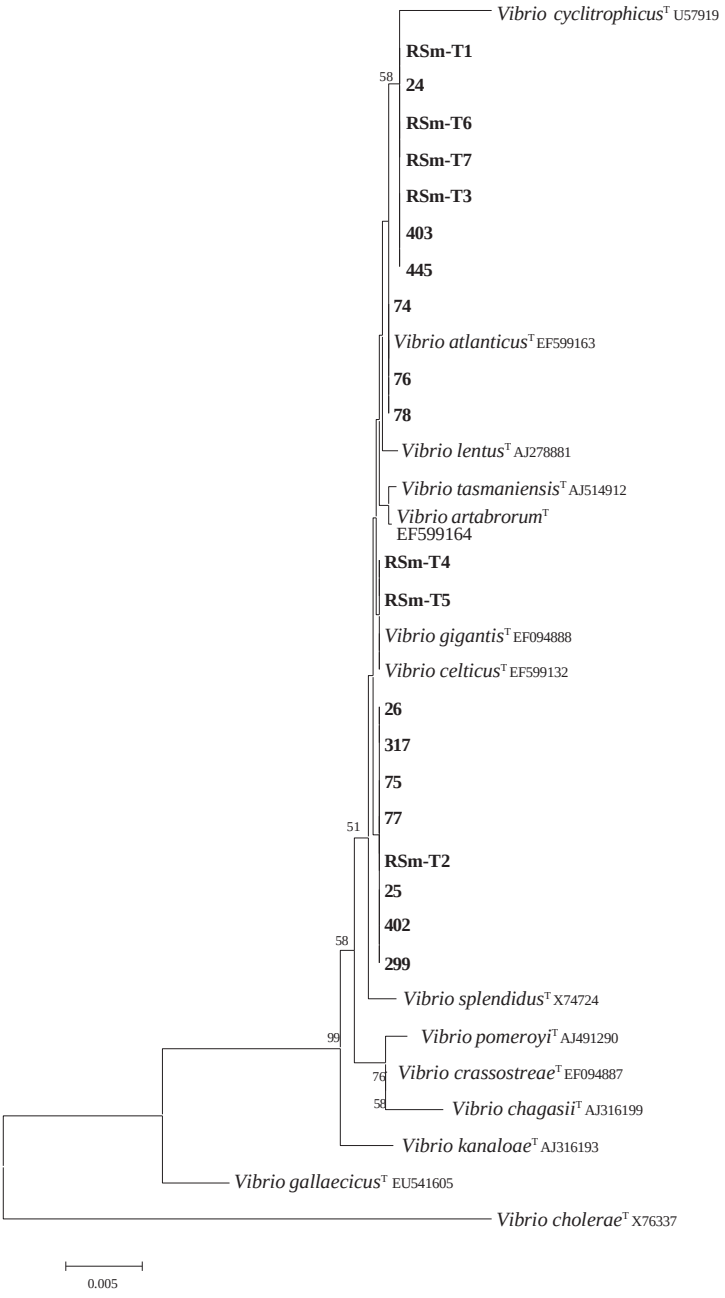

Figure 2 Phylogenetic tree of the sequences of the 16S rRNA genes for the isolates related to batch-*B*, including larval cultures and broodstock. Characteristics as Figure 1.

and release bacteria to the sea water after every renewal; this explains the initial detection of the *V. splendidus*-like bacteria in larvae and also later in sea water from culture tanks. Moreover, bacteria attached to larvae may be less exposed to the action of antibiotics.

In relation to the identification of vibrios, the presence of members of the Splendidus clade was expected, taking into account that all 13 members have been isolated from marine environments and many of them from bivalves. The high phenotypical diversity within this clade and the extremely conserved sequences of the 16S rRNA gene make further molecular studies necessary for the assignment of the isolates to any known species or the description of a new one.

Group 1 constituted phenotypically similar isolates that could not be assigned to the species

described; however, it should be taken into account that their closest relative was *V. splendidus*, reported as a pathogen in hatchery culture of bivalves, including oysters, clams, scallops and mussels (Jeffries 1982; Sugumar *et al.* 1998; G´omez-Le´on *et al.* 2005; Kesarcodi-Watson *et al.* 2009). In only 6 days, these strains became dominant in larvae and sea water, and this status was main- tained until the collapse of batch *B*. Further experiments should be carried out to ascertain whether *V. splendidus*-like bacteria are primary pathogens causing the mortalities or they are sec- ondary colonizers of hosts weakened by break- down in resistance.

In contrast, the strains included in Group 2 were not dominant and even occurred in the successful cultures. Their closest relatives, indicated by the presence of the 16S rRNA gene, were two species with no known pathogenicity for bivalves, *V. tasmaniensis* (Thompson, Thompson & Swings 2003) and *V. atlanticus* (Di´eguez *et al.* 2011). The present results appear to rule out the involvement of these species in the mortalities as they constitute a part of the normal microbiota of the cultures.

Identification of similar strains in Group 1 and Group 2 in association with gonad tissue suggests that the broodstock was an important source of the bacteria found in the larval culture. This was supported by the early detection of both types (day 0). The vertical transmission of vibrios has been demonstrated in other bivalves, such as the flat oyster, *Ostrea edulis* (Lodeiros *et al.* 1987), and the scallop, *Argopecten purpuratus* (Riquelme *et al.* 1994). Sugumar *et al.* (1998) isolated a strain of *V. splendidus*, which was similar to the strains that caused mortalities in larval cultures of the Pacific oyster, *Crassostrea gigas*, from the gonad of broodstock.

As reported here for *S. marginatus*, Riquelme *et al.* (1995) related the presence of vibrios to the failure of one *A. purpuratus* larval culture. More- over, vibrios were found in the gonad of the broodstock of the batch suffering mortalities.

In relation to the role of the antibiotics, we demonstrated that the experimental use of chloramphenicol did not guarantee the control of vibrios in the tank environment, in contrast to previous reports (Lodeiros *et al.* 1987; Jeanthon, Prieur & Cochard 1988; Campa-C´ordova *et al.* 2006). Vibrios were detected throughout the culture of batch *B*, despite treatment with antibiotic during settlement.

Chloramphenicol was commonly used in bivalve hatcheries in the last century, as it is a broad-spectrum, inexpensive antibiotic with no specific storage requirements (see reviews Le Pennec & Prieur 1977; Prado 2006).Some beneficial effects on larval cultures have been reported for bivalves, including the genera *Mercenaria*, *Ostrea*, *Pecten*, *Crassostrea* and *Argopecten* (Tubiash *et al.* 1965; Jeffries 1982; Lodeiros *et al.* 1987; Jeanthon *et al.* 1988; Robert, Miner & Nicolas 1996; Uriarte, Far´ıas & Castilla 2001; Campa-C´ordova *et al.* 2006). Nowadays, its use has been banned in animals intended for food production in many countries because it has not been possible to establish a safe level of human exposure (Hern´andez-Serrano 2005). However, the experimental use, for research purposes, in larval cultures of new species of bivalves in hatchery may provide specific information to elucidate the role of bacteria in these cultures, and human exposure to the drugs may not be relevant as the animals will be grown on to commercial size for at least 1–2 years in the sea (Uriarte *et al.* 2001).

Resistance to the antibiotic developed rapidly in Group 1 of strains in batch *B*, that is, after only 6 days of culture and antibiotic treatment. This mechanism may cause, or at least contribute to, the dominance of the *V. splendidus*-like strains during the course of the culture, via selective pressure. Resistance to chloramphenicol has been described for vibrios in aquaculture (Ho *et al.* 2000; Roque *et al.* 2001; Tendencia & de la Peña 2001; Vaseeharan *et al.* 2005; Dang *et al.* 2006), and an associated risk of reduced effective- ness of therapies has been reported, with aquacul- ture ponds becoming potential reservoirs of transferable resistance genes and the appearance of multidrug resistance (Heuer *et al.* 2009; Kümmerer 2009).

In conclusion,

(i) The bacterial populations associated with larval cultures of *S. marginatus* are an important factor in the successful culture of the razor clams, and mortalities appear to be caused by vibrios.

(ii) The Group 1 *V. splendidus*-like isolates is a good candidate for future studies of pathogenicity for razor clam larvae.

(iii) Vertical transmission of vibrios was demonstrated, which indicates the need to control the microbiota of broodstock to reduce the entry of pathogens to the culture system.

(iv) Antibiotic treatment did not guarantee survival of the larvae in hatchery cultures and caused the appearance of resistance, with the subsequent risks for the installation.

Alternative treatments should be based on knowledge and control of opportunistic pathogens combined with the enhancement of beneficial bacteria.

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