Phytoplankton production systems in a shellfish hatchery: variations of the bacterial load and diversity of vibrios

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Abstract

Aims: Outbreaks of disease caused by some *Vibrio* species represent the main production bottleneck in shellfish hatcheries. Although the phytoplankton used as food is one of the main sources of bacteria, studies of the associated bacterial populations, specifically vibrios, are scarce. The aim of the study was the microbiological monitoring of the microalgae as the first step in assessing the risk disease for bivalve cultures.

Methods and Results: Two phytoplankton production systems were sampled weekly throughout 1-year period in a bivalve hatchery. Quantitative analysis revealed high levels of marine heterotrophic bacteria in both systems throughout the study. Presumptive vibrios were detected occasionally and at low concentrations. In most of the cases, they belonged to the Splendidus and Harveyi clades.

Conclusions: The early detection of vibrios in the microalgae may be the key for a successful bivalve culture. Their abundance and diversity were affected by factors related to the hatchery environment.

Significance and Impact of the Study: This work represents the first long study where the presence of vibrios was evaluated rigorously in phytoplankton production systems and provides a suitable microbiological protocol to control and guarantee the quality of the algal cultures to avoid the risk of transferring potential pathogens to shellfish larvae and/or broodstock.

Introduction

Microalgae produced by mass culture are of high nutritional value, and they are used as the primary food source in bivalve aquaculture. Algal culture is one of the key steps in mollusc hatcheries, because large volumes of good-quality phytoplankton must be available to support the intensive production of spat, and failure in their management would be catastrophic (Brown *et al.* 1997; Helm and Bourne 2004; Sarkis 2007). The cultures are not axenic and they harbour a wide spectrum of bacterial populations that utilize metabolites and organic substances released by living or dead algal cells (Berland *et al.* 1970; Cole 1982; Riquelme *et al.* 1987; Eiler *et al.* 2007; Bruckner *et al.* 2011). Furthermore, some bacterial species may have beneficial effects, improving microalgal growth through the production of vitamins and other growth factors (Haines and Guillard 1974; Fukami *et al.* 1997; Suminto and Hirayama 1997; Croft *et al.* 2005; Rivas *et al.* 2010; Le Chevanton *et al.* 2013). However, algal cultures may also be a possible source of pathogens (Saulnier *et al.* 2009; D'Alvise *et al.* 2012; Prol-Garc´ıa *et al.* 2013). The most important larval pathogens are members of the genus *Vibrio* (*V. corallilyticus, V. neptunius, V. pectenicida, V. splendidus, V. tubiashii, V. ostreicida*), which are responsible for outbreaks of disease and the rapid loss of production batches (Tubiash *et al.* 1970; Hada *et al.* 1984; Lambert *et al.* 1998; Nicolas *et al.* 2004; Prado *et al.* 2005, 2014b; Garnier *et al.* 2007; Elston *et al.* 2008; Kesarcodi-Watson *et al.* 2009).

Considering the severe losses caused by *Vibrio* in bivalve hatcheries and the scarcity of detailed studies of the

presence of these bacteria associated with phytoplankton used as food, the present study was conducted as an evaluation of the culturable bacteria present in microalgal cultures, with special attention given to the members of this genus. Two different microalgae production systems in the bivalve hatchery were sampled weekly throughout 1-year and the bacterial load was determined. The presumptive vibrios were isolated, characterized and identified to elucidate the potential disease risk for bivalve cultures.

Materials and methods

Algal cultures

Stock cultures of the microalgae *Chaetoceros calcitrans, Chaetoceros gracilis, Chaetoceros mulleri, Diacronema lutheri, Isochrysis galbana, I. galbana T-iso, Phaeodactylum tricornutum, Skeletonema costatum* and *Tetraselmis suecica* were part of the culture collection of the Centro de Investigacións Mariñas (CIMA; Ribadeo, Galicia, NW Spain). They were kept in an isothermal chamber in 20 ml tubes at 18 1°C. Inocula of each algal species (Fig. 1) were transferred to start the monocultures in 250 ml Erlenmeyer flasks and then cultured at 20 1°C under con- stant illumination (180–220 lmol photons m^{-2} s^{-1}) in 2 l glass carboys filled with sand-filtered (1 lm) and autoclaved seawater enriched with sterilized Algal-1 med- ium (Nutricio´n Avanzada, Spain). A small portion of this volume was used to initiate mass production of phytoplankton in two different systems (Fig. 1). Algal cells produced were used to feed the shellfish larvae (during the larval rearing season between March and August) and the broodstock.

Intermediate scale cultures

Intermediate scale cultures (ISC) were carried out as described above but in 6 l glass carboys. Phytoplankton was cultured for 10 days and used to start mass produc- tion or to feed the shellfish larvae and broodstock, mix- ing previously equal proportions of *Isochrysis* sp., *Chaetoceros* sp., *Diacronema* sp., *Skeletonema* sp., *Tetrasel- mis* sp. and *Phaeodactylum* sp. in a bucket (ISCm).

Continuous culture system

A new continuous culture system (CCS) was established in the hatchery in an attempt to improve phytoplankton production. The start-up and the stabilization of CCS were carried out during the first and second year respectively. Microalgae inoculation of the CCS was performed from the monocultures produced in the ISC at late-exponential phase. Algal cells were previously checked under a microscope to ensure the purity of the culture. CCS was carried out in polyethylene bags (400 l) following the classical procedures described by Farrar (1975). Bags were filled with pasteurized seawater (30 min at 75°C) to eliminate mesophilic aerobic bacteria. Seawater was enriched with Solution C medium, which includes a mixture of mineral salts, nitrate and phosphate. Culture medium

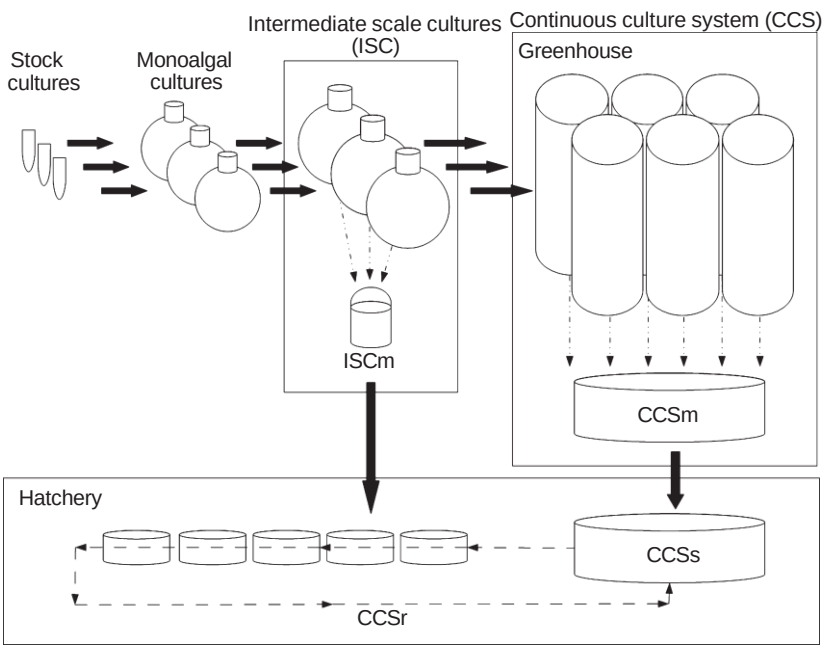

Figure 1 Diagram showing the phytoplankton production systems: intermediate scale culture (ISC) and continuous culture system (CCS). Sampling points were indicated: ISC mixing bucket (ISCm), CCS mixing tank (CCSm), storage tank (CCSs) and recirculation pipe (CCSr).

was added constantly (1 ml per litre of algal culture), whereas the supply of sodium silicate, in the diatom culture, was added twice a week. Continuous aeration was provided to prevent the algae from settling. Moreover, CO_2 addition allowed pH maintenance between 7 and 8. Bags were kept at 20 1°C and illuminated by natural and artificial light under a photoperiod regime of 18 : 6 h of light : darkness in a greenhouse. The artificial illumination was provided by vertical daylight fluorescent lamps (Philips TL-D, the Netherlands) at 180–220 lmol photons m^{-2} s^{-1}. Algae were harvested when the culture reached the exponential growth phase and the algal culture was maintained in this phase for 2 months under a dilution rate of 12·5 per day. Each species was inspected daily under a light microscope. Volumes of *Isochrysis* sp., *Diacronema* sp., *Chaetoceros* sp. and *Tetraselmis* sp. were then mixed in a tank (CCSm) at 1·4 : 1·4 : 1·9 : 1 proportions. The phytoplankton was transferred to the hatchery, placed in the storage tank (CCSs) and distributed throughout the installation by a recirculation system (Fig. 1). The remainder of the phytoplankton was returned to tank CCSs through the recirculation pipe (CCSr). The storage tank was cleaned daily, whereas CCSr and CCSm were cleaned once and twice per week respectively.

Microbiological samples

Microbiological sampling was carried out weekly during the 1-year study period (between March of the first year and April of the second year), at the points shown in Fig. 1. Samples were collected in sterile plastic container of 100 ml (Deltalab, Spain) from the mixing bucket (ISCm), in the ISC system, and from the tanks (CCSm and CCSs) and the recirculation pipe (CCSr) in the CCS. For evaluation of the systems under the least favourable conditions, the samples were collected before cleaning the systems. Samples were immediately processed *in situ* as described in Prado *et al.* (2005) and 100 ll of samples corresponding to appropriate decimal dilutions in sterile seawater was spread on Marine Agar (MA; Pronadisa-Lab Conda, Spain), for detection of marine heterotrophic bacteria (MHB), and on Thiosulphate-Citrate-Bile-Sucrose (TCBS; Oxoid, England), for detection of presumptive vibrios (PV). The TCBS and MA plates were incubated under aerobic conditions at 20 1°C for 24—48 h and 1 week respectively. Bacterial counts were expressed as colony forming units per millilitre (CFU ml^{-1}).

Algal cells were counted in the mixed samples (ISCm and CCSm) with a Bürker-Turk counting chamber and the bacterial : microalgal cell ratio was determined as a routine procedure in the hatchery. The results were expressed as number of bacteria per million phytoplankton cells.

Different types of colony on TCBS plates were selected on the basis of morphological and growth characteristics and one colony of each type was isolated, purified and maintained frozen for further studies at —80°C in Marine Broth (MA; Pronadisa-Lab Conda, Spain) supplemented with 15% glycerol (v/v). The phenotypic properties of the presumptive vibrios were determined by previously described tests (Romalde *et al.* 1990; Prado *et al.* 2005). The DNA was extracted with the Instagene kit (Bio-Rad, CA, USA). The 16S rRNA gene of the PV was amplified and sequenced using specific bacterial primers (27F and 1510R) (Lane 1991) to obtain an almost complete sequence (approx. 1400 bp). Sequences were analysed with the Lasergene Seqman (DNAstar, WI, USA) and identified using the EzTaxon-e server (Kim *et al.* 2012). Phylogenetic analyses were performed using MEGA4 software (Tamura *et al.* 2007) after multiple alignments of data by CLUSTALW (Thompson *et al.* 1994). Distances (distance options according to the Kimura two-parameter model) and clustering with the Neighbour Joining (NJ) (Saitou and Nei 1987) method were determined by using bootstrap values based on 1000 replications. Sequences were deposited in the DDBJ/EMBL/GenBank database under accession numbers HF549200–HF549247 (Figs 1 and 2; Tables S1 and S2).

Results

Bacterial counts

PV were detected on TCBS samples of microalgae from both production systems (Fig. 2; Table 1). The average counts were $5{\cdot}8 \times 10^1$ and $1{\cdot}7 \times 10^2$ CFU ml^{-1}, in ISC and CCS respectively, and some values were below the detection limits in both systems ($<10^1$ CFU ml^{-1}) (Fig. 2; Table 1). PV only grew in four samples from ISC (Fig. 2a), but were detected in a third of the CCSm samples (Fig. 2b) and in half of the recirculation system samples, CCSs and CCSr (Fig. 2c,d).

The highest levels of PV were found in samples from the CCS (Fig. 2; Table 1), mainly in the recirculation system (CCSs and CCSr) and during the larval production season. TCBS load of the pasteurized water was below the detection limits (data not shown).

In the established ISC system, the high PV concentrations in the initial samples (Fig. 2a; Table 1) were related to a vibrios contamination of the stock cultures (data not shown).

The MHB load was similar ($\approx 10^6$ CFU ml^{-1}) in both systems throughout the year (Fig. 2; Table 1), with only slightly higher values in the ISC system, which also yielded the maximum and minimum counts on MA (Table 1).

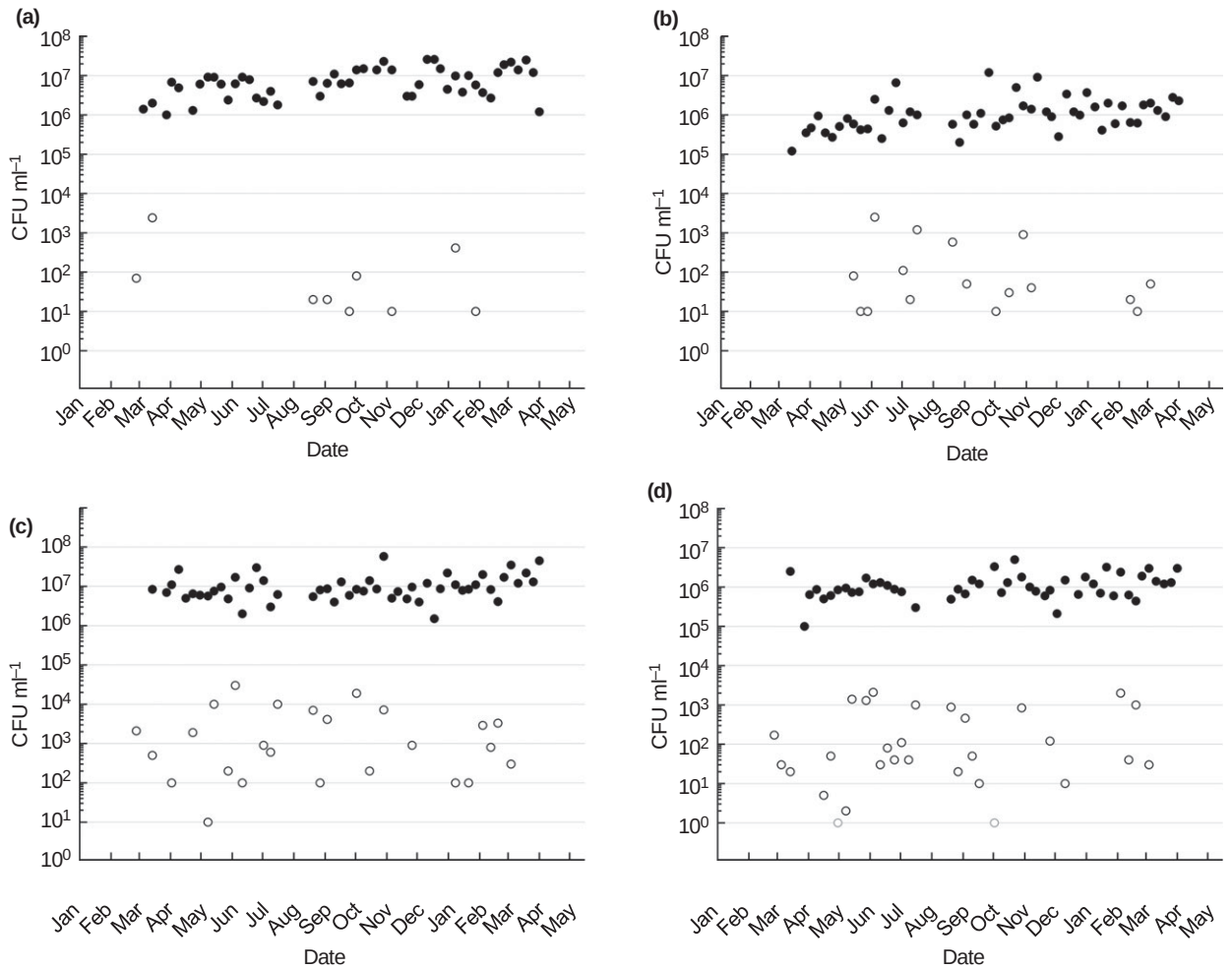

Figure 2 Bacterial counts of marine heterotrophic bacteria (filled symbols) and presumptive vibrios (open symbols) at the sampling points of the phytoplankton production systems over a 1-year period: Intermediate scale cultures (ISC)m (a), continuous culture system (CCS)m (b), CCSs (c) and CCSr (d).

The ratio of MHB per microalgal cell was approx. 1 : 1 in all cases, whereas the PV ratio was 1 : 10^6 in ISC and 23 : 10^6 in CCS (Table 2).

Identification of presumptive vibrios

A total of 48 strains were isolated from TCBS: seven in ISCm samples and 41 from CCS (seven in CCSm, 15 in CCSs and 19 in CCSr). The isolates were identified by phenotypic and molecular methods. Analysis of the simi- larities of 16S rRNA gene sequences confirmed that 44 strains belonged to the family *Vibrionaceae* and only four isolates were included in other taxonomic groups.

Family Vibrionaceae (*n* = 44)

In total, 42 strains were identified as *Vibrio* spp., whereas two isolates belonged to the genus *Photobacterium*.

Genus Vibrio. Most of the strains examined in this study shared the main phenotypic features of the genus *Vibrio*: they were Gram-negative, facultatively anaerobic, oxidase and catalase positive, motile, grew on TCBS and reduced nitrates to nitrites (Noguerola and Blanch 2008). Phylogenetic analysis enabled classification in four different groups within Splendidus clade and three groups in the Harveyi clade, whereas the isolate 2071 was identified as *V. brasiliensis* (Fig. 4; Table 3; Table S1).

Splendidus clade. Members of Splendidus clade (*n* = 32) were strongly associated with CCS (*n* = 28) and were the main type of colony or even pure cultures in these samples (Fig. 3; Table 3; Table S1).

Group A was closely related to the species *Vibrio atlanticus*, *Vibrio lentus* and *Vibrio tasmaniensis*. In CCS,

Table 1 Average, maximum and minimum counts (CFU ml^{-1}) of PV and MHB at the sampling points in the phytoplankton production systems

	ISCm		CCSm		CCSs		CCSr	
	PV	MHB	PV	MHB	PV	MHB	PV	MHB
Average	5·8 9 10^1	8·8 9 10^6	1·1 9 10^2	1·6 9 10^6	1·9 9 10^2	1·2 9 10^6	2·2 9 10^2	1·3 9 10^6
Maximum	2·4 9 10^3	2·6 9 10^7	2·5 9 10^3	1·2 9 10^7	3·0 9 10^3	5·8 9 10^6	2·1 9 10^3	5·0 9 10^6
Minimum	$<10^1$	1·0 9 10^6	$<10^1$	1·2 9 10^5	$<10^1$	2·0 9 10^5	$<10^1$	3·0 9 10^5

ISC, Intermediate scale cultures; CCS, continuous culture system; MHB, marine heterotrophic bacteria; PV, presumptive vibrios.

Table 2 Ratio of MHB and PV per million microalgal cells in the phytoplankton production systems

	ISC		CCS	
	PV	MHB	PV	MHB
Average	1·6 9 10^0	1·1 9 10^6	2·3 9 10^1	1·3 9 10^6
Maximum	3·3 9 10^1	3·1 9 10^6	5·1 9 10^2	9·2 9 10^6
Minimum	$<10^1$	1·1 9 10^5	$<10^1$	1·0 9 10^5

ISC, Intermediate scale cultures; CCS, continuous culture system; MHB, marine heterotrophic bacteria; PV, presumptive vibrios.

group A (n = 11) was only detected during stabilization of the system. This corresponded to dominant type in the recirculation system samples (CCSs and CCSr). Group B (n = 10) was the most abundant in the initial steps of the start-up of CCS. These isolates were associated with high PV counts (>10^3 CFU ml^{-1}) and constituted the main or even the only type of colony at the three sam- pling points. Group C was constituted by five strains related to the species *Vibrio hemicentroti* and the larval pathogen *V. splendidus*, and was only detected in the final CCS samples at a very low concentration (10^1 CFU ml^{-1}). Two strains, closely related to *Vibrio gigantis* and *Vibrio celticus*, constituted the group D and they were occasionally isolated in the storage tank (CCSs) as the main type of colony.

In ISC, only four vibrios were identified (one in the group A and three in the group C) and they were only detected in the first samples, coinciding with the prob- lems described previously in the algal cultures.

Harveyi clade. Nine strains belonged to the Harveyi clade and they were isolated during the start-up and stabilization of the CCS (Fig. 4; Table 3; Table S1). Three independent groups of strains were differentiated. Group E was constituted by five strains associated with the recirculation system (CCSs and CCSr) at different times. Three strains formed the group F, close to *Vibrio algino- lyticus*, and they were the predominant type in the sam- ples taken at the end of production period when the environmental temperature was high and corresponded to a unique sampling time. The isolate 2065, which was detected in the return pipe (CCSr), constituted the group G and was related to the species *Vibrio jasicida* and *Vibrio owensii*.

Genus Photobacterium

This genus includes some species formerly classified as vibrios. Strains 1319 and 1983 were isolated from CCSr samples and identified by their 16S rRNA gene sequences as members of the genus *Photobacterium* even though they showed the basic phenotypic profile of vibrios (Table 3; Table S1).

Other taxonomic groups

Only four strains, exceptionally detected in the TCBS samples plates, were identified as members of other taxonomic groups (Table 3; Table S2). Phenotypic and molecular features of the isolates 1151, 2118, 2094 and 2098 enabled their identification as members of the genera *Shewanella*, *Halomonas*, *Prolinoborus* and *Pseudomonas* respectively.

Discussion

Bivalve production in hatcheries is highly susceptible to outbreaks of disease caused by some *Vibrio* species. Monitoring the routes of entry of bacteria should be the first step to prevent bacterial proliferation in the bivalve cultures and the subsequent detrimental effects.

The results obtained throughout 1-year of sampling confirmed that the microalgae, supplied daily as food to the bivalve cultures in the hatchery, constituted an important direct source of bacteria. The MHB counts were ≈10^6 CFU ml^{-1}, which is consistent with the results obtained by other authors (Salvensen *et al.* 2000; Nicolas *et al.* 2004; Makridis *et al.* 2012). The phytoplankton is harvested at the end of the exponential growth phase when the algal division rate has slowed down and the light penetration and/or nutrients are restricted. The microalgae are then mixed and stored in tanks, where they

Table 3 Identification of the Thiosulphate-Citrate-Bile-Sucrose isolates from the phytoplankton production systems

	ISCm				CCSm				CCSs				CCSr			
Date	CFU ml^{-1}	Strain*	Group†	Identity‡ (%)	CFU ml^{-1}	Strain	Group	Identity (%)	CFU ml^{-1}	Strain	Group	Identity (%)	CFU ml^{-1}	Strain	Group	Identity (%)
16/Mar	7·0 9 10^{1}	1095$^{(+)}$	C	*V. spl* (99·6)									1·7 9 10^{2}	1151$^{(+)}$	–	*Sh. pie* (98·7)
01/Apr	2·4 9 10^{3}	1189$^{(+)}$	C	*V. spl* (99·7)												
		1190	C	*V. spl* (99·7)												
		1191	A	*V. atl* (99·8)												
04/May													5·0 9 10^{1}	1319$^{(P)}$	–	*Ph. apl* (97·8)
11/May									1·9 9 10^{2}	1465$^{(+)}$	D	*V. gig – V. cel* (100)				
01/Jun					8·0 9 10^{1}	1668$^{(P)}$	B	*V. atl – V. tas* (99·6)	1·0 9 10^{3}	1669$^{(P)}$	B	*V. atl – V. tas* (99·6)	1·4 9 10^{3}	1670$^{(P)}$	B	*V. atl – V. tas* (99·4)
15/Jun													1·2 9 10^{3}	1813$^{(P)}$	B	*V. atl – V. tas* (99·6)
22/Jun					2·5 9 10^{3}	1883$^{(P)}$	B	*V. atl – V. tas* (99·5)	tmtc§	1884$^{(P)}$	B	*V. atl – V. tas* (99·6)	2·1 9 10^{3}	1885$^{(P)}$	B	*V. atl – V. tas* (99·5)
29/Jun													1·0 9 10^{1}	1911$^{(+)}$	B	*V. atl – V. tas* (99·6)
06/Jul													8·0 9 10^{1}	1983$^{(P)}$	–	*Ph. ind* (97·4)
03/Aug					1·2 9 10^{3}	2066$^{(+)}$	F	*V. alg* (99·3)	1·0 9 10^{3}	2067$^{(+)}$	F	*V. alg* (99·5)	1·0 9 10^{3}	2064$^{(+)}$	F	*V. alg* (99·5)
														2065	G	*V. jas* (99·4)
21/Sep													4·6 9 10^{2}	2072$^{(+)}$	E	*V. alg* (99·8)
														2071	–	*V. bras* (99·8)
20/Oct									1·9 9 10^{3}	2077$^{(+)}$	D	*V. gig – V. cel* (100)				
										2078	E	*V. alg* (99·8)				
16/Nov					9·0 9 10^{2}	2085$^{(+)}$	B	*V. atl – V. tas* (99·6)								
26/Jan	4·1 9 10^{2}	2098$^{(+)}$	–	*Ps. pel* (98·5)					1·0 9 10^{1}	2097	A	*V. atl – V. tas* (99·9)				
		2094	–	*Pr. fas* (97·9)												
08/Feb									1·0 9 10^{1}	2117	B	*V. atl – V. tas* (99·6)				
15/Feb	1·0 9 10^{1}	2118	–	*H. ham* (100)												
22/Mar									2·9 9 10^{2}	2125$^{(+)}$	A	*V. len* (99·7)	1·9 9 10^{3}	2128$^{(+)}$	A	*V. atl* (100)
										2126	A	*V. atl – V. tas* (99·8)		2129	A	*V. atl* (99·8)
										2127	E	*V. alg* (99·8)				
02/Mar					2·0 9 10^{1}	2132	A	*V. atl* (99·8)	8·0 9 10^{1}	2133$^{(+)}$	A	*V. atl* (99·8)	4·0 9 10^{1}	2134	C	*V. spl* (99·8)
														2135	A	*V. atl* (99·8)
09/Mar					1·0 9 10^{1}	2166	A	*V. len* (99·7)	3·3 9 10^{2}	2167$^{(P)}$	A	*V. atl* (99·8)	1·0 9 10^{3}	2168$^{(+)}$	A	*V. atl* (99·9)
														2169	E	*V. alg* (99·8)
22/Mar					5·0 9 10^{1}	2184	C	*V. hem* (99·9)	3·0 9 10^{1}	2185	C	*V. hem* (99·9)	3·0 9 10^{1}	2187	C	*V. hem* (99·9)
										2186	E	*V. alg* (99·8)				
29/Mar													1·0 9 10^{2}	2197	C	*V. hem* (99·9)

ISC, Intermediate scale cultures; CCS, continuous culture system.

*(+) Main type of colony or (P) pure culture.

†Members of Splendidus clade (groups A, B, C, D) and Harveyi clade (groups E, F, G) (Figs 3 and 4).

‡Identities: *H. ham*, *Halomonas hamiltonii*; *Ph. apl*, *Photobacterium aplysiae*; *Ph. ind*, *Photobacterium indicum*; *Pr. fas*, *Prolinoborus fasciculus*; *Ps. pel*, *Pseudomonas pelagia*; *Sh. pie*, *Shewanella piezotolerans*; *V. alg*, *Vibrio alginolyticus*; *V. atl*, *Vibrio atlanticus*; *V. bra*, *Vibrio brasiliensis*; *V. cel*, *Vibrio celticus*; *V. gig*, *Vibrio gigantis*; *V. hem*, *Vibrio hemicentroti*; *V. len*, *Vibrio lentus*; *V. spl*, *Vibrio splendidus*; *V. tas*, *Vibrio tasmaniensis.*

§Too much to count.

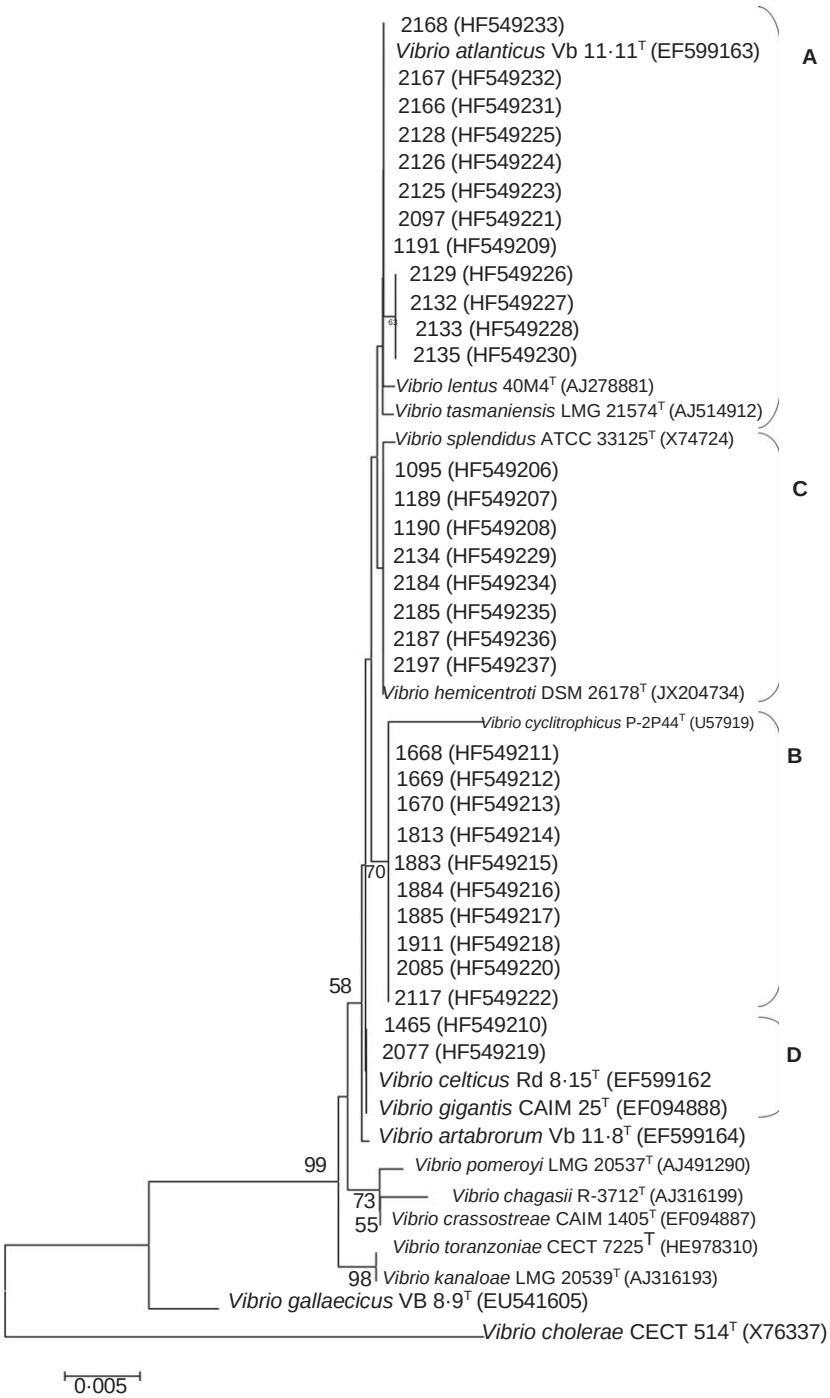

Figure 3 Phylogenetic tree based on partial 16S rRNA gene sequences of the strains included in the Splendidus clade, constructed using the NJ algorithm. *Vibrio cholerae* CECT 514^T was used as outgroup. GenBank sequence accession numbers are given in parentheses. The stability of the groupings was estimated by bootstrap percentages from 1000 replicates. Bar, 0·005 substitutions per nucleotide position.

reach the stationary phase. At this stage, growth of the MHB, including vibrios, is favoured by metabolites and organic products released by senescent algae and dead, decomposing cells (Berland *et al.* 1970; Cole 1982; Riquelme *et al.* 1987; Eiler *et al.* 2007; Bruckner *et al.* 2011). Several studies have shown that bacterial density is higher in the stationary phase than in exponential cultures and is proportional to the density of senescent microalgal cells (Nakano 1996; Grossart *et al.* 2005). These findings explain the presence of high and constant MHB levels throughout the study, regardless of the production system or the seasonal and environmental factors, and strongly indicate that the mixture of microalgae should not be stored for long periods.

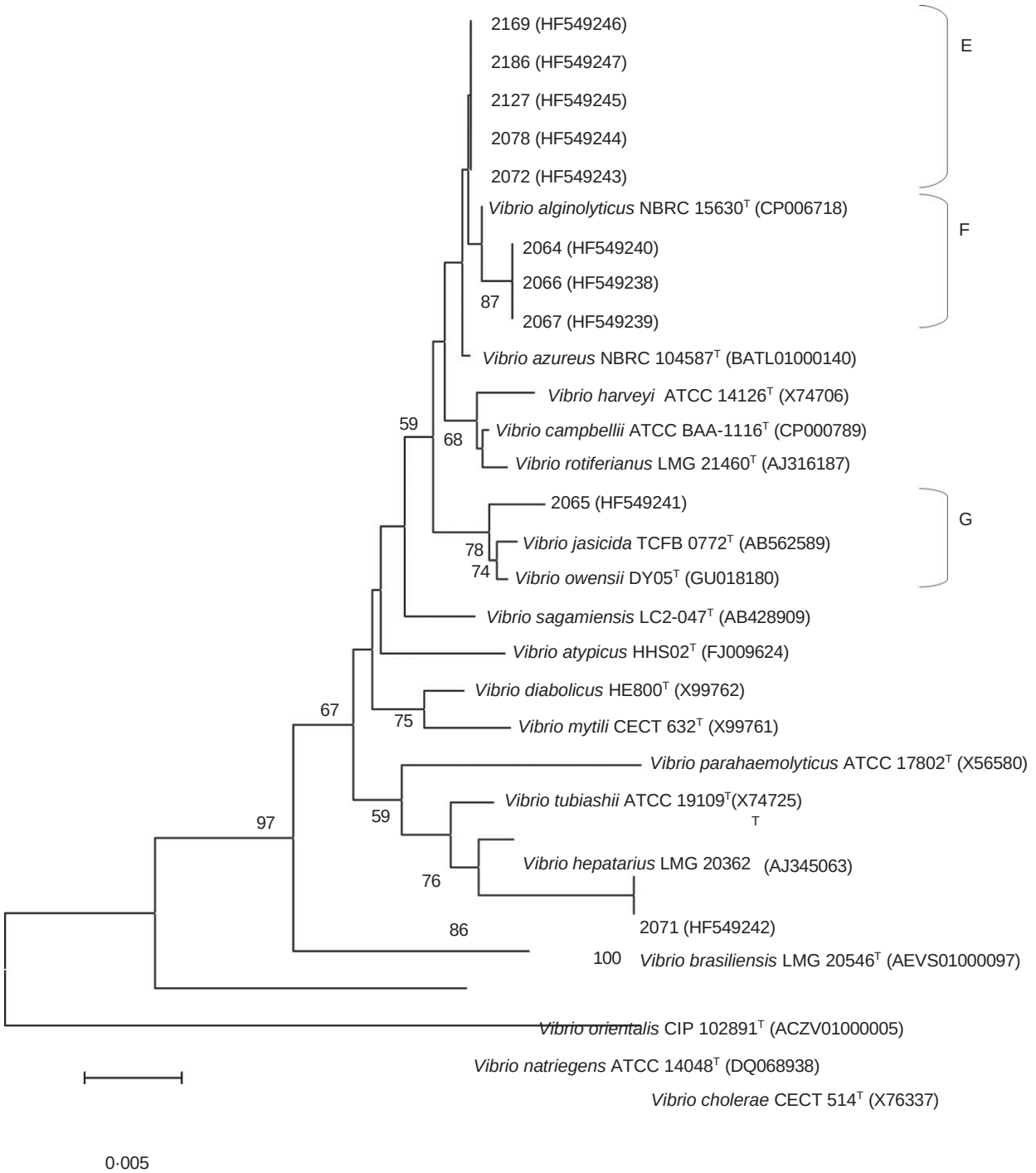

Figure 4 Phylogenetic tree based on partial 16S rRNA gene sequences of the strains included in the Harveyi clade and the isolate 2071, constructed using the NJ algorithm. Characteristics of the phylogenetic tree identical at Fig. 3.

Vibrios, if present, were never the main component of the population of culturable bacteria, and they were always in much lower numbers than the total MHB. The pasteurized water used in CCS should be taken into account as a potential source of vibrios despite these pop- ulations being under the limit of detection in TCBS plates. Viable but nonculturable state due to environmental

stress, as extreme temperatures used in the pasteurization, was reported in some *Vibrio* spp. which can recover from this state in presence of the adequate nutrients and envi- ronmental conditions that support their growth and cell division (Trevors 2011). The abundance and diversity of PV appeared to be closely related to the development of the system, and both were higher during establishment

than stabilization of the culture. The PV levels were also strongly influenced by high temperatures, as reported by Turner *et al.* (2009). In addition, vibrios were also more abundant during the periods of maximum activity in the hatchery when requirements for phytoplankton were highest. These factors and the conditions of the system itself (state of the algal cultures, high algal densities, storage time . . .) favoured the accumulation of vibrios in CCS at the peak stage of the larval production season. The PV load was also promoted by the recirculation of the phytoplankton through the pipes due to the vibrios are an important component of marine biofilms which may contribute to the attachment of bacteria to the surface and to marine organisms such as plankton (Wai *et al.* 1999). The presence of vibrios in large scale cultures as the CCS seems inevitable and the control of these populations must be established. Nonetheless, the study findings sup- ported the installation of the new continuous culture sys- tem as its higher efficiency was not related to a large increase in the ratio of MHB and PV per microalgal cell.

In the ISC samples, vibrios were found in high num- bers only during the initial monitoring period, when a vibrios contamination was observed in the stock cul- tures. The rapid detection of vibrios avoided their trans- mission to the bivalve cultures. Furthermore, the problem was resolved by renewal of the stock cultures. CCS was not affected by the vibrios contamination because it was previously started from different ISC batches and these CCS monocultures were kept during 2 months. The present findings support the need for the appropriate management of microalgal cultures, includ- ing monitoring of the bacterial diversity in the cultures, to prevent the proliferation of potential pathogens, as previously proposed by other authors (Nicolas *et al.* 2004; Elston *et al.* 2008).

The low levels of PV recorded in the microalgae cultures, consistent with the results obtained by other authors (Lewis *et al.* 1988; Salvensen *et al.* 2000; Kokou *et al.* 2012), may be related to the antibacterial activity of some microalgae such as *Chlorella* sp., *Isochrysis* sp., *T. suecica*, *Nannochloropsis* sp., *P. tricornutum* and *S. costatum*, which are able to inhibit the growth of pathogenic vibrios: *V. anguillarum*, *V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus* and *V. splendidus* (Austin *et al.* 1992; Naviner *et al.* 1999; Tendencia and de la Pena 2003; Desbois *et al.* 2008; Kokou *et al.* 2012). The biologically active compounds responsible for the inhibitory activity are more abundant at the stationary phase or at the end of the exponential growth (Borowitzka 1995). Coopera- tive strategies between microalgae and bacteria have been suggested to enhance the antibacterial effect, as reported for the action of *Nannochloropsis oculata* and bacteria of the Roseobacter clade against the pathogen *V. anguillarum* (Sharifah and Eguchi 2011). Another reason may be a low-competitive ability of vibrios at high bacterial levels. The genus *Vibrio* includes opportunistic bacteria with high growth rates, capable of proliferating during periods of increased nutrient availability, but that do not compete efficiently in crowded environments, such as stationary batch cultures, in which nutrient avail- ability is lower (Colwell 1984). However, the presence of vibrios in the microalgal cultures must be borne in mind, despite the low concentrations, because the phytoplank- ton is supplied directly to the bivalve cultures, in which conditions, the proliferation of vibrios is favoured (Prado *et al.* 2005).

The 16S rRNA gene has a rather low interspecies resolution and is not useful for species differentiation but may provide a reliable identification at clade level, and particularly in the Splendidus and Harveryi clade (Pascual *et al.* 2010; Lasa *et al.* 2013; Sawabe *et al.* 2013). In the CCS, the predominant species of the genus *Vibrio* belonged to the Splendidus clade, during the larval pro- duction season, and to the Harveyi clade, in the warmest months. The results support the close relationship between the Splendidus clade and the bivalve hatchery environment, as previously suggested Prado *et al.* (2014a) for cultures of razor clam (*Solen marginatus*). Detection of strains closely related to the larval pathogen *V. splendidus* may indicate a disease risk for larval and juvenile bivalves (Nicolas *et al.* 1996; Garnier *et al.* 2007; Kesarcodi-Watson *et al.* 2009). Further studies should determine the possible pathogenicity of these isolates and the strains related to the Harveyi clade, which also includes aquaculture pathogens such as *V. harveyi,* *V. alginolyticus* and *V. owensii* (Austin 2010; Cano- Go´mez *et al.* 2010). Only two isolates showed discrepan- cies in the results obtained from phenotypic tests and sequencing. They were identified as *Photobacterium* spp. despite of the phenotypic profile was typically of the vib- rios. The taxonomic position of the genus *Photobacterium* within the family *Vibrionaceae* is close to the genus *Vib- rio*. In fact, Sawabe *et al.* (2013) recently updated the *Vibrio* clades by multilocus sequence phylogeny and included the Damselae, Phosphoreum, Profundum and Roserbergii clades, belonging to the genus *Photobacteri- um*. In addition, the number of potential new species, with 16S rDNA similarities lower than the threshold value (98·70%) proposed by Stackebrandt and Ebers (2006), revealed the scarcity of specific studies and knowledge about the microbiota in these cultures.

From a practical point of view, the usefulness of TCBS as a routine tool in shellfish hatcheries has been demonstrated, providing a rapid overview of the culturable vibrios present in microalgal cultures despite of some bivalve pathogens as *V. pectenicida* or *V. aestuarianus*

subsp. *francensis* do not grow on TCBS (Lambert *et al.* 1998; Garnier *et al.* 2008). Indeed, most of the strains isolated from TCBS plates were identified as *Vibrio* spp., and the other taxonomic groups were detected only occa- sionally during the study.

Results obtained during 1-year of samplings confirmed the importance of the microalgae as a direct source of bacteria in bivalve hatcheries. Spatio-temporal analysis of the data set revealed that the input of MHB via the microalgae was high and constant throughout the study, regardless of the production systems and the seasonal and/or environmental factors. In general, vibrios were detected at low concentrations, and high levels were good indicators of future problems. The diversity and abundance of vibrios depended on factors related to the hatchery: the larval season, the environmental temperature, the culture conditions and the production system. In summary, the protocol for the vibrio control in the phytoplankton production systems used during the study must be implemented and adapted to the specific conditions of each hatchery to guarantee the microbiological quality of the microalgae and to avoid the risk of transferring potential pathogens to the larvae or broodstock.

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Conflict of Interest

No conflict of interest declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Differential phenotypic features of the strains included in the family *Vibrionaceae*.

Table S2 Identification and differential phenotypic features of the strains included in other taxonomic groups.