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Scallop larvae hatcheries as source of bacteria carrying genes encoding for non-enzymatic phenicol resistance

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ABSTRACT

The main aim of the study was to evaluate the role of scallop hatcheries as source of the *floR* and *cmlA* genes. A number of 133 and 121 florfenicol-resistant strains were isolated from scallop larval cultures prior to their transfer to seawater and from effluent samples from 2 commercial hatcheries and identified by 16S rRNA gene sequence analysis, observing a predominance of the *Pseudomonas*, *Pseudoalteromonas* and *Halomonas* genera and exhibiting an important incidence of co-resistance to streptomycin, oxytetracycline and co-trimoxazole. A high percentage of strains from both hatcheries carried the *floR* gene (68.4% and 89.3% of strains), whereas a lower carriage of the *cmlA* gene was detected (27.1% and 54.5% of strains). The high prevalence of *floR*-carrying bacteria in reared scallop larvae and hatchery effluents contributes to enrich the marine resistome in marine environments, prompting the need of a continuous surveillance of these genes in the mariculture environments.

1. Introduction

The culture of Chilean scallop *Argopecten purpuratus* (Lamarck, 1819) is one of the most commercially important industries of Chilean mariculture (von Brand et al., 2006). Although efficient culture techniques for scallop larvae production under hatchery-controlled conditions have been developed, Chilean hatcheries occasionally suffer episodes of massive mortalities of reared-larvae, mainly caused by bacterial pathogens (Riquelme et al., 1995, 1996; Rojas et al., 2009, 2015) leading to the intensive use of antibacterial agents during the larvae rearing period. Chloramphenicol was extensively used in most of the Chilean scallop hatcheries before it was banned due to its carcinogenic effects, whereas florfenicol is currently used in all Chilean hatcheries because its high efficacy to reduce larval mortality in the intensive culture of *A. purpuratus* (Miranda et al., 2014).

Phenicols exert its antimicrobial activity by inhibiting bacterial protein synthesis at the ribosomal level, binding reversibly with the 50S ribosome preventing the transfer of amino acids from tRNA to the growing peptide chains during protein synthesis (Schwarz et al., 2004), but the increased selective pressure as imposed by the intensive use of florfenicol in aquaculture has accelerated the development and spread of bacterial resistance to this drug. Most important mechanisms accounting for bacterial resistance to florfenicol, include efflux systems, permeability barriers and multidrug transporters (Schwarz et al., 2004).

Resistance to florfenicol in Gram-negative bacteria is mediated primarily by the *floR* gene, encoding for a specific drug exporter that confers resistance to florfenicol and chloramphenicol (Braibant et al., 2005; Schwarz et al., 2004), whereas the chloramphenicol resistance gene *cmlA* confers nonenzymatic chloramphenicol resistance via an efflux mechanism, but not confers resistance to florfenicol (Dorman and Foster, 1982; Keyes et al., 2000) despite that *floR* gene product is closely related to the CmlA protein identified in *Pseudomonas aeruginosa* (White et al., 2000).

Miranda et al. (2014) found that florfenicol was significantly more efficient than oxytetracycline to reduce larval mortality of scallop cultures at commercial scale, so it must be concluded that in the majority of Chilean scallop hatcheries the administration of florfenicol during the larvae rearing process will be continued. Recently, florfenicol resistance has been detected in a wide variety of bacterial species recovered from a commercial hatchery in Chile (Miranda et al., 2013), but despite wide use of florfenicol in Chilean scallop farming no studies have been developed to evaluate the incidence of genes encoding for resistance to phenicols among

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the resistant microbiota occurring in scallop larvae hatcheries to evaluate the feasibility of their potential spread on the marine surrounding environments.

Transfer of antibiotic resistant bacteria and resistance encoding genes to marine environment via the aquaculture industry is a significant health concern, so the present study was undertaken to obtain information on the occurrence and identification of florfenicol-resistant bacteria and the presence of the *floR* and *cmlA* genes, encoding for non-enzymatic phenicol resistance among the florfenicol-resistant strains isolated from effluents of 2 commercial scallop hatcheries as well as from untreated and florfenicol-treated larval cultures under farming conditions prior to their transfer to the marine environment.

2. Materials and methods

2.1. Sampling and processing

Two commercial hatcheries of the Chilean scallop, *A. purpuratus*, located in Tongoy bay (30°15′88″S, 71°29′42″W, hatchery 1) and Caldera bay (27°05′39″S, 70°51″52″W, hatchery 2) in the north of Chile were considered in the study. Samples of scallop larvae cultured in 5000 L (hatchery 1) and 40,000 L (hatchery 2) rearing tanks with no treatment (control) and treated with 4 mg L^{-1} of florfenicol (Centrovet™, Santiago, Chile), as well as water samples of the hatchery effluents were considered. According to the protocols of both hatcheries, water of rearing tanks is completely renewed by water exchange each 48 h, and florfenicol is administered directly to the water of rearing tanks after the water exchange process. Water used in larval rearing tanks of hatchery 1 was previously treated (25, 10 and 5 μm filtration and UV irradiation) as was previously described (Miranda et al., 2014), whereas water of larval rearing tanks of hatchery 2 was only filtered (60 μm) before use. Samples were collected twice using sterile water sampling bottles (APHA, 1992) during the week before scallop larvae were fixed on Netlon bags to be transferred to lanterns placed into coastal marine waters and processed as was previously described (Miranda et al., 2013). Homogenates and appropriate 10-fold dilutions of the scallop larvae homogenates or effluent water samples were prepared using sterile physiological saline (0.85%) (PS) and were inoculated (0.1 mL aliquots) in triplicate onto Plate count agar added with 2% NaCl (PCA2, Difco). Plates of PCA2 with and without florfenicol (30 μg mL^{-1}, Schering-Plough®, Kenilworth, NJ, USA) were used to determine the florfenicol-resistant and total culturable bacteria, respectively. All plates were incubated at 20 °C for 5 days and the bacterial numbers per mL or per g of sample were calculated as described in Miranda and Rojas (2007). Different colony morphotypes grown onto PCA2 added with florfenicol were recovered and purified in Tryptic soy agar supplemented with NaCl (2%).

2.2. Bacterial strains

A number of 133 and 121 florfenicol-resistant Gram-negative bacilli were recovered from hatcheries 1 and 2, respectively. Resistant strains were isolated from untreated (59 and 50 strains) and florfenicol-treated (56 and 39 strains) cultures of scallop larvae as well as from hatchery effluents (18 and 32 strains) to represent the larval florfenicol-resistant bacterial community from hatcheries 1 and 2, respectively. Purified strains were stored at −85 °C in CryoBank (Mast Diagnostic) vials until use.

2.3. Bacterial identification by 16S rRNA analysis

Bacterial strains were resuspended in TE buffer (Tris 0.01 M, EDTA 0.001 M, NaCl 0.15 M, pH 7.8), and cell lysis was performed using sodium dodecyl sulfate and incubation at 70 °C. The DNA were extracted with phenol/chloroform and subsequently precipitated with ethanol as previously described (Romero et al., 2002). A final purification was carried out using Wizard DNA Clean Up (Promega, Madison, WI, USA). PCR reactions were performed as described in Romero and Navarrete (2006) with a reaction mixture (30 μL) containing 0.2 mM of each deoxynucleoside triphosphate, 0.05 U μL^{-1} Platinum Taq DNA polymerase (Invitrogen, San Diego, CA, USA), 1 × polymerase reaction buffer, 2 mM $MgCl_2$, and 0.25 pmol μL^{-1} of each primer. To identify the bacterial strains, amplification of 16S rRNA from positions 28 to 1492 was performed using primers 27F and 1492R as previously described (Navarrete et al., 2010). PCR products were analyzed by polyacrylamide electrophoresis and silver nitrate staining according to Romero et al. (2002). PCR amplified 16S rDNA from the bacterial isolates were purified using Wizard PCR Preps (Promega) and then sequenced by the Macrogen USA sequencing service. The 16S rRNA gene sequences were compared with those available in the public Ribosomal Database Project II (Cole et al., 2007) (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) in order to ascertain their closest relatives.

2.4. Detection of phenicol resistance genes

2.4.1. Detection of the floR gene

The *floR* gene was detected by PCR. Genomic template was prepared by mixing 200 μL of overnight bacterial culture with 800 μL of sterile distilled water (SDW), boiling for 15 min, and then centrifuging at 16,000 g for 5 min. The supernatant was used directly as the source of template. Each 29 μL reaction mixture contained 3.0 μL 10 × PCR buffer (Invitrogen, Carlsbad, CA, USA); 1.2 μL each dNTP (1.25 mM); 1.2 μL $MgCl_2$ (50 mM, Invitrogen); 0.3 μL each primer (25 pmol μL^{-1}); 0.3 μL Taq DNA polymerase (5 U μL) and SDW to 30 μL of reaction volume. Amplification of the *floR* gene was performed in an ESCO Swift Max Pro Thermal cycler using the following cycle conditions: 96 °C for 30 s, followed by 30 cycles of 96 °C for 15 s, 52 °C for 30 s, and 70 °C for 1 min. There was a final extension at 70 °C for 5 min. PCR products were detected by electrophoresis for PAGE, and visualized by Nitrate of silver (Espejo and Escanilla, 1993). The expected size of PCR products was 215 bp. The *floR*-positive *Hafnia alvei* FE25 (Fernández-Alarcón et al., 2010) and the *floR*-negative *Hafnia* sp. FE24 strains served as positive and negative controls, respectively, and were included in each PCR run. The identity of the PCR amplicon was checked by sequencing (Macrogen USA sequencing service) and comparison with those available in GenBank using the BLASTN software (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The sequences revealed 100% matching with florfenicol export protein (*floR*) gene (EF429662) from *Escherichia coli* strain C54.

2.4.2. Detection of the cmlA gene

To detect the *cmlA* gene, the primers and protocols reported by Keyes et al. (2000) were used. Briefly, each 29 μL reaction mixture contained 1.2 μL each dNTP (5 mM); 1.2 μL $MgCl_2$ 50 mM, 0.3 μL each primer (25 pmol μL^{-1}); 0.3 μL Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and SDW to 30 μL of reaction volume. Amplification of the *cmlA* gene was performed in a PCR Esco Swift MaxPro Thermal Cycler using the following cycle conditions: 96 °C for 30 s, followed by 30 cycles of 96 °C for 15 seg, 50 °C for 30 seg and 70 °C for 1 min. There was a final extension at 70 °C for 5 min. PCR products were detected by electrophoresis for PAGE, and visualized by Nitrate of silver (Espejo and Escanilla, 1993). The expected size of PCR product was 700 bp. The *cmlA*-positive *Halomonas* sp. IF59 and the *cmlA*-negative *Pseudomonas marincola* F7 served as positive and negative controls, respectively, and were included in each PCR run. The identity of the PCR amplicon was

checked by sequencing (Macrogen USA sequencing service) and comparison with those available in GenBank using the BLASTN software (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The sequences revealed 76% matching with chloramphenicol resistance protein (*cmlA*) gene (HM175873.1) from *E. coli* strain NF903502.

2.5. Antimicrobial resistance patterns

Resistant isolates were tested for their susceptibility to 13 antimicrobials by an agar disk diffusion method as described in the Clinical and Laboratory Standards Institute (CLSI) guideline M42-A (CLSI, 2006), using Müeller–Hinton agar (Difco) supplemented with NaCl (2%). The antibacterial susceptibility patterns of resistant strains were performed using disks containing the antibacterial agents: amoxicillin (AML, 25 μg), cefotaxime (CTX, 30 μg), chloramphenicol (CM, 30 μg), florfenicol (FFC, 30 μg), streptomycin (S, 10 μg), gentamicin (G, 10 μg), kanamycin (K, 30 μg), oxytetracycline (OT, 30 μg), oxolinic acid (OA, 2 μg), flumequine (UB, 30 μg), enrofloxacin (ENR, 5 μg), furazolidone (FX, 100 μg), and sulfametoxazole-trimethoprim (SXT, 1.25 and 23.75 μg). All disks were obtained from Oxoid Ltd (Basingstoke, Hampshire, England). Bacterial strains were suspended in sterile 0.85% saline to a turbidity to match a McFarland No.2 standard (bioMerieux S.A.), diluted 1:20, and streaked on the used media. Plates were incubated for 24–48 h at 22 °C and *E. coli* ATCC 25922 was used as the control strain. Characterization of strains as resistant was stated according to standards suggested by CLSI (CLSI, 2007) or by Miranda and Rojas (2007). A number of isolates (30%) were re-examined to check reproducibility of the assay. In addition, the antibacterial resistance index (ARI) of larval and effluent samples were determined according to Hinton et al. (1985), using the formula ARI = y/nx, in which y was the actual number of resistance determinants recorded in a population of size n, and x was the total number of antibacterials tested for in the sensitivity test.

2.6. Minimum inhibitory concentrations (MIC)

Minimum inhibitory concentrations (MICs) of florfenicol (Schering-Plough) against all strains were determined by an agar plate dilution method, as recommended by the National Committee for Clinical Laboratory Standards (1997). A serial twofold dilution pattern of the antibiotic was added into Mueller–Hinton agar (Difco) added with NaCl (2%) as to obtain final concentrations ranging from 0.5 to 2048 μg mL^{-1}. Bacterial suspensions were prepared in sterile 0.85% saline and triplicate plates were inoculated by using a Steers replicator apparatus (Steers et al., 1959), delivering approximately 10^4 colony-forming units per spot, and incubated for 48 h at 22 °C. The first and the last agar plates did not contain any antibiotic in order to detect possible contamination of the isolates or antibiotic carryover. MIC was defined as the lowest concentration of florfenicol producing absence of growth at least in two of the three plates after 48 h. Reference strain *E. coli* ATCC 25922, recommended by NCCLS (2002), was used as quality control organism for verification of MIC ranges on Mueller–Hinton agar plates. MIC assays were performed twice to check reproducibility of the results.

2.7. Statistical analysis

The frequencies of resistance to the assayed antimicrobials of the selected strains from each hatchery were compared with Pearson's Chi-square test, adjusted with Bonferroni's correction and a $P < 0.05$ was considered to indicate statistical significance (Zar, 1999). Resistance frequencies between hatcheries as well as between untreated and florfenicol-treated reared larvae in each hatchery were compared. All statistical analyses were carried out using the SPSS version 12.0 computer program (Norusis, 2004).

3. Results

3.1. Total and resistant culturable bacteria

Total culturable counts from untreated and florfenicol-treated scallop larvae from both hatcheries were very similar exhibiting values of 10^6 CFU g^{-1} (Table 1), whereas resistant culturable counts commonly showed a decrease of a magnitude of 2 log (10^4 CFU g^{-1}). Comparative proportions of florfenicol resistant bacteria from hatchery 2 were slightly higher than those from hatchery 1 but the opposite was observed for untreated larval samples (Table 1). Florfenicol resistance of untreated larvae were slightly higher than those from florfenicol-treated larvae in hatchery 1 (1.3–2.0% and 0.7–0.9%, respectively), whereas the opposite was observed in the hatchery 2, because untreated larvae exhibited lower proportions of florfenicol resistance than those observed in the florfenicol-treated larvae (0.8–1.9% and 5.3–5.6%, respectively). Additionally, very similar values of total and resistant culturable counts from effluent samples from both hatcheries were observed (10^4 CFU mL^{-1}), exhibiting low levels of florfenicol resistance ranging from 0.1% to 0.4% and 0.4% to 1.4% of culturable bacteria, for hatcheries 1 and 2, respectively (Table 1).

3.2. Bacterial identification

As observed in Tables 2 and 3, a similar number of genera (13 and 11 for hatcheries 1 and 2, respectively) were identified among the florfenicol-resistant bacteria recovered from both hatcheries, but resistant microbiota from hatchery 1 exhibited a higher number of bacterial species than those from hatchery 2. Resistant microbiota from hatchery 1 was mainly comprised by the *Pseudomonas* (40.6%), *Pseudoalteromonas* (22.6%) and *Halomonas* (12.8%) genus (Table 2), whereas the resistant strains recovered from hatchery 2 mainly belonged to the *Pseudoalteromonas* (61.2%), *Idiomarina* (16.5%) and *Pseudomonas* (8.3%) genus (Table 3).

3.3. Phenicol resistance genes

The amplification products for the *floR* and *cmlA* genes carried by selected resistant strains are shown in Fig. 1. Most of the resistant strains recovered from reared scallop larvae from hatchery 1 (91 out of 133 strains) carried the *floR* gene. Among these, 31 strains carried both phenicol resistance genes, whereas an important number of strains (37 strains) were negative for both assayed resistance genes, mainly isolated from untreated reared larvae (18 strains) and hatchery effluent (12 strains) samples. Otherwise, only 5 strains recovered from untreated larvae and hatchery effluent were positive for the *cmlA* gene and negative for the *floR* gene (Table 2). In concordance with hatchery 1, a high incidence of the *floR* gene was detected among the resistant strains recovered from hatchery 2, considering that 108 out of 121 strains carried the *floR* gene and among them 57 strains carried both resistance genes (Table 3). Only 9 strains were positive for the *cmlA* gene and negative for the *floR* gene, whereas only 4 strains recovered from untreated larvae were negative for any of the assayed genes (Table 3). One of the most noticeable differences between both hatcheries is the number of resistant strains negative for both genes, because 37 strains (27.8%) from hatchery 1, whereas only 4 strains (3.3%) from hatchery 2 were negative for both studied genes. Another remarkable difference between both scallop farms

Table 1
Total and florfenicol-resistant culturable counts (mean ± SD of 3 replicates) from reared larvae and effluent from 2 commercial scallop hatcheries.

Farm	Untreated larvae Culturable count ± SD		Treated larvae Culturable count ± SD		Effluent Culturable count ± SD	
	Total (CFU g^{-1})	Florfenicol resistant (CFU g^{-1})	Total (CFU g^{-1})	Florfenicol resistant (CFU g^{-1})	Total (CFU mL^{-1})	Florfenicol resistant (CFU mL^{-1})
1	$4.43 \times 10^6 \pm 3.36 \times 10^6$ $2.75 \times 10^6 \pm 1.55 \times 10^6$	$5.89 \times 10^4 \pm 4.90 \times 10^4$ $5.57 \times 10^4 \pm 6.29 \times 10^4$	$7.86 \times 10^6 \pm 4.90 \times 10^6$ $6.35 \times 10^6 \pm 3.28 \times 10^6$	$7.41 \times 10^4 \pm 4.45 \times 10^4$ $4.52 \times 10^4 \pm 2.51 \times 10^4$	$3.69 \times 10^4 \pm 1.31 \times 10^4$ $2.52 \times 10^4 \pm 6.12 \times 10^3$	$1.53 \times 10^2 \pm 5.77 \times 10^1$ $2.00 \times 10^1 \pm 1.73 \times 10^1$
2	$9.98 \times 10^6 \pm 3.20 \times 10^6$ $5.56 \times 10^6 \pm 2.02 \times 10^6$	$1.86 \times 10^5 \pm 4.00 \times 10^4$ $4.65 \times 10^4 \pm 1.02 \times 10^4$	$4.79 \times 10^6 \pm 1.89 \times 10^6$ $2.14 \times 10^6 \pm 6.91 \times 10^5$	$2.70 \times 10^5 \pm 7.94 \times 10^4$ $1.14 \times 10^5 \pm 4.23 \times 10^4$	$7.30 \times 10^4 \pm 3.99 \times 10^4$ $1.22 \times 10^4 \pm 1.51 \times 10^3$	$3.10 \times 10^2 \pm 5.20 \times 10^1$ $1.67 \times 10^2 \pm 5.77 \times 10^1$

Table 2
Distribution of resistance genes among florfenicol-resistant bacterial strains recovered from Hatchery 1.

Species	Number of strains												Total
	Untreated larvae				Florfenicol-treated larvae				Effluent				
	floR	*cmlA*	Both	None	*floR*	*cmlA*	Both	None	*floR*	*cmlA*	Both	None	
Brachybacterium arcticum	1												1
Brevibacterium sp.					1								1
Erythrobacter sp.												1	1
Escherichia coli			1										1
Halomonas sp.	2		1	1	1		3		1	3			12
Halomonas variabilis					1		2						3
Halomonas venusta					2								2
Idiomarina sp.					6		1						7
Marinobacter litoralis				1									1
Marinobacter sp.			2										2
Marinobacter squaleniorans			1										1
Marinomonas sp.					4								4
Providencia heimbachae			2										2
Providencia rettgeri			2										2
Providencia sp.	1		1										2
Providencia vermicola												1	1
Pseudoalteromonas atlantica	1		1					2					4
Pseudoalteromonas elyakovii					1								1
Pseudoalteromonas gracilis					1								1
Pseudoalteromonas haloplanktis				1									1
Pseudoalteromonas prydzensis							1						1
Pseudoalteromonas mariniglutinosa	1				2								3
Pseudoalteromonas sp.	2		1	1	5		4	1	1		1	2	18
Pseudoalteromonas tetraodonis					1								1
Pseudomonas alcaliphila	1												1
Pseudomonas guineae				1									1
Pseudomonas knackmussii	1												1
Pseudomonas marincola	1			2				3				1	7
Pseudomonas monteilii					1		1						2
Pseudomonas plecoglossicida	1			1			2						4
Pseudomonas putida		1		3								2	6
Pseudomonas segetis	1	1	2	4			1	1				2	12
Pseudomonas stutzeri	3				1							3	7
Pseudomonas sp.	6			3	3		1						13
Psychrobacter glacincola					1								1
Psychrobacter pulmonis					1								1
Psychrobacter sp.					1								1
Vibrio inusitatus	1												1
Vibrio sp.	2												2
Total	25	2	14	18	33		16	7	2	3	1	12	133

in the prevalence of the *floR* gene among resistant strains from effluent samples, because only 3 out of 18 strains from effluent of hatchery 1 were positive for this gene (Table 2), whereas 30 out of 32 strains from effluent of hatchery 2 carried the *floR* gene (Table 3).

3.4. Antimicrobial resistance patterns

Among florfenicol-resistant strains recovered from hatchery 1, an important incidence of resistance to the antibacterials streptomycin (51.9%), oxytetracycline (54.1%), furazolidone (38.3%) and co-trimoxazole (56.4%) was observed (Fig. 2A). A similar tendency was observed for strains recovered from hatchery 2 (Fig. 2B), exhibiting a high incidence of resistance to streptomycin (47.0%), oxytetracycline (37.2%), furazolidone (27.3%) and co-trimoxazole (37.2%). Significant differences (Chi-squared test, $P < 0.05$) between untreated and treated scallop larvae from both hatcheries were detected. Among resistant bacteria from hatchery 1, strains recovered from treated larvae exhibited significantly ($P < 0.05$) higher resistance to streptomycin and kanamycin and significantly ($P < 0.05$) lower resistance to furazolidone than those from untreated larvae (Fig. 2A). Among resistant bacteria from hatchery

Table 3
Distribution of resistance genes among florfenicol-resistant bacterial strains recovered from Hatchery 2.

Species	Number of strains												Total
	Untreated larvae				Florfenicol-treated larvae				Effluent				
	floR	*cmlA*	Both	None	*floR*	*cmlA*	Both	None	*floR*	*cmlA*	Both	None	
Cobetia sp.									1				1
Halomonas boliviensis							1				1		2
Halomonas sp.	1		1						2				4
Idiomarina loihiensis	1								2		1		4
Idiomarina sp.	1	3	5						5	1	1		16
Marinobacter sp.	1												1
Mesonia algae									1				1
Microbacterium oxydans	1												1
Pseudoalteromonas atlantica	2					1							3
Pseudoalteromonas haloplanktis					1								1
Pseudoalteromonas issachenkonii							1						1
Pseudoalteromonas sp.	7		13		11		19		9		5		64
Pseudoalteromonas tetraodonis			3						1		1		5
Pseudomonas fulva										1			1
Pseudomonas marincola				1									1
Pseudomonas segetis			1										1
Pseudomonas sp.	1	3		3									7
Ruegeria scottomollicae							1						1
Shewanella algae	1		1										2
Vibrio sp.					2		2						4
Total	16	6	24	4	14	1	24		21	2	9		121

2, strains recovered from untreated larvae exhibited significantly ($P < 0.01$) higher resistance to oxolinic acid, flumequine and co-trimoxazole and significantly ($P < 0.001$) lower resistance to kanamycin than those from florfenicol-treated larvae (Fig. 2B).

When antibacterial resistance from untreated larval samples from hatcheries 1 and 2 were compared, significant differences (Chi-squared test, $P < 0.05$) for resistance to amoxicillin (18.6% and 8.0%, respectively), kanamycin (0% and 24.0%, respectively), oxolinic acid (6.8% and 20.0%, respectively), flumequine (0% and 14.0%, respectively), and furazolidone (49.2% and 28.0%, respectively) were observed (Fig. 2). Otherwise, significant differences ($P < 0.05$) between florfenicol-treated larvae from hatcheries 1 and 2 were observed only for resistance to amoxicillin (23.2% and 7.7%, respectively), kanamycin (7.1% and 56.4%, respectively), flumequine (0% and 5.1%, respectively), and co-trimoxazole (60.7% and 23.1%, respectively). Significant differences ($P < 0.05$, chi-square test) between effluent samples from hatcheries 1 and 2 were detected for resistance to cefotaxime (5.6% and 0%, respectively), kanamycin (5.6% and 28.1%, respectively), flumequine (11.1% and 28.1%, respectively), furazolidone (55.6% and 18.8%, respectively) and co-trimoxazole (72.2% and 34.4%, respectively), were detected (Fig. 2).

No significant differences in the occurrence of simultaneous resistance to various antimicrobials between strains recovered from both hatcheries were observed (Fig. 3). Florfenicol-resistant strains from both hatcheries exhibited a low incidence of multiresistance, observing that 89.8% of strains from untreated culture and 82.1% strains from florfenicol-treated showed simultaneous resistance to 3–5 antimicrobials, whereas the majority of strains from hatchery 2 were resistant to 3–6 (92.2% of strains from untreated larvae) and 3–5 antibacterials (92.3% of strains from treated larvae) (Fig. 3). Highest levels of multiresistance were detected in 3 strains from different sources, where simultaneous resistance to 9 antimicrobials were exhibited by a *Erythrobacter* sp. strain recovered from the effluent of hatchery 1, as well as a *Mesonia algae* strain and a *Pseudoalteromonas* sp. strain isolated from untreated and florfenicol-treated larvae from hatchery 2, respectively. Similar antimicrobial indexes (ARI) were observed in the florfenicol resistant microbiota isolated from both hatcheries, which strains recovered from untreated and florfenicol-treated larvae, exhibited ARI values of 0.32 and 0.34 for hatchery 1 and values of 0.34 and 0.32 for hatchery 2, whereas resistant strains from effluents of hatcheries 1 and 2 showed ARI values of 0.36 and 0.34, respectively.

3.5. Minimum inhibitory concentrations (MIC)

Resistant strains exhibited high MIC values of florfenicol, not observing remarkable differences among strains from different hatcheries and sources (Table 4). The florfenicol MIC values of resistant strains from hatchery 1 varied between 16 and 1024 μg mL^{-1}, while MIC values of strains from hatchery 2 ranged from 16 to 2048 μg mL^{-1} (Table 4). Lowest MIC_{50} values were exhibited by strains recovered from effluent of hatchery 1 (64 μg mL^{-1}), whereas highest MIC_{50} values (256 μg mL^{-1}) were observed for treated larvae from both hatcheries and untreated larvae from hatchery 1 (Table 4). Otherwise, all groups of strains from both hatcheries showed MIC_{90} values of 512 μg mL^{-1}. Highest MIC values (1024 μg mL^{-1}) were exhibited by 2 *Pseudomonas plecoglossicida* strains isolated from florfenicol-treated larvae from hatchery 1, and 3 *Pseudomonas* sp. strains isolated from untreated larvae from hatchery 2, whereas only 1 *Pseudomonas* sp. strain from untreated larvae from hatchery 2 exhibited a MIC value of 2048 μg mL^{-1}. Reference strain *E. coli* ATCC 25922, used for quality control exhibited a MIC value of florfenicol of 2 μg mL^{-1}, and agrees with the values recommended by NCCLS (2002). Resistant strains not-carrying the *floR* gene (42 and 13 strains from hatcheries 1 and 2, respectively) exhibited MIC_{50} values of 128 μg mL^{-1} for strains from both hatcheries, and MIC_{90} of 512 and 1024 μg mL^{-1}, for strains from hatcheries 1 and 2, respectively.

4. Discussion

Despite the high risk of selecting bacterial resistant strains, phenicols are the most commonly used antibacterials in pectinid hatcheries (Campa-Córdova et al., 2005; Miranda et al., 2014; Uriarte et al., 2001). It has been previously demonstrated that prophylactic use of phenicols such as chloramphenicol and florfenicol increase larval survival of reared pectinid larvae (Campa-Córdova

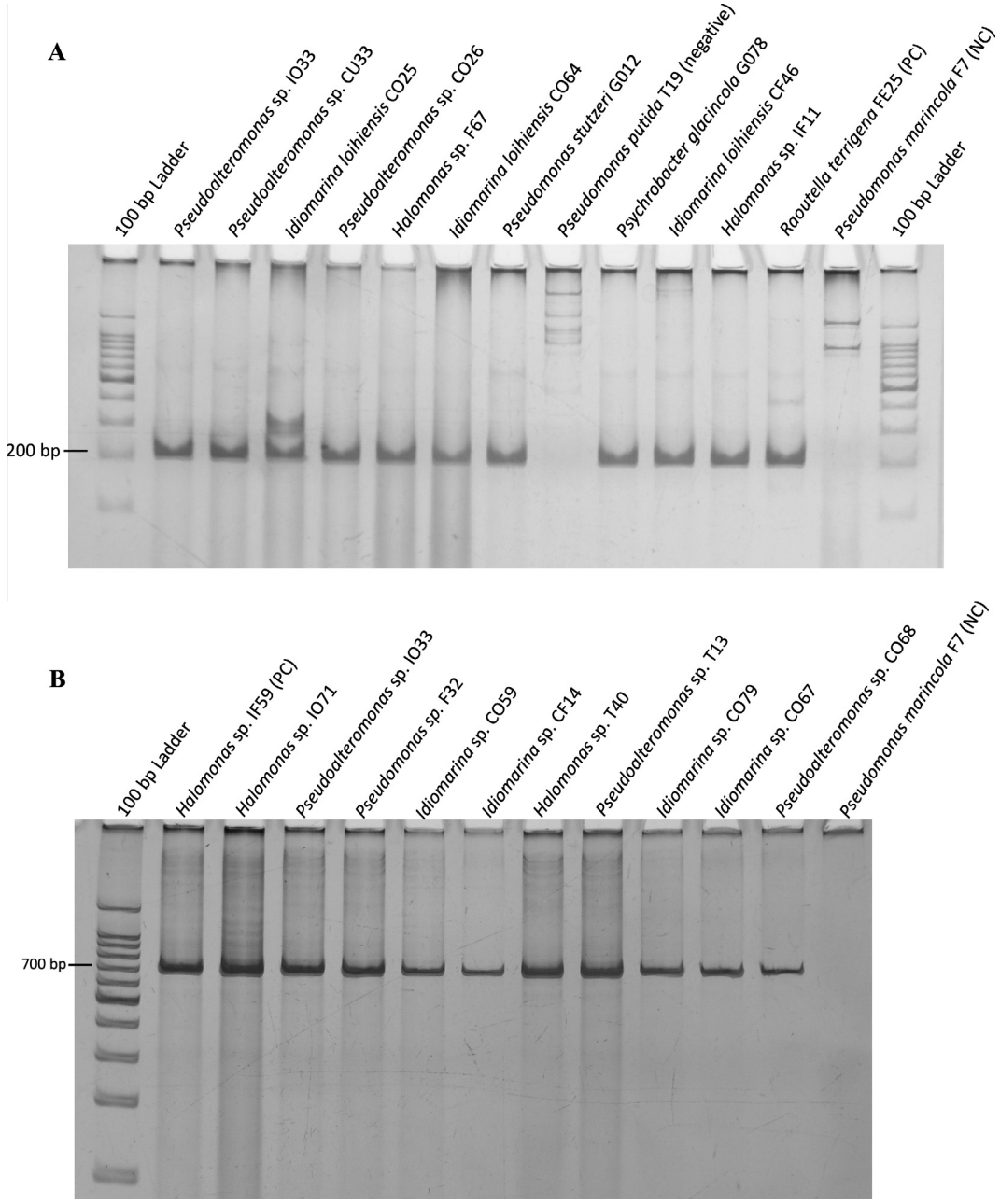

Fig. 1. PCR verification of the *floR* and *cmlA* genes in various florfenicol-resistant isolates. Gel A: Lanes from left: 100 bp ladder; strains IO33, CU33, CO25, CO26, F67, CO64, G012, T19 (negative for *floR*), G078, CF46, IF11, FE25 (*floR* positive control), F7 (*floR* negative control); 100 bp standard. Gel B: Lanes from left: 100 bp ladder; strains IF59 (*cmlA* positive control), IO71, IO33, F32, CO59, CF14, T40, T13, CO79, CO67, CO68, F7 (*cmlA* negative control).

et al., 2005; Miranda et al., 2014), so it must be concluded that use of florfenicol in scallop hatcheries will continue as a preventive practice to avoid sudden larval mortalities. Proportions of florfenicol-resistant bacteria among the reared larval microbiota were quite low but within the range of florfenicol resistant counts reported for scallop larvae and hatchery effluent using Tryptic soy agar added with NaCL (2%) (Miranda et al., 2013), even considering that in this study a different medium was used (PCA added with 2% NaCL).

Previous studies demonstrated the occurrence of the florfenicol resistance gene *floR* in the fish pathogens *Pasteurella piscicida* (Kim and Aoki, 1996) and *Edwarsiella ictaluri* (Welch et al., 2009), but it should be emphasized that in most of the studies the *floR* gene has been detected in human or terrestrial animal pathogenic species (Blickwede and Schwarz, 2004; Cloeckaert et al., 2000; Doublet et al., 2004; Du et al., 2004; Kadlec et al., 2007; Keyes et al., 2000; Poppe et al., 2006; Srinivasan et al., 2005). In a more recent study, Kuo et al. (2009) found a high incidence of harboring the *floR* and *cmlA* genes among *E. coli* strains isolated from healthy pigs.

Currently, studies reporting the incidence of the *floR* gene in aquatic environments are very scarce (Zhang et al., 2009). In a previous study of tetracycline resistance in bacteria from aquaculture in China, the *floR* gene was detected in some of these isolates (Dang et al., 2007), whereas Fernández-Alarcón et al. (2010) found that some strains associated to Chilean salmonid farms carried the *floR* gene.

This is the first report of the occurrence of the *floR* gene in bacteria associated to shellfish farming and our findings demonstrated

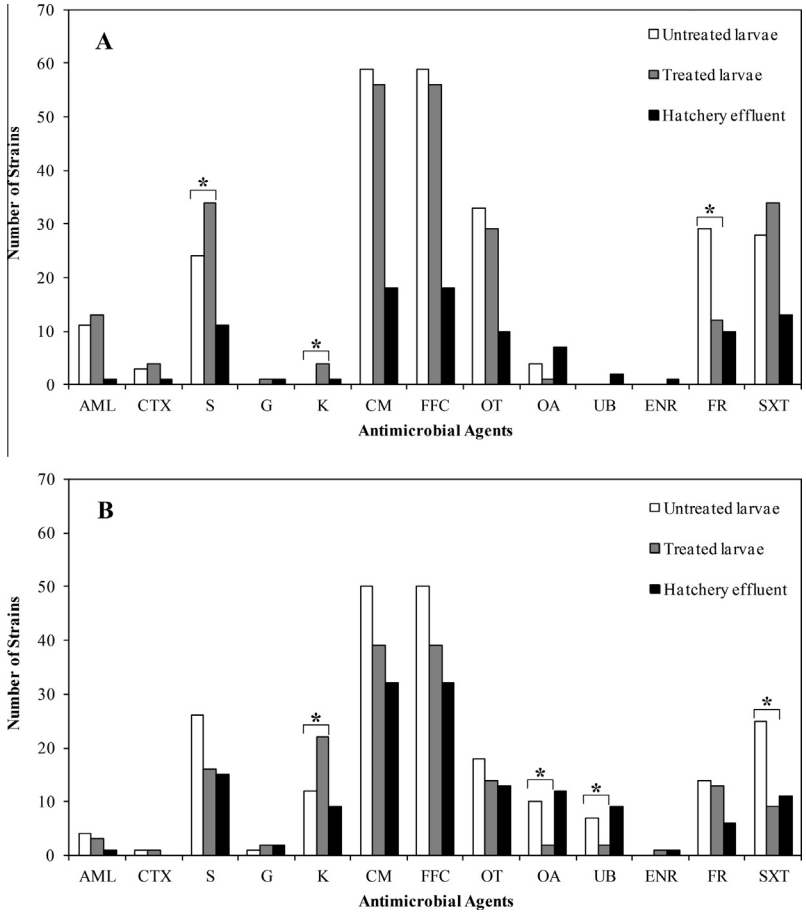

Fig. 2. Frequency of resistance to antibacterials of florfenicol-resistant bacterial strains isolated from scallop hatcheries 1 (A) and 2 (B). Antibacterial abbreviations: AML, Amoxicillin; CTX, Cefotaxime; S, Streptomycin; G, Gentamicin; K, Kanamycin; CM, Chloramphenicol; FFC, Florfenicol; OT, Oxytetracycline; OA, Oxolinic acid; UB, Flumequine; ENR, Enrofloxacin; FR, Furazolidone; SXT, Cotrimoxazole. Asterisks show significant differences ($P < 0.05$) of antibacterial resistance between untreated and florfenicol-treated scallop larvae.

that a high number of florfenicol-resistant strains isolated from 2 commercial scallop hatcheries in Chile carry the *floR* gene. Prevalence of florfenicol resistant bacteria carrying genes encoding for phenicol resistance in Chilean commercial hatcheries is of great importance due that most of scallop hatcheries have no control over their effluents addressing the high risk of resistance gene dissemination in waters surrounding farms. In addition, it must be noted that scallop seeds are transferred without sanitary regulations to growing areas at sea to obtain commercial size and the unregulated larval trading among different Chilean scallop farms is a frequent practice. In this study larval samples were taken one week before the spat was fixed to Netlon bags and then transferred to seawater in lanterns, so environmental spread of phenicol resistance genes via larval cultures appears to be of great importance but their transfer to human consumer is unlikely mainly because only scallop muscle and gonad are eaten.

Our results as well as a previous study (Miranda et al., 2013) demonstrated that even in absence of antibacterial therapy scallop larvae cultures carry an important level of resistant bacteria. Previously, antimicrobial resistance genes have been detected in aquaculture sites without antibiotic use, observing the occurrence of some resistance genes in fish farms that were not under antibiotic therapy (Dang et al., 2008; Tamminen et al., 2011). Additionally, results from other study suggested that aquaculture facilities may cause an increase in the number of tetracycline resistance genes in aquatic bacterial communities regardless of antibiotic use history (Seyfried et al., 2010). In this study, it was demonstrated that a high number of florfenicol resistant bacteria carried by reared larvae never exposed to any antibacterial harbored the *floR* gene, suggesting that this gene is widespread in scallop hatcheries, but more studies are required to know if these bacteria are introduced to the scallop culture from broodstock, influent water or other source (Riquelme et al., 1994).

Fernández-Alarcón et al. (2010) found that the majority of strains that possessed the *floR* gene were glucose-fermenting strains, whereas in this study most of the *floR*-carrying strains were non-glucose fermenters. The absence of multi-drug resistance suggests to rotate the antimicrobials used in the scallop larvae rearing process, but avoiding to replace the use of florfenicol by oxytetracycline because its lack of efficacy to reduce larval mortality in the intensive culture of scallop *A. purpuratus* (Miranda et al., 2014).

Usually *floR*-carrying bacteria exhibit high levels of simultaneous resistance against various antibacterials, and from these, ampicillin, tetracycline, streptomycin, gentamicin and potentiated sulfonamides are the most frequent (Doublet et al., 2002; Fernández-Alarcón et al., 2010). In another study, the molecular structure of the *floR* gene carried by an *Aeromonas bestiarum* strain from a freshwater stream was investigated, observing that this gene was linked to a tetracycline resistance gene (Gordon et al., 2008). It is interesting to note that Bischoff et al. (2005) suggested that in absence of specific chloramphenicol selective pressure, the *cmlA* gene is maintained by gene linkage to genes encoding resistance to other antimicrobials such as sulfamethoxazole, tetracycline and kanamycin.

Previous studies demonstrated that the presence of the *floR* gene is usually associated with high florfenicol MIC values

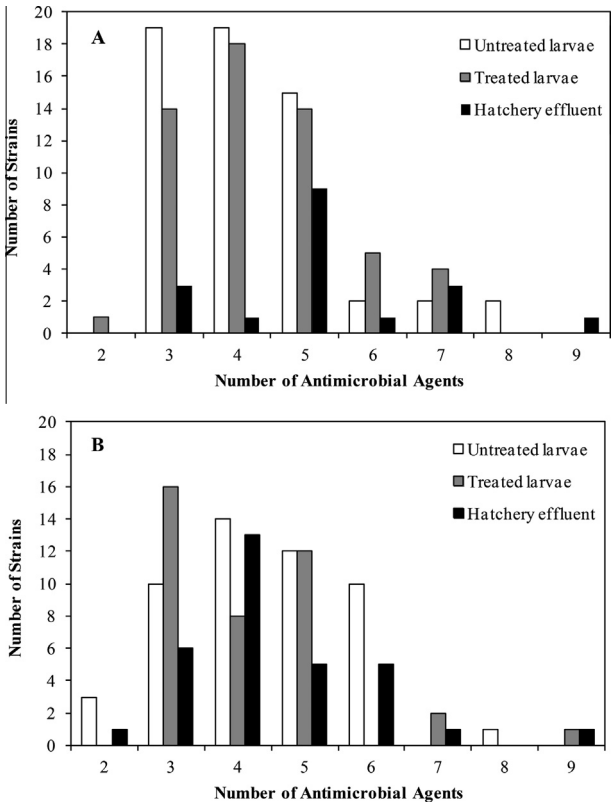

Fig. 3. Antibacterial multiresistance of florfenicol-resistant bacterial strains isolated from scallop hatcheries 1 (A) and 2 (B).

(Bischoff et al., 2002; White et al., 2000). This correlation between MIC values and carriage of the *floR* gene was also reported by Keyes et al. (2000), observing that *floR*-carrying *E. coli* isolated from sick chickens exhibited MIC values of at least 32 μg mL^{-1}, whereas 2 strains not harboring the *floR* gene exhibited MIC values of 8 μg mL^{-1}. Furthermore, Blickwede and Schwarz (2004) evidenced that all *floR*-carrying florfenicol-resistant *E. coli* from pigs exhibited MICs of florfenicol >128 μg mL^{-1}, whereas most of *E. coli* strains isolated from France and Germany harboring the *floR* gene exhibited MIC values of >128 μg mL^{-1} and some of them (5 strains) showed MIC values of 64 μg mL^{-1} (Doublet et al., 2002). In other study Li et al. (2007) found that *E. coli* isolated from diseased chickens harboring the *floR* gene had MIC values of ⩾32 μg mL^{-1}, whereas *E. coli* strains positive for both the *floR* and *cmlA* genes MIC values were elevated to ⩾64 μg mL^{-1}.

In this study, strains carrying the *floR* gene showed MIC values ranging from 16 to 1024 μg mL^{-1}, and are not in accordance with Singer et al. (2004), because MIC values of these strains were not different to those exhibited by the isolates not harboring the *floR* gene. In this study, MIC values exhibited by strains not carrying the *floR* gene were higher than the breakpoint value stated for florfenicol resistance (8 μg mL^{-1}) (Singer et al., 2004; White et al., 2000), suggesting that other resistance elements are also involved in the florfenicol resistance exhibited by these strains. Efflux pump systems are extensively observed among environmental bacteria, suggesting that a non-specific exposure to a wide variety of substances different to antibacterials could promote their expression (Alonso et al., 2001; Paulsen et al., 1996; Poole, 2005). It has been demonstrated that two (or even more) efflux systems can be over-expressed and function together in one bacterium, providing evidence that several types of multi-drug transporters may coexist together in the same bacterium with specific transporters (Lee et al., 2000; White et al., 2000). Many *Pseudomonas* species may express antibacterial intrinsic resistance mediated by different drug transporters (Li et al., 1994), indicating that the absence of the specific efflux pump encoded by the *floR* gene can be compensated by the over-expression of other multidrug efflux pumps with overlapping spectra (Lee et al., 2000; Morita et al., 2001). Furthermore, in a previous study using strains recovered from Chilean salmon farming, it was demonstrated the simultaneous expression of a florfenicol specific exporter, such as FloR and non-specific multi-drug efflux pumps (Fernández-Alarcón et al., 2010). In other study, efflux mechanisms were associated to various multi-drug resistant *Chryseobacterium* strains recovered from fish and aquatic environments and highly resistant to florfenicol, but the *floR* gene was not detected among these strains (Michel et al., 2005).

Table 4
Minimum Inhibitory Concentration (MIC) values of florfenicol (in μg mL^{-1}) of florfenicol-resistant strains isolated from scallop hatcheries.

Hatchery	Source	*n*	MIC_{50}	MIC_{90}	Range
1	Untreated Larvae	59	256	512	16–512
	Treated-Larvae	56	256	512	16–1024
	Effluent	18	64	512	16–512
2	Untreated larvae	50	128	512	16–2048
	Treated larvae	39	256	512	32–512
	Effluent	32	128	512	16–512

One aspect to be elucidated is the feasibility of the horizonthal transfer of the phenicol resistance determinants detected in this study. This study represents a further confirmation of the widespread distribution of florfenicol resistant bacteria and their resistance encoding genes in mariculture environments, evidencing a high taxonomic diversity of *floR*-carrying strains from both commercial hatcheries including many indigenous coastal marine bacteria suggests that the dissemination of the studied phenicol resistance genes is a consequence of independent horizontal gene transfer, in agreement with Rayamajhi et al. (2009), who found a high transfer of demonstrated that *floR* gene could disseminate via a high-molecular-weight plasmid and/or a putative mobile transposon (White et al., 2000), and it was also reported that *floR* is habored by an Inc plasmid (Cloeckaert et al., 2001)as well as an Inc A/C plasmid carried by the catfish pathogen, *E. ictaluri* (Welch et al., 2009). Most of studies reported that *floR* gene is commonly associated to plasmids (Blickwede and Schwarz, 2004; Cloeckaert et al., 2000; Doublet et al., 2004; Kehrenberg and Schwarz, 2005; Keyes et al., 2000), but this gene has been also detected to be integrated to the bacterial chromosome (Doublet et al., 2002; Meunier et al., 2003; Schwarz et al., 2004).

The high number of resistant bacteria carrying the *floR* and/or *cmlA* genes found in the scallop hatcheries prompts the necessity of a more restrictive attitude toward to the intensive use of florfenicol in shellfish culture, and demand further epidemiological and molecular investigations to demonstrate the spread of these genes in marine environments impacted by shellfish farming to define whether they pose a definite health risk. In agreement with Singer et al. (2006) we believe that an integrated ecological view of this resistance is urgently needed to understand the environmental conditions and factors that can contribute to the occurrence of the detected resistance, as well as to evaluate the impacts that the detected resistance genes can produce on the marine coastal environment, so becoming to a relevant reservoir of antibacterial resistant genes as was previously noted (Biyela et al., 2004; Kümmerer, 2004). In addition, future studies should focus on the feasibility of the genes conferring resistance can be transferred to other bacteria, including those of human health concern (Schwarz et al., 2006).

5. Conclusions

The present results show a high prevalence of florfenicol-resistant strains carrying *floR* and *cmlA* genes in two highly distant

hatcheries (357.82 km), demonstrating that Chilean scallop farming industry plays an important role as reservoir and potential source of florfenicol resistant bacteria and resistance encoding genes to be disseminated to surrounding marine environments. The high prevalence of *floR* and *cmlA* genes in strains isolated from prefixed scallop larvae and hatchery effluents evidences that these genes are frequent and persistent in scallop hatcheries even in the absence of use of florfenicol, suggesting that Chilean hatcheries are highly important to contribute and spread of genes encoding for resistance to phenicols in marine environments. Considering that an important number of the *floR*-carrying strains were also resistant to oxytetracycline, streptomycin and potentiated sulfonamides, the use of these antibacterials could have the ability to co-select for the florfenicol resistance, producing a selective pressure on this resistance gene and thus increasing its persistence in this environment. Otherwise, spread of these genes could potentially impact on human and animal health so further studies are needed to estimate that risk and develop strategies for coastal environment management. Other issue of great concern is the common and unregulated practice of sale and transfer of scallop among all commercial hatcheries along the country, prompting the urgent necessity of their regulation, especially when florfenicol was used during the larval rearing process.

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