Seasonal and spatial variability of antibiotic resistance genes and Class I integrons in the rivers of the Mekong delta, Vietnam

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RUNNING TITLE:

Antibiotic resistance genes show high local and temporal variability along extended stretches of rivers in the Mekong Delta, Vietnam.

30 **HIGHLIGHTS**

31 • In Vietnam, inland aquaculture massively relies on antibiotics to prevent epidemics
32 • Resistance genes were quantified along two rivers of the Mekong delta
33 • Seasonal (dry and rainy seasons) and spatial distributions were registered by qPCR
34 • BLA-*oxa1*and *sul2* reached highest abundances among bacterial communities
35 • Strong seasonal patterns and local variabilities were measured for CL1s and ARGs
36

37 **ABSTRACT**

38 Aquaculture activities are steadily expanding in Vietnam, covering an estimated 700,000 ha,
39 with 89% of these culture ponds located in the Mekong Delta. Since 2009, large-scale bacterial
40 epidemics have spread in response to this intensive cultivation. Antibiotics, even those
41 considered as a last resort, have only partially mitigated this problem. In turn, the side effects
42 of the massive use of these chemicals include the appearance of mobile genetic elements
43 associated with antibiotic resistance genes (ARGs). The large-scale emergence of a diverse
44 bacterial resistome, along with severe economic losses, has posed significant health risks to
45 local residents. In this study, the seasonal and spatial distributions of the class I integrase (CL1)
46 *intl*1 and the ARGs *sul*2 (sulfonamide), *BLA-oxa*1 (ß-lactams), and *erm*B (erythromycin) were
47 quantified from water and sediment samples collected during two consecutive seasons along
48 the Vam Co River and its tributary (Long An province, Vietnam). The results showed that CL1
49 was present in all river compartments, reaching 2.98×10^4 copies/mL and 1.07×10^6 copies/g of
50 sediment, respectively. The highest relative copy abundances to the 16S rDNA gene were
51 measured in water samples, with up to 3.02% for *BLA-oxa*1, followed by *sul*2 (1.16%) and
52 *erm*B (0.46%). Strong seasonal (dry season vs. rainy season) and spatial patterns were recorded
53 for all resistance genes. Higher amounts of ARGs in river water could be associated with higher
54 antibiotic use during the rainy season. In contrast, higher amounts of ARGs were recorded in
55 river sediments during the dry season, making this habitat a potential reservoir of transient
56 genes. Finally, the observations made in this study allowed us to clarify the environmental and
57 anthropogenic influences that may favor the dispersal and persistence of ARGS in this riverine
58 ecosystem.

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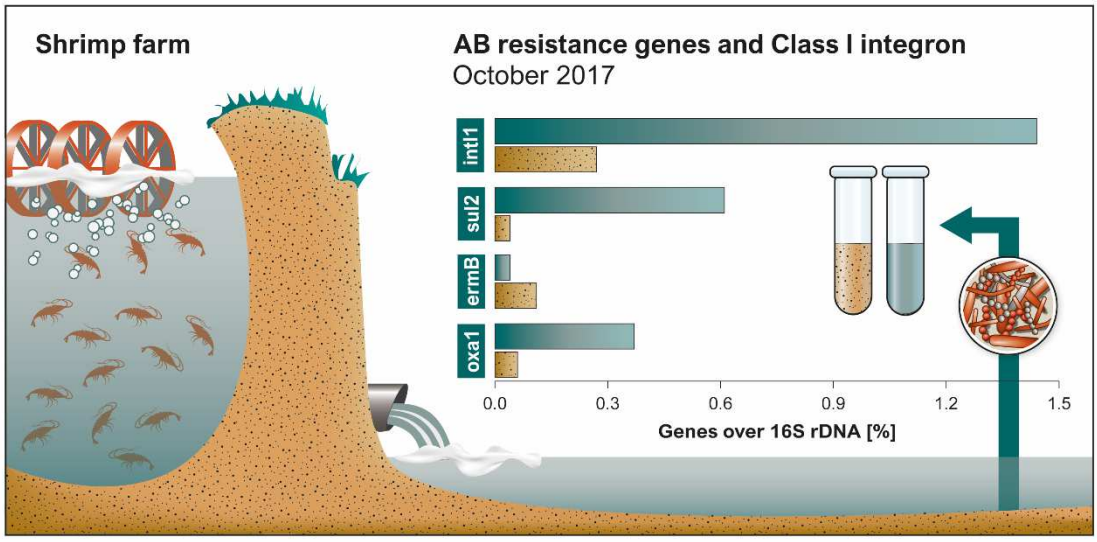
61 **ABSTRACT ART**



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63 **KEYWORDS**

64 Shrimp culture, antibiotic resistance, Mekong delta, river sediment, integrase, water quality

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1. INTRODUCTION

Aquaculture is considered one of the fastest growing food sectors and accounts for approximately 10% of all animal protein consumed worldwide (Thornber et al., 2020). According to recent FAO reports, production needs to increase by another 50% by 2050 while meeting the challenges of climate change and competition for natural resources (FAO, 2018). In 2000, the Vietnamese government implemented an agricultural diversification policy in the Mekong Delta, which shifted production from rice monoculture to a more diversified rice-based agricultural system by introducing aquaculture, horticulture, and fruit production. Aquaculture now plays an important role in the country's economy, with shrimp exports peaking at $3.85 billion in 2017 (Anh et al., 2020). White shrimp (*Litopenaeus vannamei*) and black tiger shrimp (*Penaeus monodon*) are the dominant species cultivated in ponds covering an estimated 700,000 ha. About 89% of these ponds are located in the Mekong Delta (Hai et al., 2015, Anh et al., 2020). The success of shrimp farming has provided prospects for improved livelihoods, incomes, and employment for coastal and rural communities, providing about 4 million jobs nationwide (Lan, 2013; Khanh Nguyen et al., 2019; Kruse et al., 2020). More recently, and following the new Sustainable Development Goals, Viet Nam has defined an action plan to encourage farmers to reduce environmental impacts (Ngoc et al., 2021). This governmental decision follows the increasing environmental damage caused by this type of inland aquaculture (Pham et al., 2021). The implementation of these measures is also explained by the widespread cases of bacterial and viral diseases affecting the farming ponds.

Severe disease outbreaks have been the leading cause of loss in shrimp culture in recent decades (Duc, et al., 2015; Anh et al., 2020). Six viral diseases of crustaceans have the potential to cause substantial economic losses. Of these, the "White Spot Syndrome Virus" is the leading cause of high mortality rates in shrimp ponds and hatcheries (Millar et al., 2020; Thornber et al., 2020). Since 2009, substantial economic losses have been induced by acute hepatopancreatic necrosis syndrome (AHPND) caused by *Vibrio parahaemolyticus*, a Gram-negative, halophilic, mesophilic, aerobic bacterium common in warm marine and estuarine habitats (Liu et al., 2019). Approximately 39,000 ha of shrimp ponds found in the Mekong Delta were affected by AHPND in 2011, with mortality rates up to 100%, inducing losses that were estimated to exceed $1 billion (FAO, 2013). Antibiotics (ABs), often spread directly into pond water, are the most common method to prevent and treat microbial outbreaks in shrimp aquaculture and are currently an integral part of shrimp farming procedures (Chi et al., 2017; Binh et al., 2018; Ngoc et al., 2021). More than 30 antimicrobial compounds are approved in Viet Nam, including β-lactams, aminoglycosides, macrolides, tetracyclines, polymyxins,

101 pleuromutilines, lincosamides, sulfonamides, and diaminopyrimidine (trimethoprim) (MARD,
102 2016, in Lulijwa et al., 2019). However, accurate data on antimicrobial sales and use are
103 difficult to obtain because antibiotics are available over the counter and can be of variable
104 quality (Tran et al., 2018). Due to limited access to diagnostic services, inadequate instructions
105 for use, overuse, and poor education, treatment of an infection cannot be accurately targeted,
106 with the risk of choosing an inappropriate or ineffective product (Lulijwa et al., 2019; Thornber
107 et al., 2020). Over the past decade, the overuse of ABs has been identified as one of the major
108 factors driving the increase in AB-resistant bacteria. The large-scale emergence of this bacterial
109 resistome in aquatic habitats may not only result in severe economic losses, but also pose
110 significant health risks to local communities. The spread of antibiotic resistance genes (ARGs)
111 in potential and clinically relevant human and animal pathogens is hindered by local culture
112 techniques and the use of ABs, even at low concentrations (Wistrand-Yuen et al., 2018).
113 Increasing amounts of literature articles have described the presence of ARGs in communities
114 involved in aquaculture practices and the products themselves. For example, high levels of the
115 *mcr*-1 gene conferring resistance to colistin in *Escherichia coli* have been detected within the
116 microbiome of people living in aquaculture areas in China (Shen et al., 2018). Erythromycin
117 resistance genes were recently detected by culture-independent techniques in commercial
118 shrimp purchased from aquatic markets (Liu et al., 2019). In a previous study, we isolated and
119 identified multidrug-resistant bacteria affiliated with the genera *Aeromonas, Acinetobacter,*
120 *Citrobacter, Escherichia,* and *Klebsiella* from the Vam Co River (Long An Province, Viet
121 Nam), a river receiving several channels of effluent from intensive shrimp farms (Pham et al.,
122 2018). Sequencing of 41 plasmid-transmitted ARGs showed the presence of a wide range of
123 ABs, including aminoglycosides, OXA-type ß-lactams, chloramphenicol, fluoroquinolone,
124 macrolides, rifampicin, tetracycline, trimethoprim and sulfonamide. The association of ARGs
125 with mobile genetic elements that enable their dissemination has been demonstrated worldwide
126 (Gillings et al., 2008; Gao et al., 2012; Fang et al., 2021). Previous studies in the Mekong Delta
127 have shown, for example, that *sul*2 genes have been detected in association with integrons and
128 plasmids (Pham et al., 2018). Among all mobile genetic elements, Class 1 integrons are now
129 considered key players in the dissemination of ARGs, as they can easily acquire and express
130 genes inserted into their structure as gene cassettes, expressed under a common promoter.
131 These genetic elements are usually hosted on plasmids and transposons facilitating horizontal
132 gene transfer (Preena et al., 2020). As shown by Gillings et al. (2015), Class 1 integrons provide
133 a good measure of the general level of antibiotic resistance genes. For example, recent studies

have shown that the relative abundance of the *intI*1 gene correlates significantly with total AB concentrations in water and sediment (Chen et al., 2015).

However, and despite serious potential economic losses and significant health risks to local communities, very few studies have been conducted to assess the prevalence of bacterial resistome in the Mekong Delta. The incidence of ARGs in microbial communities, their diversity, and their association with mobile elements such as integrons are all indications needed to understand the mechanisms of propagation. In this project, we sought to quantify a selection of ARGs to better understand their spatial distributions and seasonal variations in natural streams affected by inland aquaculture practices. Water and sediment samples were collected during two consecutive seasons along a tributary of the Vam Co River (Long An province, Viet Nam). *sul*2 (sulfonamide), *BLA-oxa1* (ß-lactams), *erm*B (erythromycin), and class I integrons were quantified by qPCR, normalized against 16S rDNA genes. Sampling sites included small tributaries connected to small localities and effluent channels connected to shrimp farming ponds. The results obtained so far provide detailed information to accurately understand the prevalence and variability in time and space of the inland aquaculture-induced resistome in the Mekong Delta.

2. MATERIAL AND METHODS

2.1 Sampling

Two sampling campaigns were conducted in May and October 2017 on two rivers in the Mekong Delta, Vietnam. Sampling locations (SL) 1-8 were defined along an approximately 12 km transect on the Som Vam Co Tay River (Figure SI-1). Samples SL-9 and SL-10 were located on the Vam Co River, upstream and downstream of the junction with the aforementioned tributary, to assess the potential impact of mixing of the two rivers. The section of the Som Vam Co Tay River that was selected for this study is bordered by large areas dedicated to shrimp farming. This section is particularly suitable for a detailed analysis of riparian microbial communities with potential impact from aquaculture activities. The SLs were selected based on the topology of the two rivers and were located near small tributaries and effluent outlets connected to intensive shrimp farming ponds. The Vam Co River, on the other hand, is bordered upstream by large areas dedicated mainly to agriculture (rice and

166 dragon fruit), with the presence of two major industrial parks and a regional hospital (Table
167 SI-1).
168 For all SLs, one water sample and one sediment sample were collected within 5 meters of the
169 shore (shore and sediment samples below). A second water sample was collected from the
170 middle of the river (middle sample). Water samples were collected by hand from the surface
171 of the water (approximately 20 cm deep) and stored in sterile 5-liter plastic containers.
172 Sediment samples (approximately 0-5 cm deep) were collected manually with a bucket at a
173 distance of approximately 10 meters from the river bank and transferred into sterile containers.
174 All samples were shipped to the laboratory within 8 hours and stored at 4 °C until processed.
175 Dissolved oxygen, temperature, pH, electrical conductivity, and turbidity (expressed in
176 formazine nephelometric units, FNU) were measured in the field using a YSI ProDSS portable
177 multiparameter water quality meter (Xylem, USA) (Table SI-2 and SI-3). Note that sample
178 SL-10 collected in the middle of the river (October) is missing.
179

180 **2.2 Molecular analysis**

181 DNA extraction from water and sediment samples was carried out as shown in Pham et al.
182 (2018). Quantification of the ARGs, Class I integrase and 16S rDNA genes was carried out in
183 triplicate in 10 µL reactions in a MIC qPCR Cycler (BioMolecular Systems, Australia) as
184 follows: 2.5 mL template DNA, 2.1 µL water, 0.2 µL of each primer (100mM stock) (Table
185 SI-4) and 5 µL of 2× KAPA SYBR Fast Universal qPCR kit (KAPA Biosystems, USA).
186 Samples were cycled (40 cycles) at 95 °C for 10 s, followed by an extension at 62 °C for 30 s
187 and acquisition at 72 °C for 20 s. The final melting step was carried out from 72 °C to 95 °C,
188 at a rate of 0.1 °C/s. Analysis of the results was carried out using the built-in analytical software
189 (micPCR, BioMolecular Systems, ver. 2.2). In average, efficiency (0.88 – 101.2%) and r^2
190 values (> 0.995) were determined from eight points of the serial dilutions (10^8–10^1 copies) of
191 each target gene. Based on calibration curves, Cq values were used to calculate the gene copy
192 numbers which were normalized against mass (ng) of the extracted DNA.
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3. RESULTS AND DISCUSSION

3.1 Physical and chemical measurements

Water samples collected from the Som Vam Co Tay tributary (SL-1 to SL-8) as well as those collected upstream and downstream of the junction with the Vam Co River (SL-9 and SL-10) were characterized by slightly acidic pH (pH 5.7 - 6.7) and oxygen depletion (OD 1.43 - 3.65 mg/L) (Table SI-2). No significant variation was noted between values measured in May and October, which were consistent with those measured locally in other Mekong tributaries (Strady et al., 2017). A seasonal difference was logically present in measured values for temperature, turbidity, and salinity, due to heavy monsoon rains (Table SI-3). The mean temperature of the shoreline samples decreased from 31.4 to 29.2 °C in May and October, respectively. Values for the middle samples decreased from 31.3 to 29.9 °C in the same period. Turbidity values measured in May, with an average of 18.6 and 19.5 FNU for both shoreline and middle samples were significantly lower than those measured in October. During the rainy season, higher flows increased the transport of solid particles. Turbidity values measured in October for the shoreline and middle samples averaged 145.8 and 62.4 FNU, respectively. It should be noted that the SL-3 and SL-5 shoreline samples contained large amounts of suspended particulate matter, which resulted in much higher turbidity records (542.6 and 380.0 FNU respectively). These two samples also had much higher than average salinity, with 542 and 690 μScm^{-1} respectively (Table SI-2). Typically, during the rainy season (October), high salinity and turbidity values were recorded at the junction of the river with small streams and tributaries connected to the shrimp farming ponds. Intensive shrimp farming involves the rearing of 30-60 animals/m^2, with maxima reaching up to 200 animals/m^2 (9 tons/ha) (Nguyen et al., 2019). Nutrient uptake is low under these cropping conditions and a relatively small percentage (less than 20%) of nitrogen, phosphorus, and organic carbon is recovered from the biomass (Henares et al., 2019). To maintain water quality under conditions that allow for animal growth, common practices involve frequent pumping of 2-5% of the pond volume with the removal of accumulated sludge waste from the bottom of the pond. This water is replaced by an identical volume of water accumulated in an adjacent settling tank, typically filled with relatively good quality river water pumped in at high tide. These volumes of effluent, rich in organic matter and salt, are discharged without further treatment to also compensate for heavy monsoon rains, in turn inducing eutrophication and salinization of the receiving surface water

(Lan, 2013). Bacterial cells present in the culture basins also enrich the natural microbial communities present within the river sediment and surface water (Shen, et al., 2020).

3.2 Quantitation of 16S rDNA genes

16S rDNA gene copies quantified in sediment samples showed significant differences between May and October (Figure 1). These numbers were significantly higher during the dry season (in May), ranging from 7.95×10^{7} to a maximum value of 2.14×10^{8} copies/g of sediment in SL-3 (Table SI-5). In October, copy numbers ranged from 1.77×10^{7} to 9.11×10^{7} copies/g. SL-1 only showed almost identical values in both seasons. For comparison, copy numbers measured in these sediment samples were close to those measured by Pham et al. (2018) in a previous campaign carried out in the dry season, with values ranging from 1.8×10^{7} to 2.1×10^{8} copies/g.

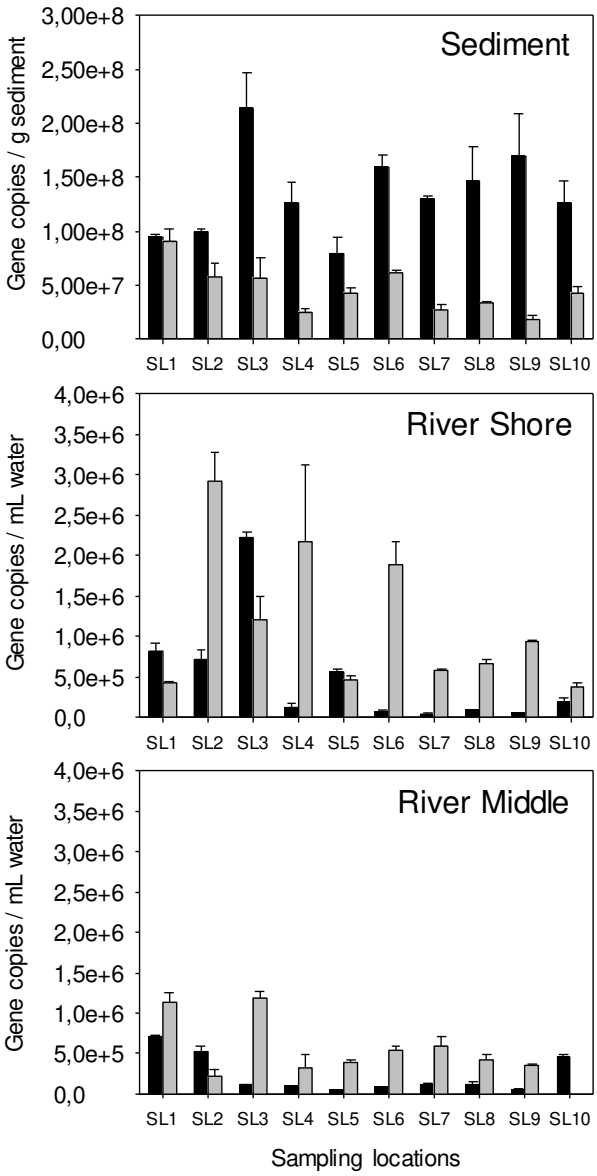

Figure 1: Copies of the 16S rDNA gene quantified in river sediment (top), river shore (middle), and mid-river (bottom) samples. Black and gray bars: samples collected in May (dry season) and October (rainy season), respectively.

Shore samples showed large seasonal differences in 16S rDNA gene copy number (Figure 1, middle). In October, variations between the lowest (SL-10: 3.71×10^5 copies/g) and highest (SL-2: 2.92×10^6 copies/g) copy numbers were spread over an order of magnitude. Conversely, the highest values (SL-2, SL-4, and SL-6) were located at the outlet of the shrimp tank effluent (Figure SI-1). These high values (>2.00×10^6 copies/mL) may be related to farming practices, with the discharge of pond water to compensate for heavy monsoon rains. Classically, the highest copy numbers were recorded during the rainy season (October), with the exception of

297 sites SL-1, SL-3 and SL-5 collected in May. Interestingly, these three sample sites were in
298 close proximity to the outlet of small tributaries. Careful observation of the topography showed
299 that these small rivers collected domestic effluent from small communities. In May, two orders
300 of magnitude separated the highest value, SL-3 (2.22×10^6 copies/mL) and the lowest, SL-7
301 (4.31×10^4 copies/mL), an indication of the presence of high spatial variability. For comparison,
302 Pham et al. (2018) measured 2.1×10^5 to 2.9×10^6 copies/mL of the bacterial 16S rDNA gene
303 for riverbank samples. These values are in agreement with those presented here. Results from
304 samples collected in the middle of the river showed a very different pattern, including less
305 seasonal variability (Figure 1, bottom). In May, the highest values were recorded for the two
306 samples collected upstream, SL-1 and SL-2 with 7.05×10^5 and 5.16×10^5 copies/mL
307 respectively, almost identical to those measured for the river shore (Table SI-5). SL-5 showed
308 the lowest value with 5.29×10^4 copies/mL. During the rainy season (October), values recorded
309 were generally higher, ranging from 2.15×10^5 (SL-2) to 1.19×10^6 (SL-3) copies/mL. The low
310 variability offered by samples collected in the middle of the river is probably the result of
311 constant mixing induced by the natural flow of the river, combined with tidal currents.
312

313 **3.3 Quantitation of Class I integrons (CL1s)**

314 The results of the quantitative analysis are shown in Figure 2 and the normalization ratios of
315 *intI*1 copies to 16S rDNA are shown in Table 1. The complete quantitative data are provided
316 in Table SI-6. The results show that *intI*1 was recovered consistently and frequently in large
317 quantities in all river samples. In the sediment samples, the highest values were found among
318 samples collected during the dry season, indicating a high seasonal pattern, reaching 1.07×10^6
319 copies/mL in sample SL-9, an order of magnitude higher than the lowest amounts measured in
320 sample SL-2 (Table SI-6). Sediment samples collected during the rainy season showed less
321 variation, with only about a threefold difference between samples SL-4 (3.70×10^4 copies/mL)
322 and SL-1 (1.98×10^5 copies/mL). These values are comparable to those recorded in river
323 sediments by studies conducted worldwide (Koczure et al., 2016; Sabri et al., 2020). However,
324 the relative proportions of cells carrying this gene-capture system in the sediments were lower
325 (0.30% and 0.27% on average for May and October, respectively) (Table 1) than those
326 measured in other river systems (Amos et al., 2018).
327 The water samples showed strong seasonal fluctuations. Only a few samples, such as SL-3 with
328 1.60×10^4 copies/mL (Table SI-6), had nearly identical copy numbers between the dry and rainy
329 seasons, with 1.60×10^4 copies/mL (Table SI-6). All other samples collected in October (shore

and middle) contained higher numbers of the CL1 gene. The differences between the dry and rainy seasons are remarkably high for samples Sl-4 through SL-10, with up to two orders of magnitude in gene copy numbers. The highest amounts were measured at the river's edge in samples SL-4 and SL-6, with 2.98×10^4 copies/mL and 2.58×10^4 copies/mL, respectively. SL-1 and SL-3 showed the highest amounts for samples collected in the middle of the river, with 2.30×10^4 copies/mL and 1.95×10^4 copies/mL, respectively.

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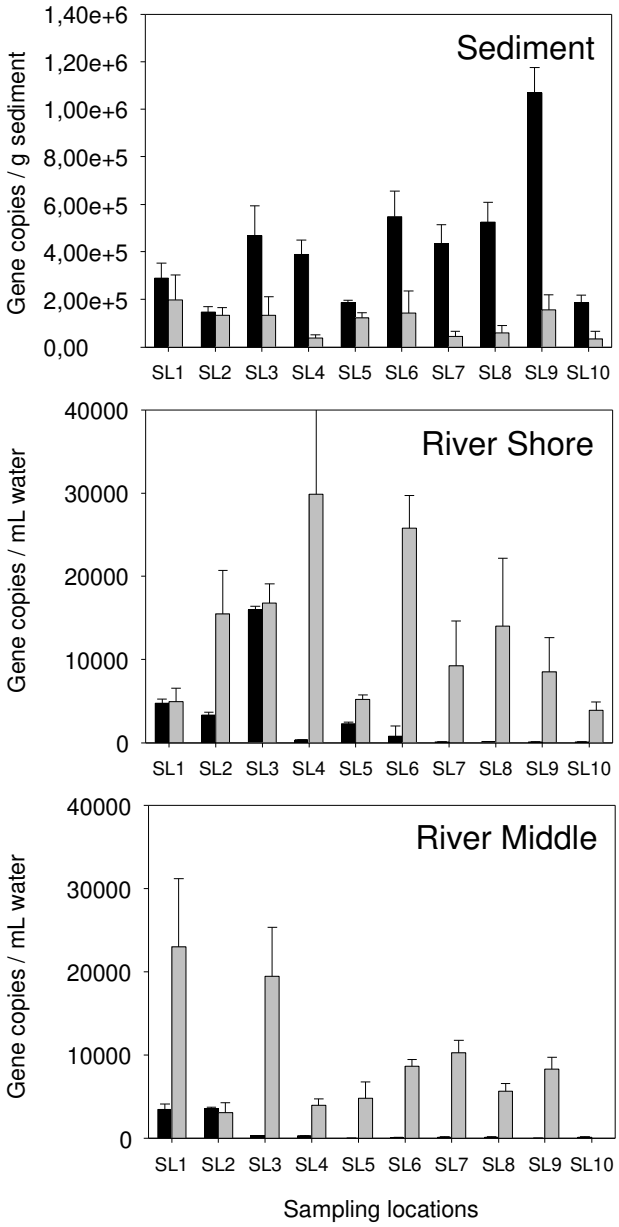

Figure 2: Copies of the *intI*1 gene quantified in river sediment (top), river shore (middle), and mid-river (bottom) samples. Black and gray bars: samples collected in May (dry season) and October (rainy season), respectively.

363 Interestingly, relative proportions of the *intI*1 gene copies (normalized to 16S rDNA gene
364 copies) followed the same seasonal pattern, with an increase in all water samples from May to
365 October (Table 1). This relative proportion increased in average from 0.38 % to 1.26% for
366 samples taken near the river shore, and from 0.22 % to 1.60% for those collected in the middle
367 of the river. Samples SL-9 taken upstream of the junction with its tributary showed the highest
368 ratio (2.29 %). The observed seasonal enrichment of bacteria carrying the CL1 gene capture
369 system was interpreted as the result of a selective pressure exerted on local communities.
370 Recent studies demonstrated that relative abundance of the *intI*1 gene correlated significantly
371 with the total concentrations of *ABs* in water and sediments (Chen et al., 2015). The seasonal
372 increase observed in our study occurred concomitantly with the use of increasing amounts of
373 ABs from August on in shrimp culture basins (Su et al., 2017; Pham et al., 2018). High rainfall
374 events affect physical and chemical parameters of the pond water, such as temperature, salinity
375 and pH, leading to deteriorating culture conditions. ABs are used in increasing quantities until
376 the capture of mature animals, classically by the end of October. *intI1* gene copies are
377 nevertheless three to four orders of magnitude lower than those found in Chinese static
378 mariculture farms, a possible indication that river flow and tide currents may play an important
379 role in the constant wash-out of ABs and CL1-bearing bacterial cells towards the ocean (Wang
380 et al., 2019).
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intI1	May 2017			October 2017		
	Edge	Middle	Sediment	Edge	Middle	Sediment
SL-1	0,58%	0,49%	0,31%	1,15%	2,01%	0,22%
SL-2	0,46%	0,69%	0,15%	0,53%	1,43%	0,23%
SL-3	0,72%	0,27%	0,22%	1,39%	1,64%	0,24%
SL-4	0,25%	0,25%	0,31%	1,37%	1,21%	0,15%
SL-5	0,40%	0,09%	0,24%	1,11%	1,23%	0,29%
SL-6	0,95%	0,12%	0,34%	1,36%	1,57%	0,24%
SL-7	0,17%	0,10%	0,33%	1,60%	1,73%	0,17%
SL-8	0,13%	0,10%	0,36%	2,11%	1,31%	0,18%
SL-9	0,16%	0,09%	0,63%	0,90%	2,29%	0,88%
SL-10	0,04%	0,03%	0,15%	1,04%	ND	0,08%
MEAN	**0,38%**	**0,22%**	**0,30%**	**1,26%**	**1,60%**	**0,27%**

sul2	May 2017			October 2017		
	Edge	Middle	Sediment	Edge	Middle	Sediment
SL-1	0,40%	0,28%	0,04%	0,93%	1,16%	0,05%
SL-2	0,40%	0,52%	0,01%	0,95%	0,67%	0,06%
SL-3	0,58%	0,20%	0,02%	0,83%	0,71%	0,04%
SL-4	0,41%	0,25%	0,02%	0,73%	0,71%	0,02%
SL-5	0,67%	0,26%	0,04%	0,49%	0,51%	0,03%
SL-6	0,38%	0,26%	0,03%	0,37%	0,34%	0,04%
SL-7	0,33%	0,32%	0,03%	0,34%	0,36%	0,04%
SL-8	0,32%	0,28%	0,04%	0,92%	0,37%	0,03%
SL-9	0,11%	0,13%	0,11%	0,35%	0,46%	0,09%
SL-10	0,22%	0,13%	0,03%	0,26%	ND	0,00%
MEAN	**0,38%**	**0,26%**	**0,04%**	**0,62%**	**0,59%**	**0,04%**

ermB	May 2017			October 2017		
	Edge	Middle	Sediment	Edge	Middle	Sediment
SL-1	0,01%	0,01%	0,00%	0,04%	0,01%	0,12%
SL-2	0,01%	0,02%	0,00%	0,01%	0,06%	0,04%
SL-3	0,01%	0,04%	0,00%	0,01%	0,01%	0,06%
SL-4	0,08%	0,01%	0,00%	0,01%	0,04%	0,27%
SL-5	0,00%	0,00%	0,00%	0,01%	0,06%	0,09%
SL-6	0,00%	0,01%	0,00%	0,02%	0,07%	0,06%
SL-7	0,46%	0,20%	0,00%	0,06%	0,12%	0,06%
SL-8	0,10%	0,00%	0,00%	0,02%	0,01%	0,13%
SL-9	0,00%	0,03%	0,00%	0,01%	0,04%	0,19%
SL-10	0,00%	0,05%	0,00%	0,07%	ND	0,11%
MEAN	**0,07%**	**0,04%**	**0,00%**	**0,03%**	**0,05%**	**0,11%**

oxa1	May 2017			October 2017		
	Edge	Middle	Sediment	Edge	Middle	Sediment
SL-1	0,11%	0,31%	0,23%	0,20%	0,22%	0,03%
SL-2	0,19%	0,46%	0,02%	0,06%	0,19%	0,04%
SL-3	0,10%	0,29%	0,08%	0,20%	0,17%	0,04%
SL-4	0,27%	0,27%	0,04%	0,22%	0,32%	0,11%
SL-5	0,17%	1,18%	ND	0,20%	0,62%	0,08%
SL-6	1,84%	2,72%	0,03%	0,10%	0,45%	0,06%
SL-7	3,02%	1,38%	0,06%	0,87%	0,53%	0,07%
SL-8	1,57%	1,24%	0,04%	0,01%	0,87%	0,03%
SL-9	1,13%	0,94%	0,10%	0,56%	0,73%	0,06%
SL-10	0,26%	0,14%	0,78%	0,53%	ND	0,04%
MEAN	**0,86%**	**0,89%**	**0,15%**	**0,29%**	**0,45%**	**0,06%**

Table 1: Relative proportions of ARGs and Class 1 integron genes normalized to 16S rDNA gene copies.

The endemic presence of this gene-capture system is now documented in all environments disturbed by human activities. This is particularly the case when these habitats are impacted by the chronic presence of ABs, heavy metals and disinfectants (Amos et al., 2018; Oliveira-Pinto et al., 2018). These substances exert constant selection pressure on microbial communities, with concomitant rapid diffusion of various mobile elements carrying AB resistance genes in a wide range of bacterial species (Gillings et al., 2014). In our study, SL-9 (river environment) showed the highest relative proportion of the *intI*1 gene among riparian bacterial communities with 2.29%. Due to its geographical location, shrimp pond effluent may not be the only cause of the massive increase observed in this section of the Vam Co River. Other sources of anthropogenic activities, such as livestock and industrial effluents, municipal and hospital wastewater may also have contributed to the observed LC1 enrichment during the rainy season.

3.4 Quantitation of antibiotic resistance genes (ARGs)

Three genes responsible for antibiotic resistance were quantified in the sediment and water samples, namely *sul*2, *erm*B, and *BLA-oxa*1. The results of these analyses are presented in Figures 3, 4, and 5, and values are provided in Tables SI-7 through SI-9. The relative proportions of ARGs normalized to 16S rDNA gene copies are presented in Table 1.

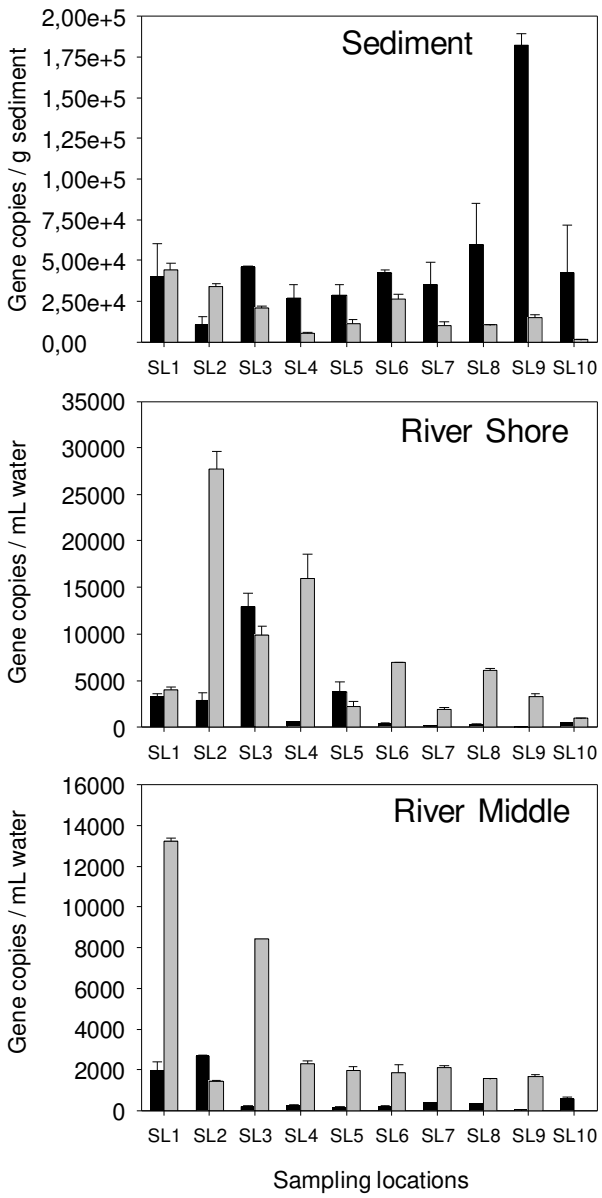

Figure 3: Copies of the *sul*2 gene quantified in river sediment (top), river shore (middle), and mid-river (bottom) samples. Black and gray bars: samples collected in May (dry season) and October (rainy season), respectively.

Analysis of the results obtained for the *sul*2 gene showed spatial and seasonal patterns closely related to those observed for the *intI*1 gene (Table 1). Quantification of this resistance gene showed higher absolute numbers in sediment samples collected in May. Similarly, SL-9 showed the highest copy numbers, with 1.82×10^5 copies/g. In October, copy numbers were generally lower, except for SL-1, with 4.41×10^4 copies/g. However, and unlike CL1, the normalized relative proportions of *sul*2 showed no variation between the two seasons, with

440 only 0.04%. This result demonstrated that this gene was uniformly maintained over the long
441 term in the sediments of both river systems, albeit in small amounts.
442 Copies of the *sul*2 gene measured in water samples (shore) were highest in October, with
443 significant local variability. SL-3 and SL-5 showed higher values only in May, with 1.29×10^4
444 copies/mL and 3.78×10^3 copies/mL, respectively. In October, SL-2 reached the highest copy
445 number with 2.77×10^4 copies/mL. Mid-river water samples showed an identical seasonal
446 pattern (Figure 3) with significantly higher copy numbers in October, particularly for SL-1 and
447 SL-3, with 1.32×10^4 and 8.43×10^4 copies/mL, respectively. As indicated for *intI*1, SL-2
448 showed the highest copy number in May with 2.70×10^3 copies/mL. The relative proportions
449 of *sul*2 gene copies in water samples, normalized to 16S rDNA gene copies, showed seasonal
450 variations (Table 1). These proportions averaged 0.38% and 0.26% for shoreline and middle
451 samples collected in May, respectively, and increased to 0.62% and 0.59% in October. SL-1
452 (middle) had the highest relative proportion of *sul*2 at 1.16%. As a reminder, this sample
453 concomitantly showed a significant increase in the proportion of *intI*1 genes, possibly
454 indicating that a significant number of *sul*2 genes were carried by mobile genetic elements, as
455 previously observed in rivers and estuaries (Sabri et al., 2020; Singh et al., 2019; Leng et al.,
456 2020). In a study conducted in (2015), Chen et al. showed a significant correlation between
457 *intI*1 gene abundance and dissolved AB concentrations, as well as with the abundance of *sul*
458 genes, a possible consequence of their embedding in the same mobile genetic elements. The
459 intensive use of sulfonamide in Viet Nam is confirmed by a 2013 study that showed that
460 approximately 12 tons of sulfamethoxazole were discharged annually from the Mekong River
461 into the South China Sea (Shimizu et al., 2013). These large quantities demonstrate the routine
462 use of sulfonamides for mitigation of bacterial diseases affecting aquaculture activities. In
463 addition, it is now recognized that bacterial strains carrying a *sul* gene also develop resistance
464 to other ABs, such as streptomycin, ampicillin, and trimethoprim, accentuating the emergence
465 of multidrug-resistant strains.
466 In the present study, the use of this class of antibiotics resulted in the prevalence of resistances
467 with a high degree of spatial heterogeneity. The results so far have confirmed that sulfonamide
468 resistances are one of the major classes of ARGs in riverine habitats affected by shrimp farm
469 effluents. However, the spread of sul2 in Mekong Delta rivers may also be correlated with
470 other environmental factors. For example, Su et al. (2020) showed an excellent correlation
471 between sul2 gene copies and COD measured in the farm ponds. Tide and river flow can
472 increase the dilution rate of bacterial cells carrying this ARG, and at the same time spread
473 these resistance genes throughout the river system. Nevertheless, the copy numbers measured

in our study were much lower than those measured in other intensive aquaculture systems. Wang et al. (2019) measured 3.5×107 to 6.5×1010 copies/mL of the sul2 gene in a Chinese salmon mariculture system, values that exceed the copy numbers measured in our study by more than 4 orders of magnitude.

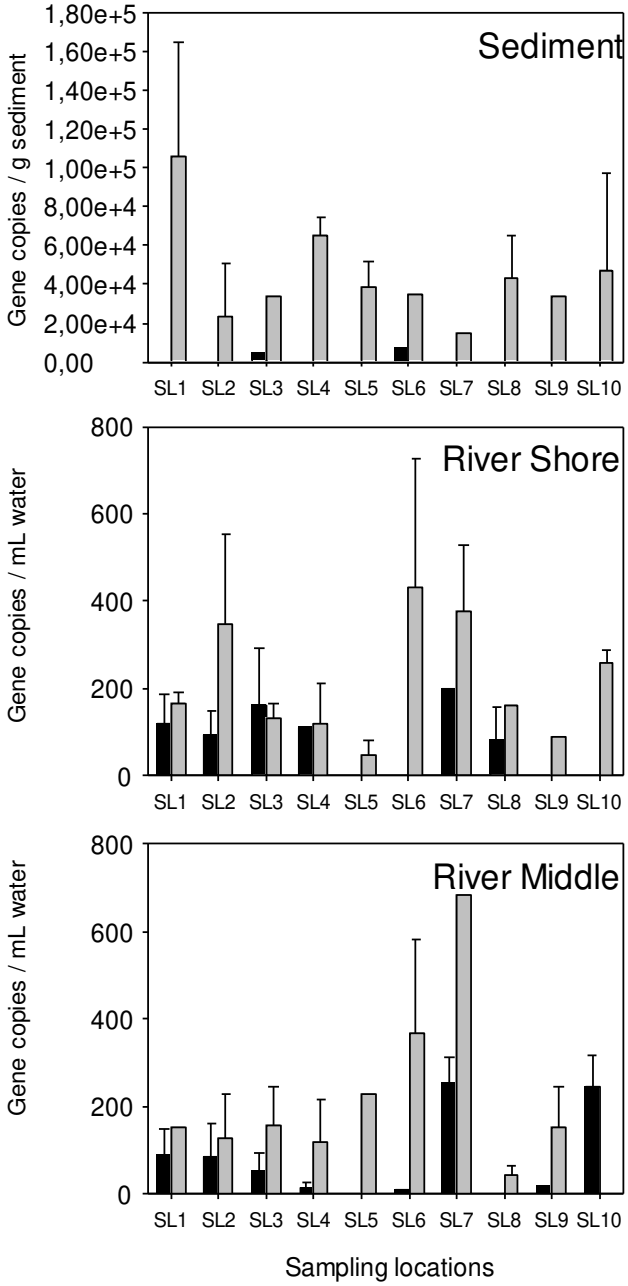

Figure 4: Copies of the *erm*B gene quantified in river sediment (top), river shore (middle), and mid-river (bottom) samples. Black and gray bars: samples collected in May (dry season) and October (rainy season), respectively.

508 Erythromycin is a macrolide on the list of compounds of critical importance to human health
509 (WHO, 2017). A corresponding resistance gene, *erm*B, was frequently detected in multidrug-
510 resistant bacterial strains characterized in a previous campaign conducted on an identical
511 section of the Vam Co River (Pham et al., 2018). Interestingly, the prevalence and distribution
512 patterns of this ARG in the sediment samples differed significantly from the results obtained
513 for *intI*1 and *sul*2 (Figure 4). The prevalence is opposite, with a significantly higher copy
514 number during the wet season (October) than during the dry season, with a maximum of
515 1.06×10^5 copies/g in sample SL1 (Table SI-8). During the dry season (May), only SL-3 and
516 SL-6 showed quantifiable values, with 4.31×10^3 and 7.33×10^3 copies/g, respectively. The
517 relative proportions of 16S rDNA genes were highest in October among the microbial
518 communities populating the river sediments, with a maximum of 0.27% for SL-4 (Table 1).
519 The seasonal trend was also strong among water samples, but with significantly lower copy
520 numbers than those measured for *intI*1 and *sul*2. SL-6 and SL-7 had the highest copy numbers
521 for both bank and media samples, with values ranging from 368 to 6845 copies/mL (Table SI-
522 8). Like many other *erm* genes, *erm*B, is considered one of the most prevalent resistance genes
523 in environmental microorganisms (Pham et al., 2018). Recently, *erm*B was detected at *ca.* 10^4
524 copies/gram of sediment, and 10^2-10^3 copies/ml in wastewater samples (Sabri et al., 2020).
525 This erythromycin resistance gene was found among the bacterial microflora populating the
526 digestive tract of *Penaeus vannamei* specimens purchased from markets (Liu et al., 2019). In
527 the present study, the strong seasonal trend shown by this ARG may indicate that the use of
528 this class of BAs was limited to the rainy season, when the risk of bacterial infections is
529 highest. Interestingly, only two sediment samples collected in May had quantifiable amounts
530 of *erm*B genes. The reasons why the prevalence of this gene was reduced in the sediment
531 samples collected in May could not be inferred from our data set. Nevertheless, previous
532 studies have shown that this ARG can be easily removed from a microbial community, as is
533 the case during biological treatment (Di Cesare et al., 2016).
534 BLA-*oxa*1 enzymes compose one of the major plasmid-mediated β-lactamases implicated in
535 amoxicillin-clavulanic acid resistance. Among these genes, class D carbapenemases consist of
536 OXA-type β-lactamases, the most versatile family of β-lactamases (Pham et al., 2018). As a
537 recall, carbapenems are used currently as last-line ABs to treat resistant bacterial infections
538 and are threatened by β-lactamases (WHO, 2017). Our results showed that copy numbers of
539 this class of ABs, considered as "antibiotics of last resort", were found in all river
540 compartments. In the sediment samples, results showed a marked seasonal pattern closely
541 related to the ones observed for *sul*2 and *intI*1 (Figure 5). Copy numbers measured during the

542 dry season were generally the highest, with a maximum of 2.19×10^5 copies/g in SL-1. Note
543 that just as it was the case with *sul*2 and *intI*1, high amounts were also measured in samples
544 from the Vam Co River, with 1.66×10^5 copies/g in SL-9. Except for SL-1 (0.23%), the relative
545 proportions calculated based on 16S RNA genes showed little local and seasonal variation,
546 with an average of 0.08% and 0.06% for May and October, respectively (Table 1). These
547 numbers indicated that the river sediment composed a stable reservoir for this ARG, albeit
548 showing low prevalence among the bacterial communities.
549 BLA-*oxa1* copy numbers measured in water samples were highest during the rainy season and
550 in samples collected near the river bank. Samples SL-4, SL-7, and SL-9 stood out with amounts
551 near 5×10^3 copies/mL. Samples collected in the middle of the river had a more homogeneous
552 pattern, with lower copy numbers, ranging from 4.12×10^2 (SL-2) to 3.74×10^3 (SL-8). In terms
553 of copy number, this ARG showed a seasonal fluctuation of low amplitude, similar to that
554 demonstrated by *erm*B. The relative proportions of BLA-*oxa1* to 16S rDNA genes revealed a
555 seasonal pattern, with higher proportions in May than in October (Table 1). Sample SL-7 had
556 a significant 3.02% of this ARG in the river water system. The persistent presence of the broad-
557 spectrum BLA-*oxa1* resistance genes, albeit in low amounts in Mekong Delta surface waters,
558 is of great concern. The higher average prevalence of this gene during the dry season cannot
559 be explained solely by hypothetical illegal use of carbapenems in aquaculture activities, as
560 these compounds have never been approved for farming in any country in the world (Bonardi
561 & Pitino, 2019). The prevalence in SL-9 could reflect the use of this class of ABs in other
562 human activities, for example in hospitals located upstream of the geographical area of interest
563 (Hoa et al., 2019). This constant presence induces a memory effect in these natural
564 compartments, which is likely to preserve and eventually transmit resistance throughout the
565 ecosystem. The most likely explanation for this memory effect is co-occurrence with other
566 ARGs, as well as metal and disinfection resistance genes (Di Cesare et al., 2016). Uncontrolled
567 use of other antibiotics possibly induces selection pressure favorable to the preservation of
568 mobile genetic elements, jointly carrying the genes responsible for carbapenem resistance
569 (Hamza et al., 2020).
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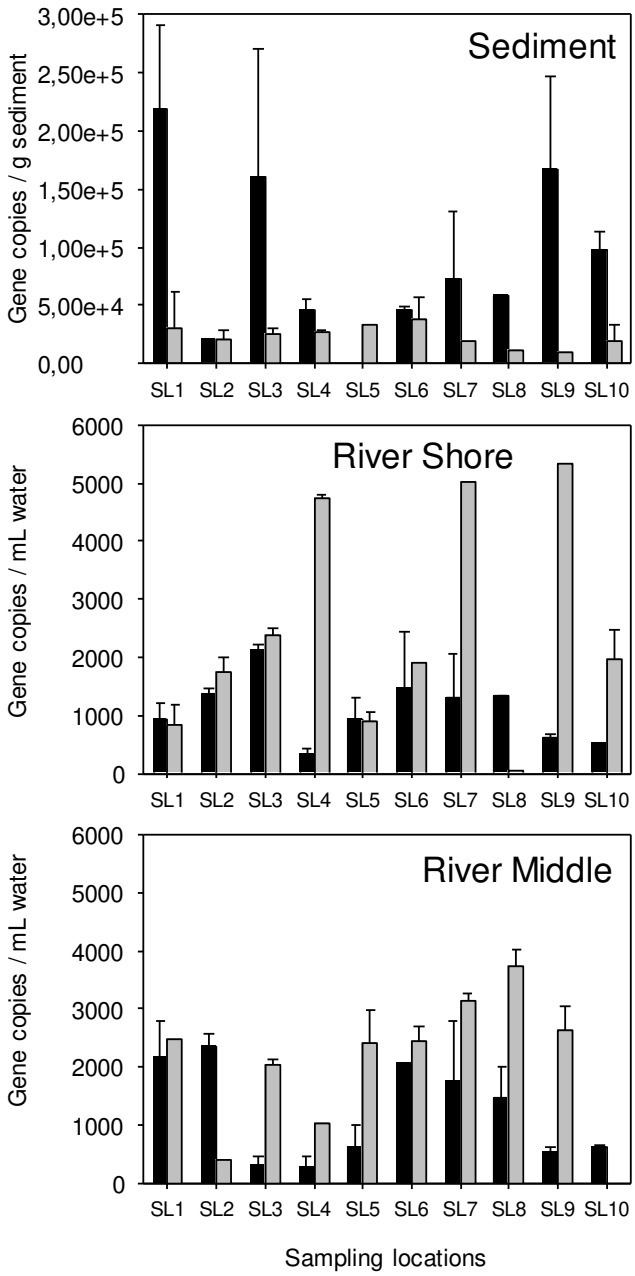

Figure 5: Copies of the BLA-*oxa*1 gene quantified in river sediment (top), river shore (middle), and mid-river (bottom) samples. Black and gray bars: samples collected in May (dry season) and October (rainy season), respectively.

4. CONCLUSIONS

Aquaculture-related activities continue to expand in Vietnam, and the outlook is favorable for shrimp production (Khanh Nguyen et al., 2019). However, the massive increase in area dedicated to shrimp monoculture also increases the risk of large-scale bacterial outbreaks. Massive use of ABs, even those considered a possible last resort, only partially mitigates this problem. Side effects of the use of these products include the appearance of large quantities of mobile genetic elements associated with resistance genes among the bacterial communities populating the culture ponds.

There is a growing awareness that these genetic elements, such as CL1s, contribute to the spread of ARGs in ecosystems adjacent to those affected by agricultural operations. In the present study, large spatial and seasonal variations were observed for ARGs and Class I integrons in the Mekong Delta river system. The results showed that the apparent distribution of ARGs and CL1s in the river system was not uniform. The prevalence of the quantified genes differed both in habitat (water vs. sediment) and in time (dry vs. wet season). The distribution of small tributaries and outfalls of cultivation basins could, logically, explain the spatial variability of ARGs, and particularly that observed in shoreline samples. A more precise location of the cultivation basins and their tributaries, as well as any other human activity involving the use of RGWs, would certainly refine the understanding of the system. In addition, seasonal variability results from the action of environmental parameters that will require more attention in the future. These parameters are likely to favor both the dispersal and persistence of the resistome in the Mekong Delta tributaries. Strong currents and tides may have played a key role in the observed seasonal decrease for resistance genes that emerged during the rainy season. Other environmental variables, such as high organic matter loads, may also have played a role (Su et al., 2020). In addition to these environmental factors, there are logically human factors, as shrimp farming is a labor-intensive activity, resulting in a high prevalence of multidrug-resistant strains among workers. Finally, the persistent presence of ARGs and CL1s along the Vam Co River and its tributary confirmed that water and sediments are routes of dissemination, highlighting the urgent development of alternative agricultural practices.

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6. DATA ACCESSIBILITY

Full data and metadata sets are available on Zenodo: https://doi.org/10.5281/zenodo.5727167

7. CONFLICT OF INTEREST DISCLOSURE

The authors of this article declare that they have no financial conflict of interest with the content of this article.

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