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.

HIGHLIGHTS

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

ABSTRACT

Aquaculture activities are steadily expanding in Vietnam, covering an estimated 700,000 ha, with 89% of these culture ponds located in the Mekong Delta. Since 2009, large-scale bacterial epidemics have spread in response to this intensive cultivation. Antibiotics, even those considered as a last resort, have only partially mitigated this problem. In turn, the side effects of the massive use of these chemicals include the appearance of mobile genetic elements associated with antibiotic resistance genes (ARGs). The large-scale emergence of a diverse bacterial resistome, along with severe economic losses, has posed significant health risks to local residents. In this study, the seasonal and spatial distributions of the class I integrase (CL1) *intl1* and the ARGs *sul2* (sulfonamide), *BLA-oxa1* (β -lactams), and *ermB* (erythromycin) were quantified from water and sediment samples collected during two consecutive seasons along the Vam Co River and its tributary (Long An province, Vietnam). The results showed that CL1 was present in all river compartments, reaching 2.98×10^4 copies/mL and 1.07×10^6 copies/g of sediment, respectively. The highest relative copy abundances to the 16S rDNA gene were measured in water samples, with up to 3.02% for *BLA-oxa1*, followed by *sul2* (1.16%) and *ermB* (0.46%). Strong seasonal (dry season vs. rainy season) and spatial patterns were recorded for all resistance genes. Higher amounts of ARGs in river water could be associated with higher antibiotic use during the rainy season. In contrast, higher amounts of ARGs were recorded in river sediments during the dry season, making this habitat a potential reservoir of transient genes. Finally, the observations made in this study allowed us to clarify the environmental and anthropogenic influences that may favor the dispersal and persistence of ARGs in this riverine ecosystem.

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,

pleuromutilines, lincosamides, sulfonamides, and diaminopyrimidine (trimethoprim) (MARD, 2016, in Lulijwa et al., 2019). However, accurate data on antimicrobial sales and use are difficult to obtain because antibiotics are available over the counter and can be of variable quality (Tran et al., 2018). Due to limited access to diagnostic services, inadequate instructions for use, overuse, and poor education, treatment of an infection cannot be accurately targeted, with the risk of choosing an inappropriate or ineffective product (Lulijwa et al., 2019; Thornber et al., 2020). Over the past decade, the overuse of ABs has been identified as one of the major factors driving the increase in AB-resistant bacteria. The large-scale emergence of this bacterial resistome in aquatic habitats may not only result in severe economic losses, but also pose significant health risks to local communities. The spread of antibiotic resistance genes (ARGs) in potential and clinically relevant human and animal pathogens is hindered by local culture techniques and the use of ABs, even at low concentrations (Wistrand-Yuen et al., 2018). Increasing amounts of literature articles have described the presence of ARGs in communities involved in aquaculture practices and the products themselves. For example, high levels of the *mcr-1* gene conferring resistance to colistin in *Escherichia coli* have been detected within the microbiome of people living in aquaculture areas in China (Shen et al., 2018). Erythromycin resistance genes were recently detected by culture-independent techniques in commercial shrimp purchased from aquatic markets (Liu et al., 2019). In a previous study, we isolated and identified multidrug-resistant bacteria affiliated with the genera *Aeromonas*, *Acinetobacter*, *Citrobacter*, *Escherichia*, and *Klebsiella* from the Vam Co River (Long An Province, Viet Nam), a river receiving several channels of effluent from intensive shrimp farms (Pham et al., 2018). Sequencing of 41 plasmid-transmitted ARGs showed the presence of a wide range of ABs, including aminoglycosides, OXA-type β -lactams, chloramphenicol, fluoroquinolone, macrolides, rifampicin, tetracycline, trimethoprim and sulfonamide. The association of ARGs with mobile genetic elements that enable their dissemination has been demonstrated worldwide (Gillings et al., 2008; Gao et al., 2012; Fang et al., 2021). Previous studies in the Mekong Delta have shown, for example, that *sul2* genes have been detected in association with integrons and plasmids (Pham et al., 2018). Among all mobile genetic elements, Class 1 integrons are now considered key players in the dissemination of ARGs, as they can easily acquire and express genes inserted into their structure as gene cassettes, expressed under a common promoter. These genetic elements are usually hosted on plasmids and transposons facilitating horizontal gene transfer (Preena et al., 2020). As shown by Gillings et al. (2015), Class 1 integrons provide a good measure of the general level of antibiotic resistance genes. For example, recent studies

have shown that the relative abundance of the *int11* 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). *sul2* (sulfonamide), *BLA-oxa1* (β -lactams), *ermB* (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

dragon fruit), with the presence of two major industrial parks and a regional hospital (Table SI-1).

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

2.2 Molecular analysis

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

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/m², with maxima reaching up to 200 animals/m² (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 ($> 2.00 \times 10^6$ 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

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

3.3 Quantitation of Class I integrons (CL1s)

The results of the quantitative analysis are shown in Figure 2 and the normalization ratios of *intI1* copies to 16S rDNA are shown in Table 1. The complete quantitative data are provided in Table SI-6. The results show that *intI1* was recovered consistently and frequently in large quantities in all river samples. In the sediment samples, the highest values were found among samples collected during the dry season, indicating a high seasonal pattern, reaching 1.07×10^6 copies/mL in sample SL-9, an order of magnitude higher than the lowest amounts measured in sample SL-2 (Table SI-6). Sediment samples collected during the rainy season showed less variation, with only about a threefold difference between samples SL-4 (3.70×10^4 copies/mL) and SL-1 (1.98×10^5 copies/mL). These values are comparable to those recorded in river sediments by studies conducted worldwide (Koczure et al., 2016; Sabri et al., 2020). However, the relative proportions of cells carrying this gene-capture system in the sediments were lower (0.30% and 0.27% on average for May and October, respectively) (Table 1) than those measured in other river systems (Amos et al., 2018).

The water samples showed strong seasonal fluctuations. Only a few samples, such as SL-3 with 1.60×10^4 copies/mL (Table SI-6), had nearly identical copy numbers between the dry and rainy 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.

Figure 2: Copies of the *intI1* 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.

Interestingly, relative proportions of the *intI1* gene copies (normalized to 16S rDNA gene copies) followed the same seasonal pattern, with an increase in all water samples from May to October (Table 1). This relative proportion increased in average from 0.38 % to 1.26% for samples taken near the river shore, and from 0.22 % to 1.60% for those collected in the middle of the river. Samples SL-9 taken upstream of the junction with its tributary showed the highest ratio (2.29 %). The observed seasonal enrichment of bacteria carrying the CL1 gene capture system was interpreted as the result of a selective pressure exerted on local communities. Recent studies demonstrated that relative abundance of the *intI1* gene correlated significantly with the total concentrations of ABs in water and sediments (Chen et al., 2015). The seasonal increase observed in our study occurred concomitantly with the use of increasing amounts of ABs from August on in shrimp culture basins (Su et al., 2017; Pham et al., 2018). High rainfall events affect physical and chemical parameters of the pond water, such as temperature, salinity and pH, leading to deteriorating culture conditions. ABs are used in increasing quantities until the capture of mature animals, classically by the end of October. *intI1* gene copies are nevertheless three to four orders of magnitude lower than those found in Chinese static mariculture farms, a possible indication that river flow and tide currents may play an important role in the constant wash-out of ABs and CL1-bearing bacterial cells towards the ocean (Wang et al., 2019).

<i>intI1</i>	May 2017			October 2017				<i>sul2</i>	May 2017			October 2017		
	Edge	Middle	Sediment	Edge	Middle	Sediment			Edge	Middle	Sediment	Edge	Middle	Sediment
SL-1	0,58%	0,49%	0,31%	1,15%	2,01%	0,22%		SL-1	0,40%	0,28%	0,04%	0,93%	1,16%	0,05%
SL-2	0,46%	0,69%	0,15%	0,53%	1,43%	0,23%		SL-2	0,40%	0,52%	0,01%	0,95%	0,67%	0,06%
SL-3	0,72%	0,27%	0,22%	1,39%	1,64%	0,24%		SL-3	0,58%	0,20%	0,02%	0,83%	0,71%	0,04%
SL-4	0,25%	0,25%	0,31%	1,37%	1,21%	0,15%		SL-4	0,41%	0,25%	0,02%	0,73%	0,71%	0,02%
SL-5	0,40%	0,09%	0,24%	1,11%	1,23%	0,29%		SL-5	0,67%	0,26%	0,04%	0,49%	0,51%	0,03%
SL-6	0,95%	0,12%	0,34%	1,36%	1,57%	0,24%		SL-6	0,38%	0,26%	0,03%	0,37%	0,34%	0,04%
SL-7	0,17%	0,10%	0,33%	1,60%	1,73%	0,17%		SL-7	0,33%	0,32%	0,03%	0,34%	0,36%	0,04%
SL-8	0,13%	0,10%	0,36%	2,11%	1,31%	0,18%		SL-8	0,32%	0,28%	0,04%	0,92%	0,37%	0,03%
SL-9	0,16%	0,09%	0,63%	0,90%	2,29%	0,88%		SL-9	0,11%	0,13%	0,11%	0,35%	0,46%	0,09%
SL-10	0,04%	0,03%	0,15%	1,04%	ND	0,08%		SL-10	0,22%	0,13%	0,03%	0,26%	ND	0,00%
MEAN	0,38%	0,22%	0,30%	1,26%	1,60%	0,27%		MEAN	0,38%	0,26%	0,04%	0,62%	0,59%	0,04%
<i>ermB</i>	May 2017			October 2017				<i>oxa1</i>	May 2017			October 2017		
	Edge	Middle	Sediment	Edge	Middle	Sediment			Edge	Middle	Sediment	Edge	Middle	Sediment
SL-1	0,01%	0,01%	0,00%	0,04%	0,01%	0,12%		SL-1	0,11%	0,31%	0,23%	0,20%	0,22%	0,03%
SL-2	0,01%	0,02%	0,00%	0,01%	0,06%	0,04%		SL-2	0,19%	0,46%	0,02%	0,06%	0,19%	0,04%
SL-3	0,01%	0,04%	0,00%	0,01%	0,01%	0,06%		SL-3	0,10%	0,29%	0,08%	0,20%	0,17%	0,04%
SL-4	0,08%	0,01%	0,00%	0,01%	0,04%	0,27%		SL-4	0,27%	0,27%	0,04%	0,22%	0,32%	0,11%
SL-5	0,00%	0,00%	0,00%	0,01%	0,06%	0,09%		SL-5	0,17%	1,18%	ND	0,20%	0,62%	0,08%
SL-6	0,00%	0,01%	0,00%	0,02%	0,07%	0,06%		SL-6	1,84%	2,72%	0,03%	0,10%	0,45%	0,06%
SL-7	0,46%	0,20%	0,00%	0,06%	0,12%	0,06%		SL-7	3,02%	1,38%	0,06%	0,87%	0,53%	0,07%
SL-8	0,10%	0,00%	0,00%	0,02%	0,01%	0,13%		SL-8	1,57%	1,24%	0,04%	0,01%	0,87%	0,03%
SL-9	0,00%	0,03%	0,00%	0,01%	0,04%	0,19%		SL-9	1,13%	0,94%	0,10%	0,56%	0,73%	0,06%
SL-10	0,00%	0,05%	0,00%	0,07%	ND	0,11%		SL-10	0,26%	0,14%	0,78%	0,53%	ND	0,04%
MEAN	0,07%	0,04%	0,00%	0,03%	0,05%	0,11%		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 *intI1* 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 *sul2*, *ermB*, and *BLA-oxa1*. 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 *sul2* 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 *sul2* gene showed spatial and seasonal patterns closely related to those observed for the *intI1* 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 *sul2* showed no variation between the two seasons, with

only 0.04%. This result demonstrated that this gene was uniformly maintained over the long term in the sediments of both river systems, albeit in small amounts.

Copies of the *sul2* gene measured in water samples (shore) were highest in October, with significant local variability. SL-3 and SL-5 showed higher values only in May, with 1.29×10^4 copies/mL and 3.78×10^3 copies/mL, respectively. In October, SL-2 reached the highest copy number with 2.77×10^4 copies/mL. Mid-river water samples showed an identical seasonal pattern (Figure 3) with significantly higher copy numbers in October, particularly for SL-1 and SL-3, with 1.32×10^4 and 8.43×10^4 copies/mL, respectively. As indicated for *intI1*, SL-2 showed the highest copy number in May with 2.70×10^3 copies/mL. The relative proportions of *sul2* gene copies in water samples, normalized to 16S rDNA gene copies, showed seasonal variations (Table 1). These proportions averaged 0.38% and 0.26% for shoreline and middle samples collected in May, respectively, and increased to 0.62% and 0.59% in October. SL-1 (middle) had the highest relative proportion of *sul2* at 1.16%. As a reminder, this sample concomitantly showed a significant increase in the proportion of *intI1* genes, possibly indicating that a significant number of *sul2* genes were carried by mobile genetic elements, as previously observed in rivers and estuaries (Sabri et al., 2020; Singh et al., 2019; Leng et al., 2020). In a study conducted in (2015), Chen et al. showed a significant correlation between *intI1* gene abundance and dissolved AB concentrations, as well as with the abundance of *sul* genes, a possible consequence of their embedding in the same mobile genetic elements. The intensive use of sulfonamide in Viet Nam is confirmed by a 2013 study that showed that approximately 12 tons of sulfamethoxazole were discharged annually from the Mekong River into the South China Sea (Shimizu et al., 2013). These large quantities demonstrate the routine use of sulfonamides for mitigation of bacterial diseases affecting aquaculture activities. In addition, it is now recognized that bacterial strains carrying a *sul* gene also develop resistance to other ABs, such as streptomycin, ampicillin, and trimethoprim, accentuating the emergence of multidrug-resistant strains.

In the present study, the use of this class of antibiotics resulted in the prevalence of resistances with a high degree of spatial heterogeneity. The results so far have confirmed that sulfonamide resistances are one of the major classes of ARGs in riverine habitats affected by shrimp farm effluents. However, the spread of *sul2* in Mekong Delta rivers may also be correlated with other environmental factors. For example, Su et al. (2020) showed an excellent correlation between *sul2* gene copies and COD measured in the farm ponds. Tide and river flow can increase the dilution rate of bacterial cells carrying this ARG, and at the same time spread 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 3.5×10^7 to 6.5×10^{10} 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 *ermB* 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.

Erythromycin is a macrolide on the list of compounds of critical importance to human health (WHO, 2017). A corresponding resistance gene, *ermB*, was frequently detected in multidrug-resistant bacterial strains characterized in a previous campaign conducted on an identical section of the Vam Co River (Pham et al., 2018). Interestingly, the prevalence and distribution patterns of this ARG in the sediment samples differed significantly from the results obtained for *intI1* and *sul2* (Figure 4). The prevalence is opposite, with a significantly higher copy number during the wet season (October) than during the dry season, with a maximum of 1.06×10^5 copies/g in sample SL1 (Table SI-8). During the dry season (May), only SL-3 and SL-6 showed quantifiable values, with 4.31×10^3 and 7.33×10^3 copies/g, respectively. The relative proportions of 16S rDNA genes were highest in October among the microbial communities populating the river sediments, with a maximum of 0.27% for SL-4 (Table 1). The seasonal trend was also strong among water samples, but with significantly lower copy numbers than those measured for *intI1* and *sul2*. SL-6 and SL-7 had the highest copy numbers for both bank and media samples, with values ranging from 368 to 6845 copies/mL (Table SI-8). Like many other *erm* genes, *ermB*, is considered one of the most prevalent resistance genes in environmental microorganisms (Pham et al., 2018). Recently, *ermB* was detected at *ca.* 10^4 copies/gram of sediment, and 10^2 - 10^3 copies/ml in wastewater samples (Sabri et al., 2020). This erythromycin resistance gene was found among the bacterial microflora populating the digestive tract of *Penaeus vannamei* specimens purchased from markets (Liu et al., 2019). In the present study, the strong seasonal trend shown by this ARG may indicate that the use of this class of BAs was limited to the rainy season, when the risk of bacterial infections is highest. Interestingly, only two sediment samples collected in May had quantifiable amounts of *ermB* genes. The reasons why the prevalence of this gene was reduced in the sediment samples collected in May could not be inferred from our data set. Nevertheless, previous studies have shown that this ARG can be easily removed from a microbial community, as is the case during biological treatment (Di Cesare et al., 2016).

BLA-*oxa1* enzymes compose one of the major plasmid-mediated β -lactamases implicated in amoxicillin-clavulanic acid resistance. Among these genes, class D carbapenemases consist of OXA-type β -lactamases, the most versatile family of β -lactamases (Pham et al., 2018). As a recall, carbapenems are used currently as last-line ABs to treat resistant bacterial infections and are threatened by β -lactamases (WHO, 2017). Our results showed that copy numbers of this class of ABs, considered as “antibiotics of last resort”, were found in all river compartments. In the sediment samples, results showed a marked seasonal pattern closely related to the ones observed for *sul2* and *intI1* (Figure 5). Copy numbers measured during the

dry season were generally the highest, with a maximum of 2.19×10^5 copies/g in SL-1. Note that just as it was the case with *sul2* and *intI1*, high amounts were also measured in samples from the Vam Co River, with 1.66×10^5 copies/g in SL-9. Except for SL-1 (0.23%), the relative proportions calculated based on 16S RNA genes showed little local and seasonal variation, with an average of 0.08% and 0.06% for May and October, respectively (Table 1). These numbers indicated that the river sediment composed a stable reservoir for this ARG, albeit showing low prevalence among the bacterial communities.

BLA-*oxa1* copy numbers measured in water samples were highest during the rainy season and in samples collected near the river bank. Samples SL-4, SL-7, and SL-9 stood out with amounts near 5×10^3 copies/mL. Samples collected in the middle of the river had a more homogeneous pattern, with lower copy numbers, ranging from 4.12×10^2 (SL-2) to 3.74×10^3 (SL-8). In terms of copy number, this ARG showed a seasonal fluctuation of low amplitude, similar to that demonstrated by *ermB*. The relative proportions of BLA-*oxa1* to 16S rDNA genes revealed a seasonal pattern, with higher proportions in May than in October (Table 1). Sample SL-7 had a significant 3.02% of this ARG in the river water system. The persistent presence of the broad-spectrum BLA-*oxa1* resistance genes, albeit in low amounts in Mekong Delta surface waters, is of great concern. The higher average prevalence of this gene during the dry season cannot be explained solely by hypothetical illegal use of carbapenems in aquaculture activities, as these compounds have never been approved for farming in any country in the world (Bonardi & Pitino, 2019). The prevalence in SL-9 could reflect the use of this class of ABs in other human activities, for example in hospitals located upstream of the geographical area of interest (Hoa et al., 2019). This constant presence induces a memory effect in these natural compartments, which is likely to preserve and eventually transmit resistance throughout the ecosystem. The most likely explanation for this memory effect is co-occurrence with other ARGs, as well as metal and disinfection resistance genes (Di Cesare et al., 2016). Uncontrolled use of other antibiotics possibly induces selection pressure favorable to the preservation of mobile genetic elements, jointly carrying the genes responsible for carbapenem resistance (Hamza et al., 2020).

Figure 5: Copies of the BLA-*oxa1* 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.

5. ACKNOWLEDGEMENTS

This research was funded partly by the Cooperation and Development Center (CODEV) at EPFL, and by the Vietnam National University Ho Chi Minh City (VNU-HCM). We

acknowledge Ho To Thi Kha Mui, Tran Phuong Anh and Pham Ngoc Tu Anh from the VNU-HCM for their technical assistance.

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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