INVESTIGATION OF SELECTED ANTIBIOTIC RESIDUES IN MARINE AQUACULTURE WASTEWATER

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INSTITUTE FOR ADVANCED STUDIES
UNIVERSITY OF MALAYA
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INVESTIGATION OF SELECTED ANTIBIOTIC RESIDUES IN MARINE AQUACULTURE WASTEWATER

ABSTRACT

The occurrence and distribution of sulfonamides, macrolides, tetracyclines, fluoroquinolones, trimethoprim, lincomycin, carbadox and four resistance genes in bacteria (*tet*(M), *sul*1, *sul*2 and *sul*3) in surface waters collected from seven main aquaculture production states in Peninsular Malaysia were investigated. Among the analytes, tetracyclines, sulfonamides and fluoroquinolones were detected at 83%, 72% and 69% frequency, respectively. Twenty-three antibiotics were present in aquaculture farms at the concentration ranging from LOQ to 957685.1 ng/L and revealed a wide distribution of antibiotics in Malaysian aquaculture farms. Moreover, the occurrence of antibiotic resistance genes in bacteria for *tet*(M), *sul*1 and *sul*2 were detected in more than 90% of the sites, indicating their ubiquitous occurrence in Malaysian aquaculture farm. According to the calculated Risk Quotient, ciprofloxacin, enrofloxacin norfloxacin and lincomycin exceeded the value of 1, and posed a high ecological risk to the relevant aquatic microorganism in Kelantan, Perak Pahang and Johor Further assessment is needed to monitor the antibiotic residues in aquaculture systems to mitigate environmental antibiotic resistance and potential transmission to humans through the food chain.

Keywords: Antibiotic residues, Aquaculture, Resistance genes, Risk assessment

PENYIASATAN ANTIBIOTIK YANG DIPILIH DI AIR SISA AKUAKULTUR MARIN

ABSTRAK

Kemunculan dan pengedaran sulfonamida, makrolida, tetrasiklin, fluoroquinolones, trimethoprim, lincomycin, carbadox dan empat gen rintangan antibiotik diperolehi dalam bakteria (*tet*(M), *sul*1, *sul*2 dan *sul*3) telah disiasat di perairan permukaan yang dikumpulkan dari tujuh negeri pengeluaran akuakultur utama di Semenanjung Malaysia. Di antara analit, tetrasiklin, sulfonamida dan fluoroquinolones dikesan pada kekerapan 83%, 72% dan 69% masing-masing. Dua puluh tiga antibiotik terdapat dikesan di ladang akuakultur pada kepekatan antara <LOQ hingga 957685.1 ng/L dan menunjukkan sebaran antibiotik yang luas di ladang akuakultur Malaysia. Di samping itu, kemunculan gen rintangan antibiotik didalam bakteria untuk *tet*(M), *sul*1 dan *sul*2 didapati mengesan melebihi dari 90% tapak penyampelan, ini mebuktikan kewujudan gen di sekeliling ladang akuakultur Malaysia. Menurut Risk Quotient yang dikira, ciprofloxacin, enrofloxacin, norfloxacin dan lincomycin melebihi nilai 1, hal ini menimbulkan risiko ekologi yang tinggi terhadap mikroorganisma akuatik yang berkenaan di Kelantan, Perak, Pahang dan Johor. Penilaian yang lebih lanjut diperlukan untuk memantau residu antibiotik dalam sistem akuakultur untuk mengurangkan rintangan terhadap antibiotik di persekitaran dan potensi penularan kepada manusia melalui rantai makanan.

Kata kunci: Akuakultur, Gen rintangan, Penilaian Risiko, Sisa antibiotik

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LIST OF SYMBOLS AND ABBREVIATIONS

AHPND	:	Acute hepatopancreatic necrosis disease
AIZ	:	Aquaculture Industry Zone
ASEAN	:	The Association of Southeast Asian Nations
ARB	:	Antibiotic resistant bacteria
ARG	:	Antibiotic resistance gene
AZM	:	Azithromycin
BFT	:	Biofloc technology
CAC	:	Codex Alimentarius Commission
CAR	:	Carbadox
CIP	:	Ciprofloxacin
Copies/16S	:	Number of copies of ARG to 16S rRNA
CTAB	:	Cetyl-trimethylammonium bromide
CTC	:	Chlortetracycline
CTM	:	Clarithromycin
CTns	:	Conjugative transposons
DHPS	:	Dihydropteroate synthase
DNA	:	Deoxyribonucleic acid
DOF	:	Department of Fisheries
DOX	:	Doxycycline
e.g.	:	For example
EC50	:	Half maximal effective concentration of a drug
ENRO	:	Enrofloxacin
ESI	:	electrospray ionization
ETM	:	Erythromycin

EU	:	European
FAO	:	Food and Agriculture Organization
FDA	:	U.S Food and Drug Administration
g	:	Gram
h	:	Hour
HCl	:	Hydrochloric acid
HGT	:	Horizontal gene transfer
HLB	:	Hydrophilic-lipophilic balance
H_2O	:	Water
i.e.	:	That is
JEFCA	:	Joint FAO/WHO Food Standard program
LC50	:	Half maximal inhibitory concentration of a drug
LC-MS/MS	:	Liquid Chromatography with tandem mass spectrometry
LIN	:	Lincomycin
LOD	:	Limit of Detection
LOQ	:	Limit of quantification
M	:	Molar
m/z	:	Mass to charge ratio
MEC	:	Measured environmental concentration
mg	:	Milligram
MIN	:	Minocycline
min/L	:	Minute per liter
mL	:	Milliliter
mm	:	Millimeter
mol/L	:	Molar per Liter
mmol/L	:	Millimolar per liter

MRL	:	Maximal residue level
n	:	Number of samples
n.d.	:	Not detected
NaCl	:	Sodium Chloride
Na_2EDTA	:	Ethylenediamine tetraacetic acid disodium
NAL	:	Nalidixic acid
ng/L	:	Nanogram per Liter
ng/mL	:	Nanogram per milliliter
ng/µL	:	Nanogram per microliter
NKEA	:	National Key Economic Area
nM	:	Nanomolar
NOEC	:	No observed effect concentration
NOR	:	Norfloxacin
OFX	:	Ofloxacin
OTC	:	Oxytetracycline
PCR	:	Polymerase chain reaction
PEC	:	Predicted environmental concentration
pH	:	Power of hydrogen
PNEC	:	Predicted no-effect concentration
PVP	:	Polyvinyl pyrrolidone
qPCR	:	Quantitative polymerase chain reaction
R^2	:	R-Squared / the coefficient of determination
RAS	:	Recirculating aquaculture system
RQ	:	Risk Quotient
rRNA	:	Ribosomal ribonucleic acid
rpm	:	Revolutions per minutes

RSD	:	Relative standard deviations
RTM	:	Roxitromycim
s	:	Second
SMA	:	Sulfadimethoxine
SMR	:	Sulfamerazine
SMT	:	Sulfamethazine
SMX	:	Sulfamethoxazole
SMZ	:	Sulfamethizole
SPE	:	Solid phase extraction
SPD	:	Sulfapyridine
SRM	:	Selected reaction monitoring
STP	:	Sewage treatment plant
STZ	:	Sulfathiazole
sul	:	Sulfonamide (for resistance gene)
TE	:	Tris- Ethylenediamine tetraacetic acid
tet	:	Tetracycline (for resistance gene)
TC	:	Tetracycline (for concentration)
TMP	:	Trimethoprim
Tris Base	:	Tris [hydroxymethyl] aminomethane
tRNA	:	Transfer ribonucleic acid
TYL	:	Tylosin
µL	:	Microliter
µm	:	Micrometre/Micron
US	:	United States
USA	:	United Stated America
USD	:	United States Dollar

v/v	:	Volume per volume
VMD		Veterinary Medicines Directorate
WHO	:	World Health Organization
%	:	percentage
°C	:	Degree Celsius

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Seafood is one of the primary sources of protein for more than 3.3 billion people in global. The annual consumption of seafood in global has increased from 9 kg in 1961 to 20.5 kg in 2018 as population growth (FAO, 2020). In order to fulfill the food demands of the growing human population, aquaculture becomes the solution provider to sustain the demand for seafood. In 2018, the global aquaculture production has reached 82.1 million tonnes with a value estimated at USD 250 billion with more than half of the amount was consumed by human (FAO, 2020). In Malaysia, the aquaculture production in Malaysia has increased 93-fold over the past 60 years between 1950 to 2019 and Malaysia was ranked as 15th top aquaculture producer globally in 2014 (DOF, 2019; FAO, 2016a).

In order to achieve sustainable food production and security, the aquaculture system has shifted to intensive system to maximize and speed up the growth of seafood for high productivity (FAO, 2016b). However, the intensification has often resulted in poor managing and monitoring and thus led to poor water quality in the farm, which subsequently causes the outbreak of disease. This has greatly affected the food quality, rate of production and development of aquaculture (Lee & Wee, 2014). With this situation, farmer tends to rely on the use of antibiotic and others supplements to control the condition (Watt et al., 2017) but this also results in indiscriminately misuse of antibiotic in aquaculture, especially in the developing countries where lack of legislation and regulation to monitor the usage of antibiotic (Chuah et al., 2016). The regulation on the use of antibiotic is varied in countries as each country have different distribution and standards to assess the levels of pollutions. In Malaysia, there is regulation and guidelines (Poison Act, 1952; Animal Act, 1953; Fisheries Act, 1985) but were only in place for the type of antibiotic allow to be used in livestock and veterinary and monitor

the maximal residue level of antibiotic presence in animal food but not in aquaculture. (Mohamed et al., 2000). Therefore, the sustainability of aquaculture in Malaysia is not assured.

Aquaculture has been identified as a potential source of antibiotic pollution in environment and serves as hotspot in promoting the emergence and dissemination of antibiotic resistance gene (ARG) and antibiotic resistant bacteria (ARB) (de Jesus Gaffney et al., 2016; Miranda et al., 2018). In aquaculture, antibiotic is commonly use as growth promoter and prophylaxis treatment to combat bacterial infection by directly subjected to the water or added to the feed (Chuah et al., 2016). In either way, antibiotic may retain in the seafood muscle or leach to the environment. Concern has arisen over the impact of residual antibiotic to the microbial community in the ecosystem, as well as the ARG and resistant bacteria brought up to table (WHO, 2018). Studies shown that exposure to traces of antibiotic can triggers the selection pressure on bacteria or pathogen to acquire resistance genes thru mutation or horizontal gene transfer (HGT) in the environment (Gao et al., 2012b; Rodrigues-Mozaz et al., 2015; Xu et al., 2015) and persist in the aquatic environment even the selection pressure has ceased (Tamminen et al., 2011). Seafood product also can act as reservoir of pathogen and bacteria which may has acquired resistance gene from aquaculture farm (Furushita et al., 2003; Watt et al., 2017). Thereby, there is a potential risk in spreading ARGs from aquaculture to human. Irrational use of antibiotic may have led to a chain reaction impact from environment to human. Therefore, it is essential to understand the relationship between antibiotic pollution and ARGs derived from aquaculture to help in reduce the emergence and spread of antibiotic residue and resistance.

In Asia countries, several studies have reported the occurrence of antibiotic in the environment, especially China (Shimizu et al., 2013; Xiong et al., 2015 Hossain et al.,

2017; Lai et al., 2018; Yan et al., 2018). However, most of the studies in Malaysia were focus on the distribution and occurrence of antibiotic susceptibility profile, ARG and ARB in aquaculture product (Chuah et al., 2016; Sing et al., 2016; Tan et al., 2017; Letchumanan et al., 2019). Studies in aquaculture farms on antibiotic residues, resistance genes and their environmental risks remained scarce in Malaysia. Hence, aquaculture farms from seven main aquaculture production states in Malaysia were examined to give us a more insightful information on the concentration and composition of antibiotic residues present in the marine aquaculture environment and understand the potential risk of antibiotics to the Malaysia environment.

CHAPTER 2: LITERATURE REVIEW

2.1 Aquaculture

Aquaculture is an activity to raise aquatic animal for the purpose of human consumption. The development of aquaculture was slow in ancient time due to the sufficient supplies of fisheries from nature and lack of information and knowledge on marine terrain and aquatic organism (Beveridge & Little, 2002). In between 14th to 19th century, aquaculture has received attention and started to develop modern aquaculture thru the knowledge of sciences due to the rise of biological sciences. Since then, aquaculture industry has started to grow (Ahmed & Thompson, 2019).

Seafood has growth recognition for its high quality and easily digested protein as well as riches of essential mineral and high quality of fatty acid. Thus, it has become the crucial source of animal protein in global diet (High Level Panel of Experts, 2014). According to Food and Agriculture Organization (FAO) (2020), fish has contributed 20% of animal protein intake to approximate 3.3 billion people in 2017 and the per capita food fish consumption in global has increased 3-fold for over six decades. As the population continues to grow, the global food fish consumption estimated to continue increase and putting pressure on the sustainability of fisheries. This condition resulted in overexploited the wild capture fisheries which the production has remained stagnant for since the late of 1980s (FAO, 2020). Therefore, aquaculture has taken place to feed the growing human population and sustain the increasing demand of seafood. The term of "Blue Revolution" was then created to describe the rapid emergence and development of aquaculture industry in global (Ahmed & Thompson, 2019).

Although, the growth of world aquaculture has slowed down since 2000. Yet, aquaculture industry still continues to grow and become the fastest growing of animal protein than other major animal protein production sector due to the fisheries production

are more efficient in converting feed to high quality of animal protein (Hua et al., 2019). From 1950 to 2018, the global fisheries production has increased 9-fold (Figure 2.1). The ratio of world aquaculture production to capture production was 1:32 in 1950, which most of the seafood supplies were contributed by wild capture. In 2018, the global fish production has peaked at 179 million tonnes with 156 million tonnes were direct consumed by human. Of the 156 million tonnes, 82 million tonnes (52%) was from aquaculture with a value estimated at USD 250 billion (Figure 2.1, FAO, 2020).

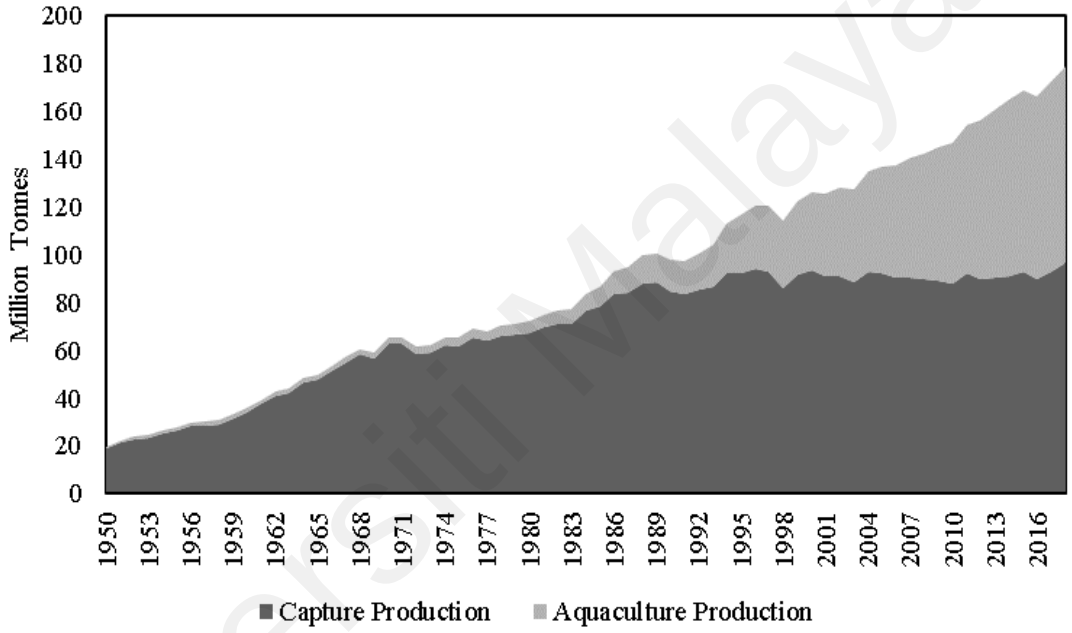

Figure 2.1: Global fisheries production from 1950 – 2018. (adapted from FAO, 2020)

As sciences and technology getting more advance, the system of aquaculture also improved from traditional small-scale culturing system to a modern large-scale culturing system. Until now, there are variety of aquaculture systems range from extensive to intensive and different of culture method such as open, semi-closed and closed system has been practiced around the world (De Silva, 2000; Bondad-Reantaso et al., 2005). In decade, the total species (including algae, reptiles and amphibians) farmed in aquaculture has increased from 472 to 598, where fin fish accounted 68% of

the total species, followed by mollusks (18%) and crustaceans (11%) (FAO, 2018). The switching of aquaculture system to intensive is a must to fulfill the rising food demand human population. High technology and intensive farming such as integrated farming, recirculating aquaculture system (RAS), Biofloc Technology (BFT) were developed in urbanization countries and practice throughout Southeast Asia (FAO, 2011; Jena et al., 2017). However, there are some farmer still practicing extensive system in rural area. Cage and pond culture which applying intensive integrated system are popular in developing countries (Petersen et al., 2002; Wartenberg et al., 2017; Van Huong et al., 2018). Open culture which practice in a pen, net, rack or sticks within a water body such as lake, estuary, coastal and offshore. Floating cages or net in the marine was often used to rear finfish, for instance, salmon in Norway, tilapia in Southeast Asia (Rico et al., 2014; Holen et al., 2018; Sumithra et al., 2019). Open culture with stick and rope are used to culture shell type species such as oyster and mussels. Whereas pond, tanks and raceways which built inland can be modified to semi or closed culture system. The different between semi-closed and closed culture system is water system. Closed culture system is a system without connection to the natural water body, together with continuous aeration system to generate oxygen. For instance, RAS, where the water was filtered and recirculating within the system without discharge wastewater to the natural water body, thus, it is known as an eco-friendly system. While a semi-closed culture system consist of inlet and outlet between the pond and local waterbody were built for water exchange, thus it is often located near to a water source such as river and estuary. Shrimp and prawn were commonly rear in an intensive pond culture system in developing countries due to the high value and short production cycle (Dierberg & Kiattisimkul, 1996; Anh et al., 2010; Bostock et al., 2010). However, the switch of intensification often come with outbreak of disease and negative effect to the environment. Despite the benefit of aquaculture brought to the global social and

economy, but the negative impact that causes by the aquaculture activities could not be ignored.

2.2 Aquaculture in Malaysia

In developing countries, Asian region dominated the global aquaculture industry with the highest production and distribution of aquaculture food fish followed by Americas, Europe and Africa region. A total land area of 3298472 km^2 which made up with 0.37% of water, as well as a total coastline of 4675 km, has given Malaysia an advantage - a natural source of fish and aquatics organism. For decade, fishery sector has been a main supply of animal protein to Malaysian due to the geographical location of Malaysia. With the increasing of income and health awareness, demand for fish and fish products have increased. In 2016, the per capita consumption of fish has reached 59 kg, which made Malaysian one of the highest consumer of fish product in the world (FAO, 2019). Despite the reliance of imported food, Malaysia still in good self-sufficiency level with 94.7 % in term of fish food (Carvalho, 2018).

From 1950 – 2019, the aquaculture production in Malaysia has significantly increased 95-fold (Figure 2.2) (DOF, 2019). In 2014, Malaysia ranked as the 15th highest aquaculture producer in the world (FAO, 2016a). By 2019, the aquaculture production has reached 412 thousand tonnes with estimated value of USD 817 million, which has remained the same from 2016. Besides, Malaysia also known to be the major producer of seaweed and marine wild capture which ranked as the seventh and 11th in the world respectively (FAO, 2018). As the aquaculture industry continue growing, Malaysian government has recognized the aquaculture industry as a potential sector to strengthen national economy. Malaysia is categorized as a net importer in fishery product (FAO, 2019). In 2017, Malaysia has imported fish and fish product at value USD 977 million, which higher than exported fishery product (USD 714 million) (FAO,

2019). Thus, to transform aquaculture industry into a higher income and sustainable industry, Malaysia government has formulated National Agro-Food policy 2011-2020 and organized few programs: National Key Economic Area (NKEA) and Aquaculture Industry Zone (AIZ) (Othman et al., 2017). By 2020, the transformation is expecting that could produce 1443 million tonnes which worth of RM 12962 million (USD 3346 million) and increase the contribution up to 50% of the total fish production (Othman et al., 2017). Moreover, with the development and expanding of aquaculture industry in the nation, opportunity of employment and income were also generated and provided to the citizen, especially in rural area. In 2019, an estimated total number of 162419 people were involved in fishery while 35824 people were engaged in aquaculture sector (DOF, 2019).

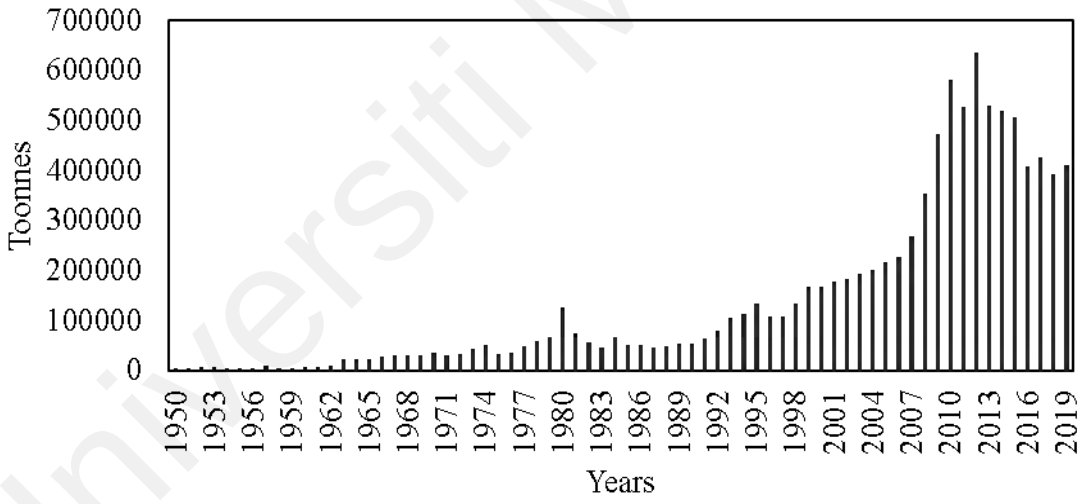

Figure 2.2: Malaysian aquaculture production from 1950 – 2019. (adapted from DOF, 2019)

As the wild marine production has exploited for long time in Malaysia, government come up with strategies by promoting intensive large-scale farming in shrimp and integrated cage net aquaculture for finfish (Othman, 1998; Othman et al., 2017). In 2019, a total of 4690302 hectares of land and water body has utilized for the development of aquaculture industry. Majority the aquaculture product excluded

seaweed was produced from Peninsular Malaysia, which contributed 86% to the total aquaculture production. The main states of total aquaculture production in Peninsular Malaysia were Perak, Penang Island, and Selangor. Perak was the main producer in total aquaculture production, it was the top in producing fish food from freshwater and second in marine and/or brackish water aquaculture with amount of 44551 tonnes and 28069 tonnes respectively. While Selangor was the second state that produces most freshwater fish food that amounted to 16620 tonnes. Less than 8000 tonnes was produced from the remaining 11 states in freshwater aquaculture. For marine and/or brackish water aquaculture, Penang Island was the main producer (32152 tonnes) followed by Perak (28069 tonnes), Johor (10554 tonnes) and Kedah (9021 tonnes). The remaining nine states were produced less than 6000 tonnes (DOF, 2019).

Shrimp was the major species produced in 2019, which contributed 13% to the total aquaculture production and 42% to the total marine aquaculture production. Hawaiian white shrimp (*Litopenaeus vannamei*) and black tiger shrimp (*Penaeus monodon*) were commonly cultured in Malaysia amounted to 38767 tonnes and 14632 tonnes at value USD 211 thousand and USD 99 thousand respectively (Figure 2.3) (DOF, 2019). Other than shrimp, various types of marine fish such as catfish, tilapia, carp, snakehead and others were also commonly cultured and exported out to neighbor countries. Among the marine fish, seabass and grouper was the main fish species farmed in Malaysia followed snapper, which constitute of 5% and 2% of total marine aquaculture production respectively (DOF, 2019). Cockles were once the main species produced and exported in marine aquaculture, which at its peak in 2002 with 78.7 thousand tonnes produced (DOF, 2002). In 15 years, the production of cockle has dropped dramatically to 14 thousand tonnes (DOF, 2019). Varies of culturation method were practiced for different species, but only three type of culture system: pond, cage and long line are commonly practiced in Malaysian marine aquaculture (DOF, 2019). Most of the shrimp

aquacultures were carried out in pond whereas fishes were mainly culture in floating cage on various water bodies such as estuary and coastal area (Figure 2.4). Long line culture system was only use for seaweeds. In 2019, there were 11506 ponds with 7494 ha and 99328 cages with 3 billion ha active in marine aquaculture, which the area of cages has rose by 3-fold in decade (DOF, 1999; DOF, 2019).

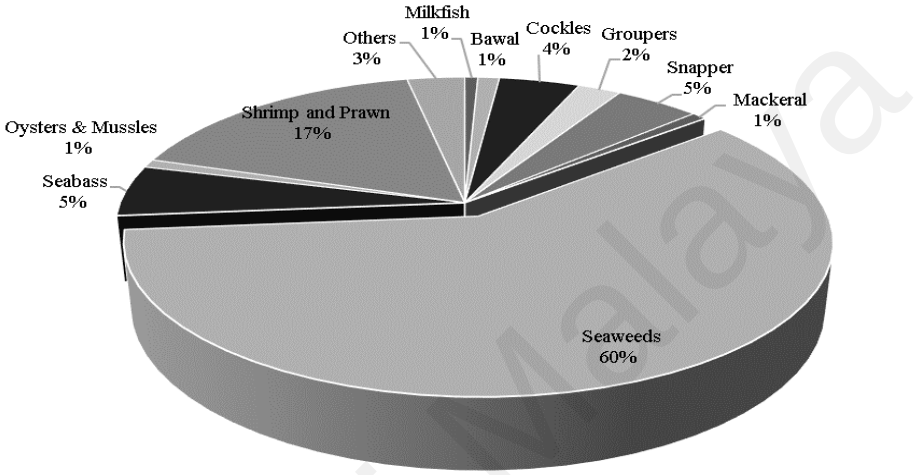

Figure 2.3: Marine aquaculture cultured species in Malaysia. (adapted from DOF, 2019)

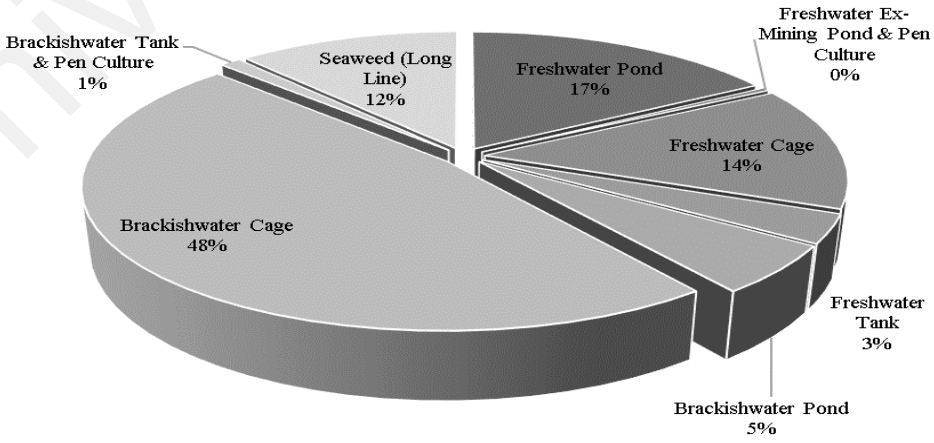

Figure 2.4: Culture system in Malaysian aquaculture. (adapted from DOF, 2019)

Freshwater aquaculture has contributed 104601 tonnes valued at USD 193 million contributed 25% of the total aquaculture production in 2019. The production has declined 1 % compared to 2014. In Malaysia, freshwater fishes were mostly cultured compared to marine fishes. Catfish was the dominant species that cultured in Malaysia freshwater aquaculture which accounted 46% of the total production of freshwater aquaculture followed by tilapia (36%) (Figure 2.5). While the other species such as Carp, *Labeo Rohita* were produced less than 9000 tonnes. Besides fish, shrimp are also cultured and produced in freshwater, but the production was relatively lower compared to marine shrimp, which amounted less than 400 tonnes. Pond, ex-mining pools and cages are the common culture system practiced in Malaysia freshwater aquaculture. The number of cages in freshwater aquaculture has increased 10 thousand in decade. But, the number of freshwater ponds has decreased to 34625 ponds (3966 hectares), which has reduced 4311 ponds in ten year, but the production was never affected (DOF, 2019).

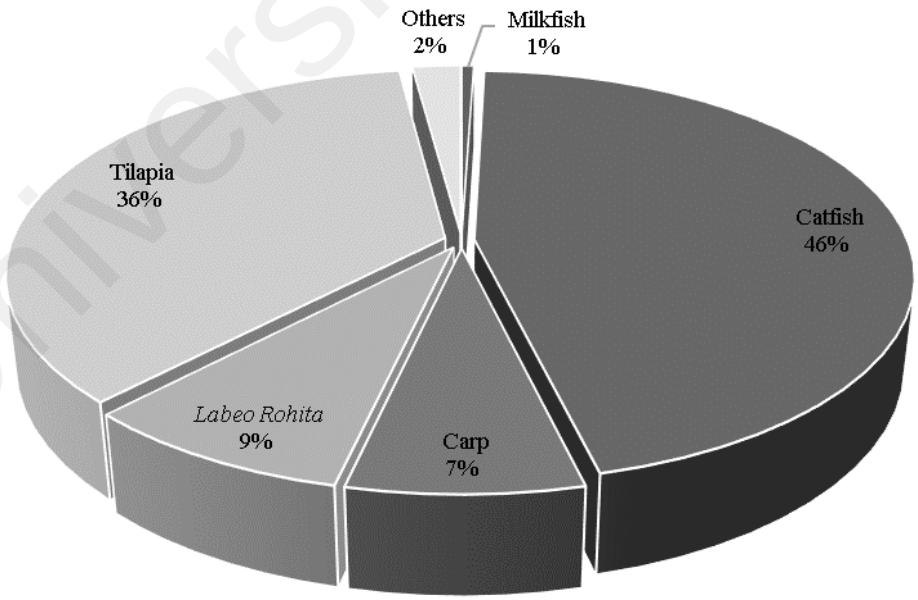

Figure 2.5: Freshwater aquaculture cultured species in Malaysia. (adapted from DOF, 2019)

2.3 Disease in Aquaculture and Usage of Antibiotic

Aquaculture culture system is continuing to increase in quantity and expand by convert to higher intensity system in order to fulfill the seafood demand. However, disease outbreak often happened in high intensity aquaculture farm due to the high stocking densities, poor management and water quality which results in stressing of the aquatic organism subsequently weaken the aquatic organism and causing them susceptible to the pathogen. More than USD 6 billion per annuum in worldwide has loses due to disease, which led to massive loss of aquatic animal (Stentiford et al, 2017).

Bacterial infection is frequently reported in shrimp and fish aquaculture farm and its greatly affecting the development of global aquaculture industry (Subasinghe et al., 2001). Besides infection causes by bacteria, virus is also posed a big threat to aquaculture followed by parasites and fungi. However, both parasites and fungi have not caused a huge loss in term of production and profit in aquaculture (Lee & Wee, 2014). *Aeromonas*, *Pseudomonas*, *Edwardsiella* and *Vibrio* are the common bacterial pathogen found in aquaculture farm from tropical countries (Yanong, 2004; Bondad-Reantaso, 2018). *Aeromonas*, *Pseudomonas* and *Streptococcus* are frequently isolated from freshwater aquatic organism, while, *Vibrio* and *Photobacterium* which are responsible to the causes of *vibriosis* disease is more common in marine aquaculture farm due to the characteristic of *Vibrio* and *Photobacterium* in marine environment which difficult to be avoided (Lee & Wee, 2014; Yanong, 2004 ; Haenen, 2017; Bondad-Reantaso, 2018). In 90s, virus infection was frequently reported in global shrimp industry such as baculovirus, white spot disease, and yellowhead disease which has brought a great impact to Thailand and Vietnam, causes loss of USD 650 million and USD100 million respectively (Chanratchakool et al., 2001). While mid-crop mortality syndrome, gill associated virus and taura syndrome virus were reported outbreaks in western countries in 90s (Australia, Panama, Costa Rica) (Lee & Wee, 2014). In 2009 a sudden outbreak

of new emergence bacteria disease - acute hepatopancreatic necrosis disease (AHPND) in China was caused by *V. parahaemolyticus* has collapsed shrimp industry across the Asia as the disease has spread to Vietnam, Thailand and Malaysia in 2010 and Mexico in 2013 (Lee & Wee, 2014; Nunan et al., 2014) that caused the production loss at estimated value at billion each year (Shinn et al., 2018). Until now, there may have disease occurring around Malaysia without our knowledge. In Malaysia, Department of Fisheries (DOF) does not has official records on the outbreak of diseases unless it causes devasting loss, thus, the data is at scarce. A general timeline of the outbreak disease in Malaysia through literature review was listed in Table 2.1.

Table 2.1: Timeline of diseases outbreak happened in Malaysia

Year	Type of outbreak
1989[a]	Disease outbreak
1990[b]	*Vibriosis* and S*treptococcus* outbreak
1994[c]	White Spot Syndrome
1997[b]	S*treptococcus agalactiiae*
1998[d]	Viral nervous necrosis
2000[e]	*Streptococcus agalactiiae*
2008[a, f]	Fungal disease, Viral nervous necrosis
2010[a, g]	AHPND, *Streptococcus* outbreak
2012[a]	Parasite infection
2013[h]	AHPND
2015[i]	AHPND, White spot syndrome

[a]. Lee & Wee (2014); [b]. Zamri-Saad et al. (2014); [c]. Oseko et al. (2006); [d]. Ransangan & Manin (2010); [e]. Siti-Zahrah et al. (2008); [f]. Ransangan et al., (2013); [g]. Abuseliana et al. (2010); [h]. The Star (2013); [i]. Whittaker (2015)

Various strategies have been established to control and minimize the loss of production and profit. For instance, prevention and treatment strategic by using vaccination or probiotic and adapting a biosecurity aquaculture system (Chuah et al., 2016; Chauhan & Singh, 2019). However, most of the farmer tends to look for a quick and costless solution to settle the bacteria disease in aquaculture which antibiotic is the first choice. Antibiotic is known to employs to treat disease in aquaculture but also to prevent the spread of infection by applied the antibiotic prophylactically as well as

feeding antibiotic as growth promotion. Among the class of antibiotic, tetracycline, fluoroquinolones, macrolide and sulfonamide coupled with trimethoprim are the most common antibiotic used in aquaculture (Figure 2.6), as they were frequently detected in water from aquaculture or water body that is near to aquaculture farm (Table 2.2).

Tetracyclines are known to be a class of antibiotic effective in against wide broad spectrum of bacteria in both Gram-positive and negative. Tetracyclines inhibit protein synthesis in bacteria by blocking the binding of aminoacyl- transfer ribonucleic acid (tRNA) molecules to 30S ribosomal subunit. and it has several semi-synthetic derivatives, which are oxytetracycline, tetracycline chlortetracycline, doxycycline and minocycline. Due to its availability in market, low toxicity and the cost is much lower compared to other broad-spectrum antibiotic (Treves-Brown, 2000; Suzuki & Hao, 2012), tetracyclines are widely use on human, plant and animal. It used to treat human skin and dental disease; used as growth promoter in livestock as well as used as prophylactic measures in plant and aquaculture (Chopra & Roberts, 2001; Thuy et al., 2011). Among the derivatives, oxytetracycline is widely used in shrimp and fish aquaculture. Moreover, it works efficiently in combating fish diseases: vibriosis (for example, *V. parahaemolyticus*) and furunculosis (for example, *Aeromonas salmonicida*) which can cause huge loss of profit to an aquaculture farm (Bermúdez-Almada & Espinosa-Plascencia, 2012). Other than oxytetracycline, other semi-derivatives – doxycycline and chlortetracycline were also used on aquaculture but not as common as oxytetracycline due to the high cost (Treves-Brown, 2000). The wide use of oxytetracycline has led to ubiquitous of oxytetracycline resistance bacteria in the aquaculture industry.

Sulfonamides – a synthetic antibiotic, has a large range of derivatives compound which are chemically related, and it inhibit the synthesis of folic acid by competing with

the substrate *p*-amino benzoic acid to bind with dihydropteroate synthase (DHPS) which is an enzyme involve in folic acid production pathway (Suzuki & Hao, 2012; Tačić et al., 2017). Sulfonamides has been widely used to treat bacterial diseases and protozoan infection in human, fish and livestock since they were the first modern antibiotic to be developed (Treves-Brown, 2000; Blahna et al., 2006). They are effectively in against fish diseases, such as *Aeromonas hydrophila*, *Edwardsilla tarda* and so on. The derivatives of sulfonamides commonly use in Asia aquaculture are sulfamethoxazole and sulfadiazine which happened detected frequently in aquaculture or environment surrounding the aquaculture farm (Le & Munekage, 2004; Suzuki & Hoa, 2012; Yuan et al., 2019). The common use of sulfonamide in aquaculture can be explained by the low cost of price, chemical stability and high environmental mobility but also it absorbs through gills which make the administration more feasible to the farmers (Treves-Brown, 2000; Suzuki & Hoa, 2012). However, the use of sulfonamide alone has reduced in aquaculture now due to the require use of doses left a little margin below of the toxicity and side effect as well as the development of bacterial resistance. But it is still commonly using together with diaminopyrimidine or also known as pyrimidine potentiators (Treves-Brown, 2000; Tačić et al., 2017). Diaminopyrimidine are often used in combination with sulfonamides to reach a synergistic effect (Wormser et al., 1982). As the combination potency of antibacterial is much greater than the potency of two antibiotics works separately. Both antibiotics acts as a competitive inhibitor in the synthesis of folinic acid pathway in bacteria, where sulfonamide inhibit the synthesis of folic acid while diaminopyrimidine inhibit the synthesis of folinic acid from folic acid. Besides, it works effective in combating bacteria due to the antibiotics in combination, which may delay the development of bacterial resistance, as bacteria will need to produce resistance for two antibiotics at the same time (Treves-Brown, 2000). Among the diaminopyrimidine antibiotics, trimethoprim was often use together with

sulfonamides, for example sulfamethoxazole and trimethoprim are widely used to treat disease in fish aquaculture in Vietnam (Rico et al., 2013; Phu et al., 2015). Both antibiotics are active in against bacteria disease in fish causes by *Vibrio*, *Aeromonas*, *Yersinia*, *Edwardsilla* but weak in against streptococci and *Pseudomonas* spp (Treves-Brown, 2000).

Fluoroquinolones are fully synthetic antibiotics that acts as an inhibitor to inhibit Deoxyribonucleic acid (DNA) synthesis by targeting DNA gyrase and topoisomerase IV which are essential for bacteria viability. It has developed a few generations with several fluorinated derivative: first generation (piromidic acid, oxolinic acid, nalidixic acid), second generation (ciprofloxacin, enrofloxacin, norfloxacin) and third generation (levofloxacin, ofloxacin). The first generation of fluoroquinolones were only limited to Gram-negative bacteria such as Enterobacteriaceae and some facultative bacteria. Modification was made on first generation and developed second generation which greatly improved the spectrum activity which included *Pseudomonas aeruginosa* and some Gram-positive bacteria, as well as increased activity against other Gram-negative bacteria (WHO, 1998; Pham et al., 2019). For instance, enrofloxacin is effective in against both Gram-negative (flavobacterium which caused columnaris disease, *Aeromonas* spp, vibriosis) and Gram-positive (Bondad-Reantaso et al., 2012; Trouchon & Lefebvre, 2016). In 1970s, first generation fluoroquinolones were widely used in Japanese aquaculture (Suzuki & Hoa, 2012). As second generation was developed, they have been extensive use in human and animals due to the increasing resistance of bacteria to other classes of antibiotic thus, they commonly serve as an alternative therapeutic measure (WHO, 1998). According to Thuy et al. (2011), ciprofloxacin, oxytetracycline and rifampicin is frequently use in shrimp larvae in Vietnam. Many studies shown that the ubiquitous occurrence of fluoroquinolones in aquaculture surface water and sediment showing that there is a change of fluoroquinolone use in Asia

aquaculture farm (Holmström et al., 2003; Le & Munekage, 2004; Takasu et al., 2011; Andrieu et al., 2015; Kim et al., 2017; Yuan et al., 2019; Han et al., 2020). However, fluoroquinolones have banned by U.S. Food and Drug Administration (FDA) to use in aquaculture as well as banned either enrofloxacin or ciprofloxacin, or both antibiotics in certain countries such as Vietnam, Taiwan, Brazil, China (Liu et al., 2017; Guidi et al., 2018; Lulijwa et al., 2019; Tsai et al., 2019). Yet, there is still detected with the present of enrofloxacin and ciprofloxacin in aquaculture surface water showing that illegal use of antibiotic is still exist (Le & Munekage, 2004, Lin et al., 2008; Andrieu et al., 2015; Zhong et al., 2018).

Macrolides and lincosamides have the same mechanism with tetracyclines which is inhibiting the synthesis of protein, but it is through the blocking of initiation of protein translation or blocking the binding of peptidyl tRNA in translocation step to the 50S ribosome (Kohanski et al., 2010). Macrolides are a medium spectrum antibiotic which actively in against Gram-positive bacteria such as *Staphylococcus* and *Streptococcus* sp. and Gram-negative cocci as well as anaerobic bacteria (*Brachyspira*, *Fusobacterium*, *Clostridium*). Besides, they also known to be effective in against diseases caused by *Mycoplasma* sp (European Medicines Agency, 2010). While lincosamides has activity against Gram-positive bacteria and many anaerobic bacteria (Serrano, 2005). There are some microorganisms (*Escherichia coli*, *Salmonella spp*, other Enterobacteriaceae and non-fastidious Gram-negative non-fermentative bacteria) that are intrinsically resistant to macrolides as the outer membrane prevents the macrolide to reach ribosomal in the cytoplasm (Vaara, 1993). Moreover, most of the seafood diseases were caused by Gram-negative bacteria thus, macrolide and lincosamides were less likely chosen to use in aquaculture compared to the others antibiotic class mentioned above. Only three antibiotics belongs to macrolides are commonly used in aquaculture – erythromycin, spiramycin and josamycin (Treves-Brown, 2000). In recent, Shiogiri et al. (2017) has

proposed the use of azithromycin as a potential therapeutic antibiotic to tilapia due to it greater effectiveness against Gram-negative bacteria as compared to erythromycin in terms of pharmacokinetic.

The global consumption of antibiotics in food animals in 2010 was estimated at 63151 tons and it is estimated a rise of 67% (105596 tonnes) usage of antibiotics by 2030. In 2010, the top five countries that contributed the usage of antibiotics in food animal production were China (23%), United States (US) (13%), Brazil (9%), Germany (3%) and India (3%). Antibiotic classes that commonly used in global livestock such as poultry, cow and pig, are β-lactam followed by tetracyclines, sulfonamides and macrolides (Van Boeckel et al., 2015). In Asia, tetracyclines and sulfonamides are the most common antibiotic used in animals which contributed 32% and 17% of the reported quantities of antibiotic used in animals by 17 Asia countries (Lavilla-Pitogo, 2017). However, the practice of consuming antibiotic in Malaysia livestock is different from the global and Asia, where macrolides was frequently used (191800 kg) followed by polypeptides (73800 kg), tetracycline (73800 kg), penicillin (61800 kg) and sulfonamide with trimethoprim (18000 kg) (Zakaria, 2017). According to the National Pharmaceutical Regulatory Agency, 688 veterinary drugs were registered, 67% antibiotic products were registered for livestock usage. Macrolides has the greatest number of products registered (84) followed by sulfonamide and sulfonamide together with trimethoprim (55), fluoroquinolone (54) and tetracycline (51) (Hassali et al., 2018).

However, the annual amount of antibiotics used on aquaculture in the world are scarce, as there are only few developed countries (e.g. Norway and Chile) monitoring the quantity of antibiotics used in fish but not crustaceans (The Norwegian Veterinary Institute, 2016; Miranda et al., 2018). In Norway, usage of antibiotics in aquaculture has reduced from 1 mg/kg to 0.34 mg/kg of fish, a total of 523.4 kg of antibiotic was

prescribed to salmon aquaculture in 2014. In a decade, the usage of antibiotics in Chile has increased tremendously from 143.2 tonnes to 382.5 tonnes (Miranda et al., 2018). Lozano et al. (2018) reported that 393.9 tons of antibiotics were used in salmon farming where florfenicol contributed 92.2%, oxytetracycline 6.7% and 1% from erythromycin and amoxicillin. While for crustaceans, there is no information on the usage of antibiotics, this may be due to most of the diseases encountered by crustaceans are mainly viruses. The recent bacteria disease that causes severe loss in shrimp is Vibriosis which is caused by *Vibrio* spp. Oxytetracycline and fluoroquinolones are commonly used to treat infected shrimp (Ibrahim et al., 2020). Lulijwa et al. (2019) revealed that 67 antibiotics were used in 11 major aquaculture production countries in the period of 2008 – 2018. Among the 11 countries, oxytetracycline, sulfadiazine and florfenicol were frequently used in 73% of the countries. While 55% were using sulfadimethoxine, erythromycin, amoxicillin and enrofloxacin. In Asia, a high variety of antibiotics were used in Vietnam and China aquaculture compared to Korea, Bangladesh, India and Philippines. While Thailand and Japan have reduced the usage of antibiotics in aquaculture. For Malaysia, there is no specific data on the use of antibiotics on aquaculture, but the amount of antibiotic sold/used in livestock has been recorded in DOF. In 2015, 532370 kg of total antibiotic was used as growth promoter and treatment in Malaysia where macrolide and tetracycline are the most antibiotic used in veterinary (Zakaria, 2017).

According to FDA (2020), only oxytetracycline, florfenicol, sulfamerazine and sulfadimethoxine combined with ormetoprim are approved to be used in aquaculture. Of course, different countries also have their own approved list of antibiotics used in aquaculture by their government authorities. For instance, Ministry of Agriculture of the People's Republic of China which only has 13 antibiotics are authorized for aquaculture use included doxycycline, enrofloxacin, florfenicol, flumequine, neomycin, norfloxacin,

oxolinic acid, sulfadiazine, sulfamethazine, sulfamethoxazole, sulfamonomethoxine, thiamphenicol and trimethoprim (Liu et al., 2017) whereas, in Norway, only allowed six type of antibiotics (florfenicol, flumequine, oxolinic acid, oxytetracycline, oxolinic acid-flumequine and a combination of sulfonamides with trimethoprim) are allowed to use in aquaculture (Burridge et al., 2010; Midtlyng et al., 2011). While in Malaysia, tetracycline, oxytetracycline, chlortetracycline, oxolinic acid, erythromycin, sulfonamide and sulfamerazine are allowed to be used with recommended maximal residual limit and withdrawal period in aquaculture industry (ASEAN, 2013). However, in 2017, FDA has rejected the entry of Malaysian shrimp to the US due to banned antibiotics being detected in food products, this indicated illegal use of banned antibiotics (FDA, 2019). Thus, establishment of a proper system to monitor the usage of antibiotics is critically important to study the antibiotic contamination from aquaculture to the environment which might be related to the emergence and spreading of ARGs and bacteria in the aquatic ecosystem.

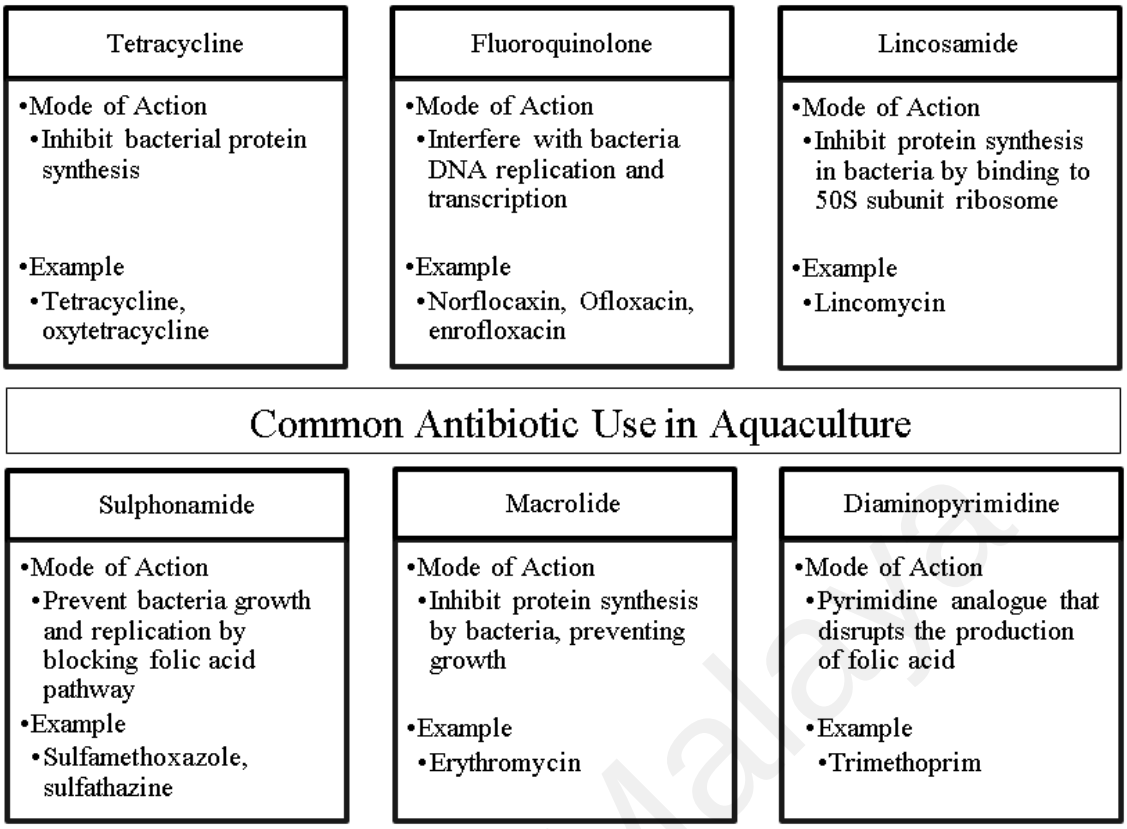

Figure 2.6: Common antibiotic uses in aquaculture and its mechanism.

2.4 Antibiotic Residue in Aquaculture

The rampant usage of antibiotic has led to the frequent presence of antibiotic in the environment and raised the public concern over antibiotics in the nature environment or water system. In recent studies, antibiotic was frequently found to be present in various water bodies (river, sewage water treatment, lake), plants and animals (Wang et al., 2017; Lye et al., 2019; Tran et al., 2019; Sabri et al., 2020). However, antibiotic that brought to the environment could be traced back to many source such as effluent from sewage wastewater treatment plant, pharmaceutical industrial production, run-off from anthropogenic activities (aquaculture and agriculture) and direct expose to environment during application of antibiotic to plant and animal.

Various studies have been carried out to investigate the level of antibiotic contamination in marine and freshwater aquaculture pond farm, mariculture, cage farm

along the river and the area around the aquaculture. Few studies detected antibiotics in aquaculture concentration at range from below limit of quantification (<LOQ) to 10^6 ng/L (Table 2.2 & Table 2.3). The composition of antibiotic used in both marine and freshwater aquaculture shown not much different to each other. The reported antibiotic in both aquacultures were comparable as sulfonamides (sulfamethoxazole, sulfadiazine, sulfamethazine) and quinolones (norfloxacin, ciprofloxacin, enrofloxacin) were frequently detected due to their mechanism work effectively in a wide broad spectrum of bacteria in both Gram-positive and negative. While tetracycline was frequently reported in marine aquaculture compared to freshwater aquaculture. Besides, it is noticeable that the concentration of antibiotic in freshwater aquacultures were generally detected higher than marine aquaculture. In general, the concentration of sulfornamides, tetracycline, quinolones and macrolides were detected ranged from <LOQ – 210 ng/L, <LOQ – 359 ng/L, <LOQ - 287 ng/L and <LOQ – 68.8 ng/L respectively. While for marine aquaculture, the detection was ranged from <LOQ – 291 ng/L, <LOQ - 158.6 ng/L, <LOQ - 190 and <LOQ – 45.8 ng/L respectively. Up to 10^3 ng/L of sulfadiazine, sulfamethazine, sulfamethoxazole, N-acetlysulfamethazine, oxytetracycline, enrofloxacin, erythromycin, florfenicol and lincomycin were detected in China and Vietnam freshwater aquaculture (Rico et al., 2014; Chen et al.,2018; Harada et al., 2018; Wang et al., 2018a; Zhong et al., 2018).While for marine aquaculture, only three studies from China (He et al., 2012; Chen et al., 2015) and Vietnam (Le & Munekage, 2004) was detected with extremely high concentration of oxytetracycline (15163 ng/L) and quinolone (up to 10^6 ng/L). Overall, antibiotics were found present with high concentration in freshwater aquaculture. The rate of water exchange in an aquaculture farm could have caused the high variation of concentration in freshwater and marine aquaculture. Freshwater aquaculture for instance, lake has poor water exchange which caused the stocking density is much higher than marine aquaculture where has stronger

water flow rate that caused strong dilution and fast dissipation rate to open sea (He et al., 2012). Some studies also suggested that seawater may cause fast degradation of certain antibiotic due to the formation of cations (Christian et al., 2003; Le & Munekage, 2004). However, marine aquacultures also can run in a close aquaculture system where a pond on land are fill with marine water. This system has poor water exchange rate and leads to the accumulation of antibiotic residues in the pond, for instance a shrimp pond in Vietnam has detected concentration up to 10^6 ng/L (Le & Munekage, 2004).

By comparing regionally, differ concentration levels were detected in different regions, and this could be explained by the practice of administration of antibiotics to different types of infected culture organisms or different types of culture system. In China aquaculture, fluoroquinolones and sulfonamides (5 out 6 cases) are the most frequently detected antibiotic. In general, the detection of antibiotic concentration in China aquaculture were ranged from <LOQ to 200 ng/L. Up to 10^3 ng/L of norfloxacin, tetracycline, sulfadiazine, sulfamethazine, lincomycin, florfenicol were reported in Pearl River, Beijiang River and Hailing Island where the locations are known to carry out anthropogenic activities. While high concentration of oxytetracycline were reported in Hailing Island at concentration range from 17.8 – 15163 ng/L. Vietnam, one of the top countries that use variety of antibiotic in aquaculture has detected high concentration of fluoroquinolones which reach up to 10^6 ng/L in shrimp aquaculture (Le & Munekage, 2004). In 2012 and 2016, the Ministry of Agriculture and Rural Development, Vietnam has banned enrofloxacin and ciprofloxacin for aquaculture use. However, detection of enrofloxacin and ciprofloxacin in catfish farms in the Mekong River at concentration ranged from not detected to 680 ng/L, indicating illegal use of these antibiotics. According to Ibrahim et al. (2020), Thailand has reduced the usage of antibiotics in aquaculture but there is still antibiotic report to be present in aquaculture and effluent of aquaculture in which erythromycin and tetracycline were detected from the effluent of

aquaculture with concentration <LOQ except oxytetracycline which range from <LOQ to 187 ng/L. High concentration of enrofloxacin and tetracycline were found in tilapia cage farm along the Tha Chin River ranged from <LOQ – 3050 ng/L. According to Rico et al. (2013; 2014) and Baoprasertkul et al. (2012), enrofloxacin, norfloxacin, tetracycline, oxytetracycline, amoxicillin, penicillin and sulfonamides with trimethoprim were frequently detected in Thailand aquaculture farm., indicated the common use of antibiotics in the period of 2012 – 2014. While, Portuguese, Taiwanese, Korean and Bangladesh aquaculture has detected most of the antibiotics at concentrations below 20 ng/L which is slightly higher than antibiotics present in lake and seawater. Among the four countries, sulfonamides were commonly used for aquaculture except Portugal. Whereas fluoroquinolones were not used in Bangladesh, this may be due to the cost of the antibiotic. For Europe and America, there is not many studies have reported antibiotic residue in aquaculture this may be due to the reduction of antibiotic usage in aquaculture and strict regulations, especially Norway and Chile (Sapkota et al., 2008; Midtlyng et al., 2011). To my best knowledge, there are only four countries that are Italy, France, Brazil and Chile were found present of antibiotic in aquaculture's sediment and aquatic product in past 15 years (Table 2.2 & Table 2.3). High concentration of florfenicol, flumequine, oxolinic acid and oxytetracycline were detected in Italy and France, this could be due to both Europe countries follows Norway's policy that those four antibiotics are authorized for aquaculture use. While for Brazil, only oxytetracycline and florfenicol are authorized to be use in aquaculture but the present of tetracycline indicated the illegal use of antibiotic (Monteiro et al., 2015; 2016). According to Sapkota et al. (2008), there is a significant reduction on antibiotic usage in Japanese aquaculture from 1990 - 2007, and until now there is no antibiotic has been reported in the present of Japanese aquaculture industry. Lulijwa et al. (2019) suggested that this could be Japan has followed the step of Norway by replacing the use

of antibiotic to vaccines or probiotic to treat and prevent the outburst of disease. Although many studies have reported the presence of antibiotics in different water bodies, there is a lack of studies on the source of the antibiotic residue from aquaculture especially in Malaysia.

Antibiotic can be directly added to water or indirectly by mixing with feed. An estimation of 75% of the antibiotics fed to the aquatic organisms are excreted back into the water in feces, as aquatic organisms do not metabolize antibiotic effectively (Zhang et al., 2018). Free antibiotic that does not consume by aquatic organisms together with those unmetabolized antibiotics may have leached or transported to environment and settle to the bed of water body or retain in the tissue of aquatic organism. The fate of the antibiotic ended in the tissue of aquatic organisms or in the environment, either roaming in aqueous water or adsorb to soil/sediment/particle. This will depend greatly on the physiochemical properties of the antibiotic and the environment condition they are in, the metabolized rate of antibiotic in different aquatic organism (species, size, growth stage) and the practice of antibiotic used (type and dosage) (Kim & Carlson, 2007; Kümmerer, 2009).

The partition coefficient of octanol/water (K_{ow}) has been used to characterize the lipophilicity of a compound and it value has shown correlation with water solubility, bioaccumulation properties (bioconcentration factor) in living organism as well as the sorption to soils or sediments (Chiou et al., 1977; Patil, 1991). Thus, K_{ow} is a useful parameter to assess the studies of the environmental fate of a chemical pollutant. A compound with high K_{ow} value suggesting the compound is very hydrophobic, high soil or sediment adsorption coefficient and high bioconcentration factor. For antibiotic compound, the commonly detected sulfonamides in aqueous environment have a low log K_{ow} (<1.68, Kuang et al., 2020) value which explained sulfonamides prone to stay

mobile and has low potential to be absorbed by sediment and thus distribute and disseminate widely in the aquatic environment. While tetracycline, fluoroquinolone and macrolides have greater K_{ow} value which means these antibiotics tend to stay immobile and adsorb on soil or sediment particle (Gao et al., 2012a). Eventually, antibiotics will accumulate in soil which may exert selection pressure to soil microorganism community and promote the development of ARB and ARG.

By comparing, the composition of antibiotic in aquaculture water has higher diversity than in the sediment (Table 2.2. & Table 2.3). Among the four antibiotic classes, sulfonamides have relatively low concentration in both marine and freshwater aquaculture. This has lent some support to sulfonamides has low log K_{ow}, small soil or sediment adsorption coefficient. Although, sulfonamides were detected in low concentration, however, the frequent detection of sulfadiazine and sulfamethoxazole in sediment could have because they are the most apt to accumulate in sediment compared to the other sulfonamides compounds. The predominant antibiotic in sediment were varied in region. In China, tetracyclines followed by quinolones and erythromycin were dominated in marine sediment. While erythromycin has domineering in Korea aquaculture environment. For freshwater aquaculture, enrofloxacin and ciprofloxacin was detected at higher concentration in China' sediment which ranged from 44 – 446 ng/g followed by doxycycline (34.3 ng/g) and make them dominated in sediment. For Europe country, flumequine, oxolinic acid and oxytetracycline were the predominant antibiotic in sediment as these three antibiotics are legally authorized to use in aquaculture. The high concentration of quinolone in water and sediment shown that quinolone dominated in Vietnamese marine and freshwater aquaculture. The distribution of antibiotic in solid or aqueous phase cannot be predicted solely by K_{ow}. Some antibiotic has complicated structure with different functional groups, or some may have multiple ionization site which may changed the efficiency on absorption.

Therefore, many other factors will need to be consider such as physical or chemical characteristics of an antibiotic, highly complex dynamic environment condition, co-exist of other pollutant, weather condition, the practice of antibiotic used and the management of a farm (Kim & Carlson, 2007; Kümmerer, 2009; Maskaoui & Zhou, 2010; Dong et al., 2019).

Most of the studies shown that the concentration of antibiotic residue in water was higher than it retained in tissue muscle. As this is expected due to the aquatic organism body system are not effective enough to fully metabolized antibiotic, an estimate of 75% unmetabolized antibiotic will be excreted out from body in feces and return back to the environment (Zhang et al., 2018). In most reported studies, the detection of antibiotic residue in muscle was less than10 ng/g (Table 2.2 & Table 2.3) which has the same concentration range with Done et al. (2015) where reported five antibiotics (ormetoprime, sulfadimethoxine, virginiamycin and oxytetracycline) at 0.3 – 8.6 ng/L in different varieties seafood that purchased from 11 countries. While minority were detected at a range of 24.75 – 43.51 ng/g which are enrofloxacin (24.75 ng/L; Han et al., 2020), trimethoprim (24.9 ng/L; Chen et al., 2015), tetracycline (32.4 ng/L; Monteiro et al., 2016) and norfloxacin (43.51 ng/L; He et al., 2012). Besides, few studies were reported the present of antibiotic residue in aquatic product has exceeded the set of MRLs. According to Uchida et al. (2016), sulfonamides (130 – 5800 ng/g), trimethoprim (53 – 1000 ng/g), ofloxacin (149 ng/g), enrofloxacin (180 – 355 ng/g) and ciprofloxacin (196 ng/g) were frequently violated the MRLs in Vietnam. While enrofloxacin, oxolinic acid, sulfamethoxazole and trimethoprim were found present in freshwater fish and shrimp that has violated the MRLs in Korea (Kang et al., 2018). Brazil, the only western country was reported oxytetracycline has exceeded the set MRLs in freshwater fish which the concentration detected up to 1379 ng/g (Monteiro et al., 2015; 2016). In China, the present of 15090 ng/g of erythromycin was found in

shrimp has overly exceeded the set of MRLs which at 600 ng/g (Chen et al., 2015), as well as the enrofloxacin in fish sample was detected up to 2200 ng/g (Chen et al., 2018). Although most of the contaminated aquatic food was below the MRLs and has less potential to pose health risk to human. However, the presence of multiple antibiotics residue may pose a potential risk to humans. Large variation of antibiotic concentrations in aquatic organism was observed in different growth stages because of feed supply or disease treatment, thus the withdrawal period is important to allow the aquatic organism to excrete out the antibiotic out before harvest.

Table 2.2: List of antibiotics detected in marine aquaculture farm in global.

Country	Location	Type of farm[#]	Antibiotic detected		
			Water (ng/L)	Sediment (ng/g)	Muscle(ng/g)
China	Pearl River[a]	F	Norfloxacin (4140 – 6620)	Norfloxacin (1.88 – 11.20) Ciprofloxacin (0.76 – 2.42)	Norfloxacin (1.95 – 43.51) Ciprofloxacin (<LOQ – 2.16) Enrofloxacin (0.65 – 1.71)
	Hailing Island[b]	F	Sulfadiazine (1.3 -1.5) Sulfameter (<LOD) Sulfamethoxazole (0.9 – 5.9) Sulfamonomethoxine (<LOD) Enfloxacin (1.9) Ofloxacin (0.8) Oxytetracycline (17.8) Methacycline (2.1) Erythromycin (1.4 – 7.7) Salinomycin (6.4 – 7.5) Trimethoprim (3.5 – 3.8)	n.a.	Sulfadiazine (1.6) Sulfamethoxazole (1.1) Sulfaquinoxaline (1.0) Sulfamerazine (0.2) Sulfadimethoxine (<1.9) Sulfathiazole (0.8) Sulfisoxazole (2.3) Ciprofloxacin (1.4) Enfloxacin (1.6) Fleroxaxin (1.4) Ofloxacin (1.0) Salinomycin (8.0) Trimethoprim (24.9)
	Hailing Island[b]	S	Sulfadiazine (1.3 – 1.4) Sulfameter (<LOD) Sulfamethoxazole (0.4 – 3.0) Sulfamonomethoxine (<LOD) Ciprofloxacin (186) Enrofloxacin (2.3) Ofloxacin (1.2) Oxytetracycline (15163) Methacycline (2.3) Tetracycline (2305) Erythromycin (3.3) Salinomycin (6.8 – 12.9) Trimethoprim (1.3 – 36.9)	Erythromycin (0.8)	Sulfadiazine (1.9) Ciprofloxacin (4.2) Enrofloxacin (1.5 - 149) Oxytetracycline (32.1) Clarithromycin (0.3) Erythromycin (2498 – 15009) Salinomycin (8.5) Trimethoprim (2.3)
	Hailing Island[b]	F, S, M	Sulfadiazine (1.3) Sulfameter (<LOD) Sulfamethoxazole (0.8) Sulfamonomethoxine (<LOD) Ofloxacin (0.8) Methacycline (2.1) Erythromycin (10.3) Salinomycin (7.0) Trimethoprim (3.8)	Enrofloxacin (2.6) Ofloxacin (1.2) Erythromycin (4.8)	Only salinomycin detected Mollusks (13.0) Crustaceans (3.8) Fish (7.7)

Table 2.2: continued.

Country	Location	Type of farm[#]	Antibiotic detected		
			Water (ng/L)	Sediment (ng/g)	Muscle (ng/g)
China	Mariculture[c]	F	Sulfadiazine (17-291.2) Sulfapyridine (3.7 – 47.9) Sulfamethoxazole (7.4 – 69.5) Sulfathiazole (n.d. – 121.5) Sulfamerazine (<LOQ – 2.3) Sulfamethazine (7.3 – 63.9) Sulfaquinoxaline (3.69 – 19) Tetracycline (n.d. – 54.7) Oxytetracycline (n.d. – 158.6) Doxycycline (n.d. – 28.7) Chlortetracycline (n.d. – 27.4)	Sulfadiazine (n.d. – 0.4) Sulfapyridine (n.d. – 0.2) Sulfamethoxazole (n.d. – 0.4) Sulfathiazole (n.d. – 0.1) Sulfamerazine (0.002 – 0.1) Sulfamethazine (<LOQ – 20.8) Sulfaquinoxaline (0.001 – 0.1) Tetracycline (0.9 – 25.3) Oxytetracycline (0.4 – 39.5) Doxycycline (0.3 – 40.6) Chlortetracycline (0.1 – 0.9)	n.a.
	Yellow Sea[d]	F	Trimethoprim (2.46 – 7.32) Sulfamethazine (0.15 – 0.26) Sulfamethoxazole (n.d. – 3.43) Oxytetracycline (12.61 – 41.03) Doxycycline (0.69 – 3.73) Ofloxacin (n.d. – 3.25) Norfloxacin (1.26 – 3.78) Ciprofloxacin (37.29 – 61.26) Enrofloxacin (125.96 – 995.02) Sarafloxacin (0.19 – 0.71) Erythromycin (n.d. – 0.55) Roxithromycin (n.d. – 1.26)	Sulfadiazine (0.29 – 0.44) Trimethoprim (0.09 – 6.58) Sulfamethazine (0.8) Sulfamethoxazole (0.09 – 2.55) Sulfaquinoxaline (n.d. – 0.16) Oxytetracycline (59.78 – 1478) Tetracycline (n.d. – 7.43) Doxycycline (n.d. – 0.18) Enrofloxacin (869 – 895) Sarafloxacin (0.44 – 0.46)	Trimethoprim (0.75) Sulfamonomethoxine (0.19) Sulfamethoxazole (0.16) Oxytetracycline (3.95) Tetracycline (0.86) Doxycycline (0.85) Ofloxacin (0.38) Ciprofloxacin (9.62) Enrofloxacin (24.75)

Table 2.2: continued.

Country	Location	Type of farm[#]	Antibiotic detected		
			Water (ng/L)	Sediment (ng/g)	Muscle (ng/g)
	Yellow Sea[d]	S	Sulfadiazine (n.d. – 0.49) Trimethoprim (0.31 – 7.77) Sulfamethazine (n.d. – 0.64) Sulfamethoxazole (n.d. – 1.81) Sulfaquinoxaline (n.d. – 0.40) Oxytetracycline (n.d. – 34.36) Tetracycline (n.d. – 0.72) Doxycycline (n.d. – 1.65) Ofloxacin (n.d. – 25.10) Norfloxacin (n.d. – 8.73) Ciprofloxacin (n.d. – 46.26) Enrofloxacin (n.d. – 103.93) Sarafloxacin (0.30 – 1.13) Erythromycin (n.d. – 0.83) Roxithromycin (n.d. – 0.57)	Sulfadiazine (n.d. – 0.19) Trimethoprim (n.d. – 0.46) Sulfamethoxazole (n.d. – 0.09) Oxytetracycline (n.d. – 30.74) Doxycycline (n.d. – 0.26) Ofloxacin (n.d. – 0.47) Norfloxacin (n.d. – 0.84) Ciprofloxacin (n.d. – 0.40) Enrofloxacin (n.d. – 14.40) Sarafloxacin (n.d. – 0.76)	Sulfadiazine (0.46) Sulfamethazine (0.09) Oxytetracycline (0.48) Tetracycline (9.65) Doxycycline (0.80) Ciprofloxacin (0.68) Enrofloxacin (0.11) Sarafloxacin (0.75) Roxithromycin (0.12)
	Hangzhaou Bay[e]	F, S	Sulfadiazine (0.86 – 2.67) Sulfamonomethoxine (11.16 – 31.56) Sulfamethoxazole (3.48 – 15.17) Norfloxacin (15.2 – 115.28) Tetracycline (9.89 – 34.11) Oxytetracycline (12.35 – 38.33) Florfenicol (0.48 – 47.97)	Sulfamonomethoxine (0.55 – 2.03) Norfloxacin (5.20 – 13.60) Enrofloxacin (0.23 – 2.05) Tetracycline (0.84 – 5.61) Oxytetracycline (0.75 – 4.36) Florfenicol (0.10 – 2.05)	n.a.

Table 2.2: continued.

Country	Location	Type of farm[#]	Antibiotic detected		
			Water (ng/L)	Sediment (ng/g)	Muscle (ng/g)
	Beibu Gulf [o]	S	Sulfacetamide (0.87 – 1.03) Sulfadiazine (0.73 – 24.42) Sulfamethazine (0.88 – 1.62) Sulfamethoxazole (3.30) Sulfadiazine (1.88) Trimethoprim (7.16) Norfloxacin (4.31 – 97.3) Ciprofloxacin (5.42 – 182) Enrofloxacin (1.69 – 2.31) Ofloxacin (1.71 – 1.91) Enoxacin (2.61 – 59.3) Clarithromycin (0.40) Roxithromycin (1.61 – 10.9) Erythromycin (0.62 – 45.8)	Sulfamethoxazole (1.59 – 25) Trimethoprim (0.2 – 2.82) Norfloxacin (1.2 – 52.5) Ciprofloxacin (0.41 – 4.28) Enrofloxacin (0.88 – 25.4) Enoxacin (0.84 – 4.48) Azithromycin (0.16 – 0.25) Clarithromycin (0.02) Roxithromycin (0.02) Erythromycin (0.55)	Sulfadiazine (0.16 – 0.76) Sulfamethoxazole (6.46 – 8.21) Trimethoprim (0.10 – 0.14) Norfloxacin (1.22 – 4.40) Ciprofloxacin (0.33 – 2.51) Enrofloxacin (0.38 – 1.07) Ofloxacin (0.23 – 1.52) Enoxacin (0.54) Clarithromycin (0.12 – 0.14) Roxithromycin (0.12) Erythromycin (0.20 – 0.74)
Vietnam	CanGio & CaMau [f]	S	Trimethoprim (80000 – 2030000) Sulfamethoxazole (40000 – 5570000) Norfloxacin (60000 – 6060000) Ofloxacin (10000 – 2500000)	Trimethoprim (9.02×10^6 – 7.34×10^8) Sulfamethoxazole (4.77×10^6 – 8.20×10^8) Norfloxacin (6.51×10^6 – 2.62×10^9) Ofloxacin (1.81×10^6 – 4.26×10^8)	n.a.
	Hanoi [g]	S	Sulfamethoxazole (n.d – 914) Sulfamethazine (n.d. – 2) Trimethoprim (n.d. – 85) Erythromycin (<LOQ – 4) Lincomycin (n.d. – 10) Oxytetracycline (<LOQ)	n.a.	n.a.
	Hanoi [h]	S	Ofloxacin/Levofloxacin (179 – 190) Norfloxacin (<LOQ) Ciprofloxacin (<LOQ) Lomefloxacin(<LOQ)	n.a.	n.a.

Table 2.2: continued.

Country	Location	Type of farm[#]	Antibiotic detected		
			Water (ng/L)	Sediment (ng/g)	Muscle (ng/g)
Bangladesh	Cox's Bazar, Shatkhira & Khulna [i]	S	Sulfamethoxazole (n.d. – 16.77) Trimethoprim (n.d. – 11.39) Tylosin (n.d. – 0.16) Erythromycin (n.d. – 3.91)	n.a.	n.a.
Taiwan [j]	-	F	Sulfamethoxazole (229) Sulfathiazole (7) Sulfamonomethoxine (2) Sulfadimehoxine (21) Oxytetracycline (12) Chlortetracycline (11)	n.a.	n.a.
Taiwan [k]	-	F	Sulfadiazine (n.d. – 6.3) Sulfamethoxazole (n.d. – 19.6) Sulfamethazine (n.d. – 24.5) Sulfamonomethoxine (n.d. – 26.1) Sulfadimethoxine (n.d. – 3.8) Lincomycin (n.d. – 57.9) Erythromycin (n.d. – 24.9)	n.a.	n.a.
	-	S	Lincomycin (n.d. – 2.9)	n.a.	n.a.
	-	F, S	Sulfamethoxazole (n.d. – 16.7) Lincomycin (n.d. – 9) Trimethoprim (n.d. – 9.4) Oxytetracycline (n.d. – 75) Ciprofloxacin (n.d. – 16.3) Erythromycin (n.d. – 18.5)	n.a.	n.a.

Table 2.2: continued.

Country	Location	Type of farm[#]	Antibiotic detected		
			Water (ng/L)	Sediment (ng/g)	Muscle (ng/g)
Korea [l]	-	F	Enrofloxacin (n.d. – 0.88) Ciprofloxacin (n.d. – 1.52) Ofloxacin (n.d. – 54.5) Norfloxacin (n.d. – 3.04) Sulfadiazine (n.d. – 5.69) Sulfathiazole (1.53 – 126) Erythromycin (n.d. – 1.02) Lincomycin (n.d. – 47.8) Trimethoprim (n.d. – 13.3)	Sulfadiazine (n.d. – 3.13) Sulfathiazole (4.17 – 8.02) Sulfamerazine (n.d. – 0.905) Erythromycin (n.d. – 48.1) Trimethoprim (n.d. – 10.3)	Enrofloxacin (n.d. – 0.261 Ciprofloxacin (n.d. – 3.16.) Norfloxacin (n.d. – 0.781 Sulfadiazine (n.d. – 0.487) Sulfathiazole (n.d. – 0.174) Trimethoprim (n.d. – 0.0402)
Italy	Central region of Italy [m]	F	n.a.	Flumequine (0.4 – 0.6) Oxytetracycline (0.2 – 0.8)	n.a.
Chile	Calbuco archipelago [n]	F	n.a.	Flumequine	n.a.

n.d.= not detected; n.a. = not analyze; - = unknown
< LOQ = below limit of quantification,
< LOD = below limit of detection,
F = fish, S = Shrimp/Prawn/Crab, M = Mollusk
[a]. He et al. (2012); [b]. Chen et al. (2015); [c]. Chen et al. (2017); [d]. Han et al. (2020); [e]. Yuan et al. (2019); [f]. Le & Munekage (2004); [g]. Shimizu et al. (2013); [h]. Takasu et al. (2011); [i]. Hossain et al. (2017); [j]. Lin et al. (2008); [k]. Lai et al. (2018); [l]. Kim et al. (2017); [m]. Lalumera et al. (2004); [n]. Buschmann et al. (2012), [o]. Zhang et al. (2018)

Table 2.3: List of antibiotics detected in freshwater aquaculture farm in global.

Country	Location	Type of farm[#]	Antibiotic detected		
			Water (ng/L)	Sediment (ng/g)	Muscle (ng/g)
China	Beijiang River[a]	F	Sulfadiazine (n.d. – 7418) Sulfamethazine (n.d. – 1940) Sulfamethoxazole (n.d. – 29.6) Sulfamonomethoxine (n.d. –1.57) Sulfachlorpyridazine (n.d. – 57.4) Erythromycin (n.d. – 68.8) Enrofloxacin (n.d. – 20.4) Ciprofloxacin (n.d. – 27.3) Ofloxacin (n.d. – 114) Oxytetracycline (n.d. – 89.4) Tetracycline (n.d. – 303) Chlortetracycline (n.d. – 359) Florfenicol (n.d. – 1282) Lincomycin (n.d. – 1643) Trimethoprim (n.d. – 226)	n.a.	Enrofloxacin (0.31 – 5.16) Florfenicol (15.80)
	Guangdong[b]	F	Sulfametoxydiazine (7.58 – 17.9) Sulfamethazine (0.67 – 3.60) Sulfamethoxazole (12.1 – 33.7) Chlortetracycline (29.1 – 48.6) Oxytetracycline (22.5 – 43.5) Doxycycline (20.3 – 98.6) Enrofloxacin (16.9 – 21.3) Ciprofloxacin (22.1 – 32.8) Norfloxacin (26.4 – 27.8)	Sulfametoxydiazine (1.31 – 4.20) Sulfamethazine (2.99 – 5.63) Sulfamethoxazole (3.06 – 3.44) Chlortetracycline (0.53 – 2.44) Oxytetracycline (0.48 – 3.21) Doxycycline (4.06 – 34.3) Enrofloxacin (262 – 446) Ciprofloxacin (0.68 – 7.72) Norfloxacin (0.51 – 2.29)	n.a.

Table 2.3: continued.

Country	Location	Type of farm[#]	Antibiotic detected		
			Water (ng/L)	Sediment (ng/g)	Muscle (ng/g)
	Tai Lake[c]	F, S	Sulfadiazine (12.38) Sulfamethoxazole (98.21) Sulfachloropyridazine (6.96) Sulfamonomethoxine (58.45) Sulfaquinoxaline (0.5) Norfloxacin (3.61) Enrofloxacin (5.08) Tetracycline (48.31) Oxytetracycline (7.79) Chlortetracycline (3.84) Erythromycin (1.68)	n.a.	n.a.
	Lake Guchenghu[d]	S	Sulfamethoxazole (n.d. – 21.7) Sulfadiazine (n.d. – 654) Trimethoprim (4.4. – 24.5) Roxithromycin (n.d. – 0.2) Leucomycin (n.d. – 8) Clarithromycin (n.d. – 76.2) Erythromycin (1.3 – 2450) Azithromycin (n.d. – 24.2) Ciprofloxacin (n.d.- 46.5) Lincomycin (n.d. – 4.4)	n.a.	n.a.
	Panyu [r]	F	Sulfamethazine (4.5 – 120) Sulfamethoxazole (n.d. – 210) Sulfaquinoxaline (n.d. – 32) Enrofloxacin (n.d. – 100) Erythromycin (80 – 1400) Clarithromycin (n.d. – 0.12) Trimethoprim (n.d. – 180) Lincomycin (5.9 – 16)	Sulfamethazine (<LOD) Sulfaquinoxaline (n.d. – 1.9) Norfloxacin (2.3 – 36) Ofloxacin (1.6 – 2.0) Ciprofloxacin (2.0 – 230) Enrofloxacin (6.8 – 44) Erythromycin (<LOQ) Trimethoprim (n.d. – 2.5)	Norfloxacin (3.1 – 11) Enrofloxacin (1.8 – 2200) Erythromycin (3.5 – 12)

Table 2.3: continued.

Country	Location	Type of farm[#]	Antibiotic detected		
			Water (ng/L)	Sediment (ng/g)	Muscle (ng/g)
Portugal	River Caima [e]	F	Norfloxacin (n.d. – 75.1) Ciprofloxacin (<LOQ – 19.1) Oxytetracycline (<LOQ – 11.9) Enrofloxacin (< LOQ – 9.3)	n.a.	n.a.
Vietnam	Mekong River [f]	F	Enrofloxacin (50 – 680) Ciprofloxacin (<LOQ – 250)	Enrofloxacin (2590) Ciprofloxacin (529)	n.a.
	Mekong River [g]	-	Sulfamethoxazole Sulfadimidine Sulfadiazine Trimethoprim Cephalexin	n.a.	n.a.
	Red River delta [h]	F	Sulfamethoxazole (n.d. – 914) Trimethoprim (n.d. – 85) Erythromycin (n.d. – 0.28)	n.a.	n.a.
	Hanoi [i]	F	Ofloxacin/Levofloxacin (117) Norfloxacin (<LOQ – 6.4) Ciprofloxacin (<LOQ) Lomefloxacin (<LOQ – 35.9)	n.a.	n.a.
	Hanoi [i]	S	Ofloxacin/Levofloxacin (<LOQ – 75.8) Norfloxacin (<LOQ – 43.1) Ciprofloxacin (<LOQ) Lomefloxacin (<LOQ)	n.a.	n.a.
	Mekong River [j]	F	Sulfamethoxazole (2 – 135) Sulfadiazine (1 – 108) Trimethoprim (1 – 330) Enrofloxacin (1 – 59)	n.a.	n.a.
	Hanoi [k]	F	Sulfamethoxazole (7.3 – 2017) Sulfamethazine (9.3 – 6621) N-acetylsulfamethazine (0.7 – 3005) Sulfadiazine (0.9 – 474) Ofloxacin (0.2 – 0.9) Nalidixic acid (4.3 – 17.3) Trimethoprim (0.8 – 78)	n.a.	n.a.

Table 2.3: continued.

Country	Location	Type of farm[#]	Antibiotic detected		
			Water (ng/L)	Sediment (ng/g)	Muscle (ng/g)
	Thai Binh [k]	F	Sulfamethoxazole (1.5 – 189) Sulfamethazine (1.8 – 5.5) N-acetylsulfamethazine (0.3 – 26) Ofloxacin (1.0 – 2.7) Trimethoprim (4.7 – 56)	n.a.	n.a.
	Can Tho [k]	F	Sulfamethoxazole (1.9 – 14) Sulfamethazine (10 – 165) N-acetylsulfamethazine (3.8 – 49) Sulfadiazine (6.6 – 113) Trimethoprim (2.0)	n.a.	n.a.
	Can Tho [k]	-	Sulfamethoxazole (0.6 – 642) Sulfamethazine (2.0 – 14.3) N-acetylsulfamethazine (1.3 – 5.3) Trimethoprim (1.5 – 3.3)	n.a.	n.a.
	Hanoi [l]	-	Sulfamethoxazole (10 – 57) Trimethoprim (16 – 593) Clarithromycin (10 – 13) Ofloxacin (500) Norfloxacin (126 – 188) Ciprofloxacin (363)	n.a.	n.a.
	Tha Chin [m]	F	Oxytetracycline (3050) Enrofloxacin (n.d. – 1590)	n.a.	n.a.
Thailand	Khon Kaen [i]	F	Ofloxacin/Levofloxacin (<LOQ – 96.2) Norfloxacin (16.2 – 287) Ciprofloxacin (<LOQ) Lomefloxacin (13.2 - 111)	n.a.	n.a.
	Khon Kaen [i]	S	Ofloxacin/Levofloxacin (<LOQ) Norfloxacin (20.4 – 38.0) Ciprofloxacin (<LOQ) Lomefloxacin (<LOQ - 179)	n.a.	n.a.

Table 2.3: continued.

Country	Location	Type of farm[#]	Antibiotic detected		
			Water (ng/L)	Sediment (ng/g)	Muscle (ng/g)
	Kohn Ken [j]	S	Erythromycin (<LOQ) Tetracycline (<LOQ) Oxytetracycline (<LOQ)	n.a.	n.a.
Bangladesh	Rajshahi, Jessore & Mymensingh [o]	F	Sulfamethoxazole (n.d. – 20.02) Sulfadiazine (n.d. – 17.97) Sulfamethazine (n.d. – 11.71) Sulfamethizole (n.d. – 10.81) Trimethoprim (n.d. – 241.67) Tylosin (n.d. – 39.34)	n.a.	n.a.
Philippines	Laguna Lake [p]	F	Sulfamethoxazole (27.8 – 41.6) Sulfamethazine (8.3 – 16.2) Trimethoprim (1.7 – 2.5) Oxytetracycline (<LOQ) Lincomycin (2.2 – 2.6)	n.a.	n.a.
Italy	River Oglio & River Ticino [Q]	F	n.a.	Flumequine (0.1 – 1.1) Oxytetracycline (0.4 – 246.3)	n.a.
France	Elorn Rivers	F	Flumequine (n.d.) Oxytetracycline (n.d.) Florfenicol (n.d.) Oxolinic acid (n.d.)	Flumequine (2000) Oxolinic acid (112)	
Brazil	Paraná and Grande rivers [t]	F	n.a.	n.a.	Oxytetracycline (15.6 – 1231.8) Tetracycline (7.7) Florfenicol (521.2 – 528)
	Paraná and Grande rivers [u]	F	n.a.	n.a.	Oxytetracycline (10.9 – 1298.7) Tetracycline (11 – 32.4) Florfenicol (10.4 – 524.7)

n.d.= not detected; n.a. = not analyze; - = unknown
< LOQ = below limit of quantification, < LOD = below limit of detection,
* mean concentration
# F = fish, S = Shrimp/Prawn/Crab, M = Mollusk
[a]. Zhong et al. (2018); [b]. Xiong et al. (2015); [c]. Song et al. (2016); [d]. Wang et al. (2018a); [e].Pereira et al. (2015); [f]. Andrieu et al. (2015); [g]. Nakayama et al. (2017); [h]. Hoa et al. (2011); [i]. Takasu et al., 2011; [j]. Giang et al. (2015); [k]. Harada et al. (2018); [l]. Thai et al. (2018); [m]. Rico et al. (2014); [n]. Shimizu et al. (2013); [o]. Hossain et al. (2017); [p]. Suzuki et al. (2013); [q]. Lalumera et al. (2004); [r]. Chen et al. (2018); [s]. Pouliquen et al. (2009); [t]. Monteiro et al. (2015); [u]. Monteiro et al. (2016)

2.5 Antibiotic Resistance Genes in Aquaculture

Some studies suggested that aquaculture could be a potential source in spreading and disseminating ARGs (Harnisz et al., 2015; Shen et al., 2020). Discriminate use of antibiotics in aquaculture, which eventually releases to the environment may be able to induce the emergence of ARB and ARGs (Zhang et al., 2009; Suzuki et al., 2017). Even at subinhibitory concentration level, the antibiotic might have effects on the selection and dissemination of ARGs to microorganism communities in aquatic environments (Gullberg et al., 2011). Thus, aquaculture has been known to be designated as "genetic reactors" or "hot spots for ARG" and expected to be one of the sources in contaminating aquatic environments with antibiotics, ARB and ARG (Cabello et al., 2013). Moreover, the risk of exposing ARGs to humans remains unclear. Therefore, it is important to monitor the level of ARGs contamination in aquaculture systems, in order to mitigate the transmission of ARGs in the environment as well as humans.

To date, 61 tetracycline (*tet*) resistance genes have been determined and grouped into three types of resistance mechanisms: active efflux pump (34), ribosomal protection (13), enzyme inactivation (13) and one unknown (Roberts, 2019). The distribution of *tet* resistance genes have been extensively studied in environmental, clinical and food settings (Liu et al., 2014; Chuah et al., 2016; Amador et al., 2019; Roberts, 2019). Genes encoding *tet* resistance in both Gram-positive and Gram-negative bacteria are frequently detected in aquaculture environments and aquatic organisms (Akinbowale et al., 2007; Nonaka et al., 2007; Nguyen et al., 2017; Martins et al., 2019). The presence of these *tet* resistance genes is due to broad-host-range plasmids, transposons and conjugative transposons (CTns) that may play a significant role in dissemination of resistance among environmental and clinically important species (Chopra & Roberts, 2001; Kim et al., 2004; Agersø et al., 2007; Han et al., 2015; Harnisz et al., 2015). Among the 61 *tet* resistance genes, the most widely distributed *tet*

gene is *tet*(M) which belongs to ribosomal protection mechanism, where *tet*(M) has been identified to be associated in 80 different genera in both gram positive and negative bacteria because *tet*(M) thought to be associated with wide host range of CTns belonging to the Tn*1545* – Tn*916* compare to other *tet* genes (Kim et al., 2004; Robert, 2019). While *tet*(B) – an efflux protein which carried by a Tn*10* transposon is known to be the most widely distributed in Gram-negative bacteria which have found to be present in 35 Gram-negative genera (Robert, 2019). As time passed, mobile element that carry *tet* gene has been evolving and acquired high number of different ARGs and resistance genes for heavy metal which increase the transfer of co-resistance genes (Giovanetti et al., 2003; Wood & Garner, 2015; Robert, 2019). Moreover, a study shown that low concentration of tetracycline has shown to promote the transfer of *tet*(M) transposons and plasmid, indicating higher potential dissemination of *tet*(M) in aquaculture where tetracycline was commonly used (Facinelli et al., 1993).

Tetracycline genes in aquaculture have been found to be commonly possessed one or more *tet* genes from efflux pumps and ribosomal protection mechanisms in Gram-negative bacteria. Several studies have been carried out in different countries to study the occurrence and distribution of ARGs in the environment that affected by aquaculture activity and the results were different from each other (Table 2.4). Most of the studies that using the culturable method and conventional PCR were mainly focused on the most frequent detected *tet* resistance genes which are *tet* [(A), (B), (D), (E)] from efflux pump mechanism and *tet*(M) from ribosomal protection mechanism (Seyfried et al., 2010; Tamminen et al., 2011; Piotrowska et al., 2017). Huang et al. (2017) suggested that *tet*(A) could act as a potential indicator of *tet* genes in aquaculture. While the recent occurrence of ribosomal protection gene: *tet*(M), *tet*(S) and *tet*(W) were reported mostly in Asia country, for instance Japan, Korea, Singapore, Sri Lanka, Taiwan and Thailand aquaculture (Petersen & Dalsgaard, 2003; Kim et al., 2004; Nonaka et al., 2007; Ng et

al., 2018; Kim et al., 2012; Liyanage & Manage, 2019; Suzuki et al., 2019).The detection of *tet* genes in China has shown varying results in different regions. Mariculture in Southeast China has detected the presence of *tet* [(M, O, W, Q, A)] which is dominant with ribosomal protection mechanisms (Chen et al., 2017). Similarly, Gao et al. (2012b), Xiong et al. (2015), Su et al. (2017) and Wu et al. (2019) were also found a high prevalence of *tet*[(M, O, W, Q, T, A, B, X)] in different type of water bodies aquaculture in China where high variety of *tet* genes from ribosomal protection mechanism was detected compared to efflux pump mechanism. However, there are few studies that show a different profile of *tet* genes in China aquaculture. For instance, Dang et al. (2006), Dang et al. (2009), Huang et al. (2017), Yuan et al. (2019) and Shen et al. (2020) detected a more diverse *tet* gene that under efflux pump mechanism (*tet* [(A, B, C, E, G, H)]) than ribosomal protection mechanism (*tet* [(M, O and W)]). While, high incidence of efflux pump genes *tet*[(A, B, C, E, G and H)] were detected in Finland, Swedish and Chilean fish farm, US aquaculture farm and Vietnam shrimp farm and Korea coastal aquaculture (Miranda et al., 2003, Seyfried et al., 2010; Tamminen et al., 2011; Shah et al., 2014; Jang et al., 2018, Pham et al., 2018).

Culture-independent is a new approach to detect and quantify ARGs using real-time polymerase chain reaction (PCR). Recently, several studies have adopted this approach to quantify ARGs in aquaculture and the relative abundance. Studies shown that *tet* genes from efflux pump [*tet* (A) and (B)] and ribosomal protection [*tet*(M), (W) and (O)] were frequently detected in global aquaculture farm and the relative abundance was found at a range from 10^{-7}- 10^{-1} copies/16S ribosomal ribonucleic acid (rRNA) gene copies (copies/16S) (Table 2.4). A threshold was suggested by Graham et al. (2011) to indicate an area as pristine or contaminated with *tet* gene, if the normalized ratio of *tet* gene to 16S rRNA are fall between 10^{-8}- 10^{-6} copies/16S it is a typical pristine area whereas highly contaminated area is to expect to have a normalized ratio $>10^{-4}$

copies/16S. This shown that most of the studies found that the environment surrounding aquaculture farm and aquaculture farm were contaminated with *tet* genes. In Korean, *tet* genes were detected at relative abundance range from 10^{-7}- 10^{-2} copies/16S in coastal aquaculture farms where *tet*(B) and *tet(*D) were the predominant *tet* genes (Jang et al., 2018). *tet* (W) was the only *tet* gene tested with a relative abundance at 10^{-5} copies/16S in Singapore aquaculture farm (Ng et al., 2018). In China, *tet*[(M), (O), (W), (S), (Q) & (X)] had the relative abundance of 10^{-5} - 10^{-3} copies/16S in an aquaculture farm (Gao et al., 2012b; Xiong et al., 2015). While in Swedish and Finnish fish farms, a relative abundance of 10^{-4} - 10^{-2} copies/16S was quantified in sediment (Tamminen et al., 2011). An estuary aquaculture in Hangzhou Bay, *tet*[(A), (B), (C), (M), (O) and (H)] detected in a total relative abundance of 10^{-2} - 10^{-1} copies/16S (Yuan et al., 2019).

Sulfonamides are the oldest and widely used antibiotic on human, veterinary, livestock and aquaculture (Houvinen et al., 1995; Sköld, 2000). The frequent used of sulfonamides has led to widespread resistance. Sulfonamides interrupt the folic acid pathway by targeting enzyme DHPS to suppress the production of DHPS (Suzuki & Hao, 2012; Tačić et al., 2017). The development of resistance against sulfonamides in bacteria can be acquired through the mutation in *folP* genes encoding DHPS or the acquisition of *sul* genes (*sul*1, *sul*2 and *sul*3) which is an alternative DHPS gene. The *sul* genes produce distinct DHPS between themselves, although *sul*1, *sul*2 and *sul*3 has an estimated 50% similarity on nucleotide sequence (Sköld, 2000, Rolbiecki et al., 2020). Though, the DHPS product of *sul* genes show low affinity for the substrate *p*-amino benzoic acid but still inhibit sulfonamide from binding (Sköld, 2000). Of the two developments of sulfonamides resistance mechanism, *sul* genes are the most prevalent mechanism compared to mutation in *folp* gene (Enne et al, 2002; Hoa et al., 2008). Mobile genetic element such as transposons, integron, play an important role in disseminating antibiotic resistance genes (Partridge et al., 2018). *sul*1 was mostly found

associated with class 1 integrons at the 3' end and on large conjugative plasmids whereas, *sul*2 usually located on either a small non conjugative plasmids or large transmissible multi-resistance plasmids. While *sul*3 was proposed to be linked to non-classic class 1 integrons (Sköld, 2000; Enne et al, 2002; Domínguez et al., 2019). Sulfonamides is commonly use together with trimethoprim to give greater potency of antibacterial effect. Thus, the association of *sul* genes with mobile genetic elements together with *dfr* genes (a genes that encoding dihydrofolate reductase enzyme which causes resistance to trimethoprim) has higher possibility in promoting the emergence and dissemination of sulfonamide and trimethoprim resistance in environment (Domínguez et al., 2019).

High prevalence of sulfonamide resistance has been reported to be present in Gram-negative bacteria which mostly attributed by plasmid-borne *sul*1 and *sul*2 (Sköld, 2000; Domínguez et al., 2019; Rolbiecki et al., 2020). On both approaches, the dissemination of sulfonamides resistance genes shown the similar result where *sul*1 and *sul*2 is reported more often than *sul*3 in aquaculture in different region (Table 2.4). In China, three *sul* gene were found to have the relative abundance of 10^{-5} - 10^{-2} copies/16S (Gao et al., 2012b; Xiong et al., 2015; Gao et al., 2018). In Singapore, *sul*1 and *sul*2 were detected at relative abundance of 10^{-4} and 10^{1} copies/16S respectively (Ng et al., 2018). Both *sul*1 and *sul*2 were found present in Korea aquaculture farms at relative abundance at 10^{-6}- 10^{-4} copies/16S (Jang et al., 2018). While Taiwan has abundance of *sul*1 and *sul*2 at 10^{-4} and 10^{-2} copies/16S respectively (Suzuki et al., 2019). *sul*1 and *sul*2 were detected at 10^{-5} - 10^{-4} copies/16S and 10^{-8} - 10^{-6} copies/16S were observed in US where the area with anthropogenic activity going on (Pruden et al., 2006). In most studies, the detection frequency of *sul* genes were as following: *sul*1>*sul*2>*sul*3 (Hoa et al., 2008; Shah et al., 2014; Xiong et al., 2015; Jang et al., 2018; Yuan et al., 2019; Shen

et a., 2020). This could be due to the association of *sul*1 with the class 1 integron which has wide dissemination (Gündoğdu et al., 2011).

Identification of ARG has been carried out in a classical way by observing the phenotypic effects on the culturable bacteria. However, a high percentage of environment bacteria are known to be non-culturable or yet to be cultured (Lloyd et al., 2018). Thus, quantitative analysis of ARGs was conducted in this study, to evaluate the abundance of ARGs in the entire microorganism community in aquatic environments. Many studies have assessed the abundance of ARGs for sulfonamide and tetracycline due to the common and long use of these antibiotic in aquaculture. Thus, *sul* and *tet* genes were frequently detected present in aquaculture (Table 2.4). Among *tet* genes, *tet*(M) was known of having the broadest host range and distributed widely in natural environment (D'Costa et al., 2011; Roberts et al., 2012) as well as the origin of *tet*(M) has been reported to be ancient (Kobayashi et al., 2007). Moreover, in recent resitome analysis, *tet*(M) was reported as the most predominant and ubiquitous in fish farms (Muziasari et al., 2014). Furthermore, the different composition of *tet* and *sul* genes were occurred in aquaculture farms on different geographical regions suggesting that the variation may be affected by the farming systems, practices, antibiotic dosages used and bacterial community composition at different densities. Thus, as a potentially predominant source of *tet*(M) and *sul* genes distribution, an inclusive monitor of aquaculture is needed in Malaysia.

Table 2.4: Detection of ARGs in global aquaculture.

Location	Source	Resistance genes																			
		tet gene																	*sul* gene		
		A	*B*	*C*	*D*	*E*	*G*	*H*	*K*	*L*	*M*	*W*	*O*	*T*	*X*	*Q*	*S*	B/P	*sul*1	*sul*2	*sul*3
Culture Independent																					
Korea	W[a]	√	√	-	√	√	√	√	-	-	√	-	-	-	√	√	-	√	√	√	-
Poland	W[b]	√	-	√	-.	-	-	-	-	√	-	-	√	-	-	-	-	-	-	-	-
Singapore	W[c]	-	-	-	-	-	-	-	-	-	-	√	-	-	-	-	-	-	√	√	-
Japan	W[d]	-	-	-	-	-	-	-	-	-	√	-	-	-	-	-	-	-	√	√	√
Taiwan	W[d]	-	-	-	-	-	-	-	-	-	√	-	-	-	-	-	-	-	√	√	-
China	W[e]	-	-	-	-	-	-	-	-	-	√	√	√	-	√	√	√	-	√	√	√
	W[f]	√	√	-	-	-	-	-	-	-	√	√	√	-	-	√	-	-	√	√	√
	W[g]	-	-	-	-	-	√	-	-	-	-	√	-	-	√	-	-	-	√	-	-
	W[h]	n.d.	√	-	-	-	-	-	-	-	√	√	√	-	√	√	n.d.	-	√	√	√
	W[i]	-	-	-	-	-	-	-	-	-	-	√	√	-	√	√	-	-	√	√	-
	W[j]	√	√	√	-	-	-	√	-	-	√	-	√	-	-	-	-	-	√	√	√

Table 2.4: continued.

Location	Source	Resistance genes																			
		tet gene																	*sul* gene		
		A	*B*	*C*	*D*	*E*	*G*	*H*	*K*	*L*	*M*	*W*	*O*	*T*	*X*	*Q*	*S*	B/P	*sul*1	*sul*2	*sul*3
	S[e]	-	-	-	-	-	-	-	-	-	√	√	√	-	√	√	n.d.	-	√	√	√
	S[f]	√	√	-	-	-	-	-	-	-	√	√	√	-	-	√	-	-	√	√	√
	S[g]	-	-	-	-	-	√	-	-	-	-	√	-	-	√	-	-	-	√	-	-
	S[k]	-	-	-	-	-	-	-	-	-	√	√	√	√	-	-	-	-	√	√	-
	S[i]	-	-	-	-	-	-	-	-	-	-	√	√	-	-	√	√	-	√	√	-
	S[j]	√	√	√	-	-	-	√	-	-	√	-	√	-	-	-	-	-	√	√	√
	M[i]	-	-	-	-	-	-	-	-	-	-	√	√	-	-	n.d.	n.d.	-	√	√	-
Finland	S[l]	√	-	√	-	-	-	√	-	-	√	-	-	-	-	-	-	-	-	-	-
	S[m]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	√	√	n.d.
Swedish	S[l]	n.d.	-	√	-	-	-	n.d.	-	-	√	-	-	-	-	-	-	-	-	-	-
Culture Dependent																					
Poland	W[b]	√	√	√	√	√	√	-	√	√	√	-	√	-	√	√	√	-	-	-	-

Table 2.4: continued.

Location	Source	Resistance genes																			
		tet gene																*sul* gene			
		A	*B*	*C*	*D*	*E*	*G*	*H*	*K*	*L*	*M*	*W*	*O*	*T*	*X*	*Q*	*S*	B/P	*sul*1	*sul*2	*sul*3
Japan	W[n]	-	-	-	-	-	-	-	-	-	√	-	-	-	-	-	√	-	-	-	-
	W[o]	-	-	-	-	-	-	-	-	-	√	-	-	-	-	-	-	-	-	-	-
South African	W[p]	√	√	-	√	√	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sri Lanka	W[q]	√	√	-	-	-	-	-	-	-	√	-	-	-	-	-	√	-	-	-	-
Egypt	W[r]	√		√	√	√	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chile	W[s]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	√	√	-
Denmark	W[t]	√	-	-	√	√	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	W[u]	√	-	-	√	√	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Korea	W[v]	-	-	-	-	-	-	-	-	-	√	-	-	-	-	-	-	-	-	-	-
Vietnam	W[w]	√	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	W[x]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	√	√	-
	W[y]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	√	√	√

Table 2.4: continued.

Location	Source	Resistance genes																			
		tet gene																*sul* gene			
		A	*B*	*C*	*D*	*E*	*G*	*H*	*K*	*L*	*M*	*W*	*O*	*T*	*X*	*Q*	*S*	B/P	*sul*1	*sul*2	*sul*3
China	W[z]	-	√	-	√	√	-	-	-	-	√	-	-	-	-	-	-	-	-	-	-
	W[aa]	√	√	-	√	√	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	S[k]	-	-	-	-	-	-	-	-	-	√	√	√	√	-	√	-	-	√	√	n.d.
Singapore	S[c]	-	-	-	-	-	-	-	-	-	-	√	-	-	-	-	-	-	√	√	-
Finland	S[l]	-	-	-	-	√	√	-	-	-	-	√	-	-	-	-	-	-	-	-	-
Swedish	S[l]	-	-	-	-	√	n.d.	-	-	-	-	√	-	-	-	-	-	-	-	-	-

W=Water;
S=Sediment;
M=Muscle,
n.d. = not detected,
"- " = not tested

[a]. Jang et al. (2018); [b]. Harnisz et al. (2015); [c]. Ng et al. (2018); [d]. Suzuki et al. (2019); [e]. Xiong et al. (2015); [f]. Chen et al. (2017); [g]. Shen et al. (2020); [h]. Wu et al. (2019); [i]. Su et al. (2017); [j]. Yuan et al. (2019); [k]. Gao et al. (2012b); [l]. Tamminen et al. (2011); [m]. Muziasari et al. (2014); [n]. Kim et al. (2004); [o]. Nonaka et al. (2007); [p]. Jacobs & Chenia (2007); [q]. Liyanage & Manage (2019); [r]. Ishida et al. (2010); [s]. Domínguez et al. (2019); [t]. Agersø et al. (2007); [u]. Schmidt et al. (2001); [v]. Kim et al. (2012); [w]. Pham et al. (2018); [x]. Hoa et al. (2008); [y]. Hoa et al. (2010); [z]. Dang et al. (2009); [aa]. Dang et al. (2006)

2.6 Risk Associated with Aquaculture

2.6.1 Environment

The use of antibiotic in aquaculture has turned aquaculture become one of the potential sources of antibiotic pollution to the environment (Figure 2.7). In aquaculture, antibiotics are directly added to the feed or water, or thru injection (Okocha et al., 2018). After the antibiotic has consumed by the seafood, some antibiotic will be metabolized inside the seafood body. Eventually, those unmetabolized antibiotic will be excreted out through urine and feces and return to the water. Free antibiotic that does not consume by the seafood may retain in the aquaculture water and has the potential leached to the environment from effluent and sewage system or to the agriculture field where the water is recycled to use between aquaculture and agricultural activity. While some unconsumed antibiotic-pellet and feces-contained unmetabolized antibiotic may reach to sediment and persist in the aquaculture or wash by the water current to a distance location. Although most of the studies detected antibiotic residues in the aquaculture and surrounding environment were below than the inhibitory concentration level (Table 2.2). However, some studies have shown that with low concentration of antibiotic can exert selection pressure to promote the development and dissemination of ARGs and ARB in the environment (Gullberg et al., 2011). Eventually, may results in alter the microbial community in the environment. Besides the emergence and spreading of ARG and ARB, the residue of the antibiotic present in the environment may also has to potential imposed toxic to the non-target selected species such as zooplankton and phytoplankton. Studies have shown that present of antibiotic might disturb the production of chlorophyll (Song et al., 2016) and development of zooplankton at the early stage (Park & Kwak, 2018), which in turn caused the changes in food chain and ultimately led to imbalance of aquatic ecosystem. Furthermore, indiscriminately misuse

of antibiotic in aquaculture may induce the selection of pathogenic bacteria resistance to multiple antibiotics, which can reduce the effectiveness in treating bacterial infection.

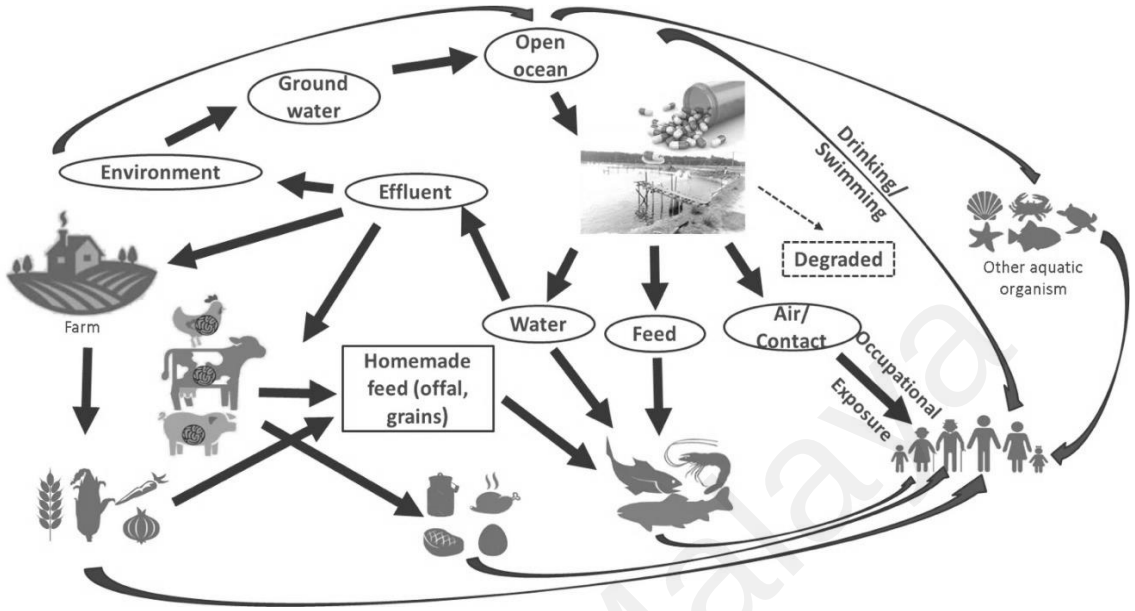

Figure 2.7: The route of exposure to antibiotic from farm to table.

Eutrophication is one of the risks associated with aquaculture activity. In closed aquaculture system, the excretory waste and unconsumed food accumulate in pond culture has caused high level of phosphorus and nitrogen which deplete the oxygen level and enhance grow of algae and phytoplankton, thus created harmful algal and phytoplankton blooms, and massive dead of aquatic animal (dead zone) (Chislock et al., 2013). Another environment concern associated with aquaculture is the changes of biodiversity by the farmed fish to the wild fish. As farmed fish escaped to the wild may end up competing for the food resource with the wild fish and predating the wild prey, which potentially affect the food availability, displacing the wild, disrupt the natural balance of an ecosystem and change of food web (Diana, 2009). Besides, the escaped farmed fish that carrying unknown or severe disease can be also transmitted to wild aquatic organism and causes the extinction of local species if the condition goes outbreak and uncontrollable (Naylor et al., 2005; Madhun et al., 2015). Another major

impact is destruction of habitat, which caused by the clearing of wetlands (Barange et al., 2018). In order to expand and develop aquaculture industry, wetland destruction and deforestation were carried out to utilize the unused land, especially for the development of shrimp aquaculture where inland pond is needed to build. Destruction of habitat could cause the loss of wetlands, eventually may lead to the lose the protection of shoreline from erosion and increased the occurrence of flood and drought at the local site (Ahmed & Glaser, 2016).

2.6.2 Human

Ultimately, the usage of antibiotic in aquaculture can bring negative impact from environment to human (Figure 2.7). The application of antibiotic in the farm has led to the retention of antibiotic residue in the seafood muscle and this has raised the awareness on food security and human health concern. If human consumed antibiotic contaminated seafood product has the potential to result in, development of ARB, allergy and toxicity which may led to morbidity or fatal (Okocha et al., 2018). The condition even can go worst if expose to antibiotic for long period, for instance farmer who has directly contact or inhaled antibiotic during the application of antibiotic to the farm, severe adverse effect may stimulate based on the type of antibiotic exposed by the farmer (Chuah et al., 2016). Figure 2.8 shown the refusal of shrimp from FDA due to the shrimp was found present with banned antibiotic, which is chloramphenicol and nitrofurans. Accidentally, consumed banned antibiotic may cause severe adverse effect - bone marrow depression on human. Thus, chloramphenicol and nitrofurans are banned to be used as human and veterinary medicine (Morris et al., 2012).

Other than antibiotic residue in muscle, the seafood product that infected with disease may carried resistance pathogenic bacteria inside the gut of seafood product which encourage the direct transfer the resistance gene from animal to human. Seafood

product also can act as a reservoir for resistance gene. Some studies shown evidence of resistance gene can be exchange between bacteria from aquatic environment and terrestrial environment, as well as pathogenic bacteria from animal and human, thru a broad range of mechanism such as plasmid and transposon (Peterson & Kaur, 2018). Besides contaminated seafood product, there is a potential risk for ARB to be transmitted to human thru occupational exposure during handling the antibiotic contaminated infected seafood (Addis et al., 2011).

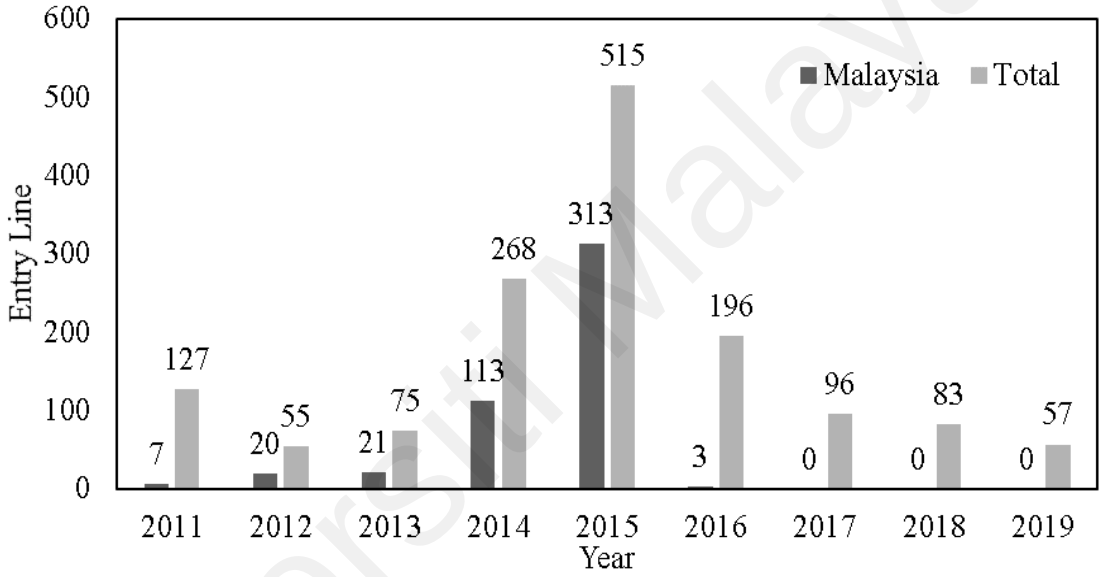

Figure 2.8: FDA refusals of Malaysian shrimp's entry lines for antibiotic contamination from 2011-2019. (adapted from FDA, 2019)

2.7 Legislation

Most of the developed countries (e.g. EU, US) have established and enforced the act and policy in regulate and monitor the use of antibiotic in aquaculture. For instance, Codex Alimentarius Commission (CAC) and the European Union (EU) (Commission regulation No 37/2010) (European Commission, 2010). World Health Organization (WHO) and Food and Agriculture Organization together have created the CAC in 1963. While Joint FAO/WHO Food Standard program (JEFCA) is served as advisory body to

WHO, FAO and CAC (FAO, 2021). The purpose is to protect the health of consumer and set a food security standard to serve as a reference for global. The Codex consists of the practice, guidelines and recommendation of withdrawal time, prohibited antibiotic as well as the maximum residue levels (MRLs) of veterinary medicine. Till now there is more than 70 veterinary drugs have established MRLs in Codex (CAC, 2018). While others producing countries have adopted the Codex MRLs to set up their own antibiotic regulation and MRLs. Most of the producing countries, the application and usage of antibiotic were regulated by the nation legislation, which governed by government agency. For instance, China governed by Ministry of Agriculture of the People's Republic of China, while Vietnam is Ministry of Fisheries (Lulijwa et al., 2019).

In 2013, The Association of Southeast Asian Nations (ASEAN) has set up their own guidelines for the use of chemical and antibiotic in aquaculture for Southeast Asia countries (ASEAN, 2013). In Malaysia, the regulation and guidelines were only in place for livestock, veterinary and monitor the maximal residue level of antibiotic presence in animal food but not the dosage of antibiotic use in treating aquaculture product. According to the Poison Act, 1984 (Revised 1989), all drugs are required to be registered under the Drug Control Authority. However, the regulation is only used in human medicine. Moreover, The Animal Act, 1953 (Revised 2006) does have the regulation for animal farm, livestock industries, but it does not apply to fish nor has a regulation to monitor the usage of antibiotic on livestock. Even though there is a Fisheries Act 1985 for aquaculture, still it does not have the regulations to control, monitor and standardize the use of antibiotic and others drug in the aquaculture. National Pharmaceutical Regulatory Agency is an agency under the Ministry of Health Malaysia which responsible for the registering and licensing of human and veterinary pharmaceutical product and also provided with MRLs of drug residue in food. According to National Pharmaceutical Regulatory Agency (2020), only 25 antibiotics

and sulfonamides can use on aquaculture with recommended MRLs in aquaculture product. Recently, tetracycline and enrofloxacin were announced and being banned to use on aquaculture (The Sun Daily, 2020). In the past five years, FDA has been refusing the entry of Malaysian shrimps into US, due to the contamination of banned antibiotic (Figure 2.8). The number of refusals has increased throughout the year. This shown that monitoring and enforcement of antibiotic usage in aquaculture is illusory and non-existent. With no proper guidelines, regulation and monitoring, limited information on distribution and composition of antibiotic in Malaysia's aquaculture farm and the potential risk of residual antibiotic toward the ecosystem remains unclear.

2.8 Research Questions

a) What are the type and current levels of antibiotic pollution present in the marine aquaculture wastewater?

b) Do the antibiotic residues in the marine aquaculture wastewater pose an environment risk?

2.9 Research Objectives

a) To determine the concentration of selected antibiotic residues in marine aquaculture wastewater.

b) To assess whether the selected antibiotic residues in marine aquaculture wastewater pose potential environment risk.

CHAPTER 3: MATERIALS AND METHODS

3.1 Sampling

One time sampling was conducted to collect water samples from 29 aquaculture farms which known to be the seven main aquaculture production states (Perak, Selangor, Pahang, Kelantan, Penang Island, Malacca and Johor) (Figure 3.1) in Peninsular Malaysia (DOF, 2019). Location of the sampling sites are summarized in Table 3.1. Water samples were collected using a stainless-steel bucket and passed through a 20 µm mesh net then stored into a clean two L amber glass bottle for antibiotic residue quantification. While two L sterile glass bottle was used to collect water sample for antibiotic resistance genes quantification. Samples were kept in ice box with ice and transport back to laboratory for further processing.

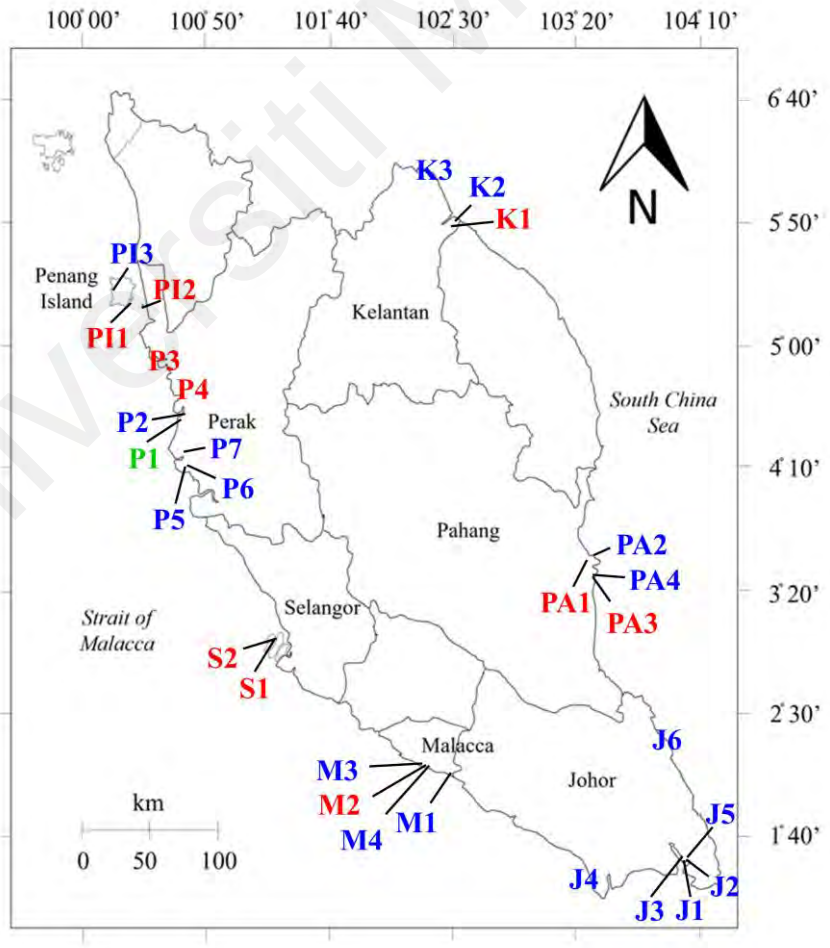

Figure 3.1: Map of sampling sites. (Blue font: Prawn, red font: fish, green font: prawn and fish)

Table 3.1: The location of the sampling sites.

Sampling Site		Location	Type of Aquaculture Product
Johor	J1	01°32.100'N,104°03.280'E	Prawn
	J2	01°32.233'N,104°03.300'E	Prawn
	J3	01°32.383'N,104°03.250'E	Prawn
	J4	01°26.283'N,103°35.200'E	Prawn
	J5	01°32.516'N,104°07.016'E	Prawn
	J6	02°22.700'N,103°52.557'E	Prawn
Malacca	M1	02°06.350'N,102°29.110'E	Prawn
	M2	02°08.090'N,102°23.480'E	Fish
	M3	02°08.020'N,102°22.370'E	Prawn
	M4	02°08.020'N,102°24.110'E	Prawn
Selangor	S1	03°01.348'N,101°16.462'E	Fish
	S2	03°01.286'N,101°16.663'E	Fish
Kelantan	K1	05°51.817'N,102°29.767'E	Fish
	K2	05°52.139'N,102°29.550'E	Prawn
	K3	06°07.504'N,102°21.773'E	Prawn
Perak	P1	04°31.764'N,100°39.139'E	Prawn, Fish
	P2	04°33.999'N,100°40.405'E	Prawn
	P3	04°51.704'N,100°34.238'E	Fish
	P4	04°45.903'N,100°37.214'E	Fish
	P5	04°11.016'N,100°39.467'E	Prawn
	P6	04°12.733'N,100°39.100'E	Prawn
	P7	04°17.800'N,100°41.267'E	Prawn
Pahang	PA1	03°35.102'N,103°23.842'E	Fish
	PA2	03°36.098'N,103°24.203'E	Prawn
	PA3	03°26.419'N,103°25.655'E	Fish
	PA4	03°26.589'N,103°25.752'E	Prawn
Penang Island	PI1	05°18.383'N,100°18.917'E	Fish
	PI2	05°19.020'N,100°19.340'E	Fish
	PI3	05°22.560'N,100°11.340'E	Prawn

3.2 Antibiotic extraction and quantification of antibiotic residue in water

3.2.1 Chemical and standards

Twenty-six selected antibiotics belonging to six antibiotics classes: diaminopyrimidine, fluoroquinolones, macrolides, sulfonamides, tetracyclines and others (lincomycin, carbadox) were analyzed. They are trimethoprim (TMP), ciprofloxacin (CIP), enrofloxacin (ENRO), ofloxacin (OFX), norfloxacin (NOR), nalidixic acid (NAL), carbadox (CAR), lincomycin (LIN), azithromycin (AZM), clarithromycin (CTM), erythromycin-H^2O (ETM), roxitromycin (RTM), tylosin (TYL), sulfadimethoxine (SMA), sulfapyridine (SPD), sulfathiazole (STZ), sulfamethoxazole

(SMX), sulfamethazine (SMT), sulfamerazine (SMR), sulfamethizole (SMZ), doxycycline (DOX) , minocycline (MNC), chlortetracycline (CTC), oxytetracycline (OTC) and tetracycline (TC). SPD, RTM, TC, MNC and NOR were purchased from Sigma-Aldrich Co. (St. Louis, Mo, USA). SMX, SMR, SMA, SMT, TMP, CTM, OTC, CTC, NAL, and CAR were purchased from Wako pure chemicals Co. (Osaka, Japan). STZ, SMZ, ETM, TYL, and LIN were purchased from Honeywell Riedel-de Haen Co. (Seelze, Germany). AZM and DOX were purchased from LKT laboratories Co. (St Paul, USA) and ICN Biomedicals Co. (Santa Ana, USA), respectively. OFX and ENRO were purchased from Hayashi pure chemicals Co. (Osaka, Japan). Oxytetracycline-$^{13}C_1$,d_3, sulfamethoxazole-d_4, clarithromycin-d_3, roxithromycin-d_9 and norfloxacin-d_5 were used as surrogate standards and were purchased from Hayashi pure chemicals Co. (Osaka, Japan). All the antibiotic standards were prepared in methanol with respective concentration and stored in freezer.

All solvents were in HPLC grade. Methanol, acetonitrile, formic acid (> 99.5%) and Ethylenediamine tetraacetic acid disodium (Na_2EDTA) were obtained from Wako Pure Chemicals (Osaka, Japan). Ultra-pure water was prepared by Milli-Q ultrapure water system (Millipore, Bedford, MA, USA).

3.2.2 Antibiotic extraction and quantification

One to two L water samples were precisely taken and filtered through pre-combusted GF/F (Satorius, Göttingen Germany) with nominal pore size of 0.7µm. The filtrates were adjusted to pH 3 with 3 mol/L sulfuric acid (Appendix A) and 0.2 g of Na_2EDTA was added as chelating agent. Solid phase extraction (SPE) was conducted with Oasis Hydrophilic-lipophilic balance (HLB) cartridges (500 mg, Waters, UK) and the Supelco Visiprep SPE system (Supelco, USA) to concentrate the target antibiotic. Prior to extraction, the cartridge was precondition with 6 mL of methanol, 6 mL of ultra-pure

water and 6 mL of 10 mmol/L acidified Na_2EDTA buffer (Appendix A). The filtrates were pass through the SPE cartridge at a flow rate of 10 min/mL. After the filtrates were loaded, the cartridge was washed with 10 mL of acidified ultra-pure water and dried under nitrogen flow for 30 minutes. The analyte was then eluted with 4 x 2 mL methanol containing 0.1% (v/v) formic acid. The eluent was combined and spiked with 50 µL of surrogate mixture consisting of sulfamethoxazole-d_4, clarithrobycin-d_3, roxithromycin-d_9, and oxytetracycline-$^{13}C_1$, -d_3, norfloxacin-d_5 (500 ng/mL each, in methanol). The eluent was then concentrated to around 0.5 mL in a rotary evaporator and transferred to a 4 mL amber plastic vial. The eluent in the vial was evaporated to complete dryness under a nitrogen stream at 80 ºC and dissolved in an appropriate volume (20 mL – 100 mL) of H_2O/acetonitrile (94:6 v/v) containing 0.1 % formic acid, providing preconcentration factor of 10 to 100. A 20 µL aliquot was injected into a liquid chromatograph (Accela, Thermo Fisher Scientific, Yokohama, Japan) equipped with a tandem mass spectrometer (LC-MS/MS) (Quantum Access, Thermo Fisher Scientific, Yokohama, Japan). The antibiotics were separated in an Xterra MS C18 (2.1 mm i.d. 50 mm; particle size: 2.5 µm; Waters) with a guard column (Xterra MS C18; 2.1 mm i.d. 20 mm; particle size: 3.5 µm; Waters), using a binary gradient system (solvent A: 0.1% formic acid in H_2O; solvent B: acetonitrile), at a flow rate of 0.2 mL/min. The run started at 5% B for 5 min, followed by a 11min linear gradient to 95% B, after which the initial conditions were reestablished, and the column was equilibrated for 17 min. Analytes were quantified in selected reaction monitoring (SRM) mode with positive electrospray ionization (ESI) in positive mode. The m/z values of the precursor ion (Q1) and two monitored product ions (Q3) are listed in Appendix B.

To identify antibiotics, the retention times and the area ratios of the two product ions in each sample with the average retention time and peak ratios of standards in all measurements were compared. The criteria difference between samples and the standard

was within 0.3 min for the retention time and 20% for the area ratio of the two product ions. External calibration curves (area of individual components as a function of their concentrations) were used for quantification. Calibration lines of the individual antibiotics, with 7 concentration levels (1, 3, 5, 10, 30, 50 and 100 ng/mL), were used on a routine basis. The linearity of the calibration curve in this range was confirmed ($R^2 > 0.99$). Final concentrations of most of the samples in the vials were within the range of the calibration lines. When the final concentrations were lower than the lowest standard concentration (1 ng/mL), the concentration in the sample was calculated by interpolation from the calibration lines between 1 ng/mL and the origin. The concentrations of the target antibiotics were corrected against the recovery of the surrogates as indicated in Appendix C.

3.2.3 Analytical performance

Based on successive dilution of the standard mixture solution, 0.03 ng/mL was determined as lowest concentration of reliable detection for all the target antibiotics except for TYL and TC where 0.3 ng/mL was lowest concentration of reliable detection. Considering highest preconcentration factor (i.e., 100), the limit of detection (LOD) was determined at 0.3 ng/L of sample water for all the target antibiotics except for TYL and tetracyclines with 3 ng/L of LOD. Procedural blanks were run for each set of sample analysis and were used to calculate the LOQ. The LOQ was defined as 10 times the procedural blank value. LOQ were normally 2 ng/L for the target antibiotics except for TYL and tetracyclines with 20 ng/L of LOD.

Reproducibility was determined by triplicate analysis of effluent from a sewage treatment plant (STP). Relative standard deviations (RSD) of concentrations of the target compounds were < 11%. For the aquaculture water samples, solid phase extraction was done on-site without spiking the surrogates which were spiked after

elution of target compounds from SPE cartridge. That is extraction efficiency on SPE was not corrected, though loss during evaporation, transfer and matrix effects on LC-MS/MS analysis were corrected by using recovery. Thus, the extraction efficiency was checked by the analysis of sewage effluent with or without native standards before SPE. Recoveries, i.e., extraction efficiencies ranged from 78 % to 132 %. This shown that the reported concentrations were reliable with this range of accuracy.

3.3 Antibiotic resistance genes

3.3.1 DNA extraction from membrane filter

For ARGs quantification, total DNA of natural bacterial assemblages were trapped on 0.2 µm polycarbonate membrane filter (Merck Millipore, Germany) and extracted according to Suzuki et al. (2013). The total DNA trapped on polycarbonate membrane filter was cut into small pieces and placed into a sterile 2 mL microcentrifuge tube. Prior the DNA extraction, shredded membrane was treated with 1 mL of cetyl-trimethylammonium bromide (CTAB) buffer (Appendix D) and allowed to freeze overnight at -20°C. The freeze tube that contained treated shredded-membrane was thawed under room temperature for 5 -15 min followed by adding in 4 µL beta-mecaptoethanol (final concentration of 0.4% (v/v)). The treated shredded-membrane was then vortex and incubated at 65°C for 15 min. The membrane-contained microcentrifuge tube was then placed in a rotating platform for 20 min at room temperature after added in 900 µL of chloroform:isoamyl alcohol (24:1). The homogenized membrane-contained microcentrifuge tube was then spun at 12500 rpm at 4°C for 15 min to form a two-layer liquid. The aqueous layer (top) was collected without disturbing the interface and transferred into a new sterile 2 mL microcentrifuge tube. Extraction was repeated by adding in 900 µL chloroform:isoamly alcohol (24:1) to the new microcentrifuge tube which contains previous extracted aqueous layer and spun again. The aqueous layer was extracted to a sterile 1.5 mL microcentrifuge tube and

added with 90 µL of 3M of sodium acetate (Appendix E) and mix well by inverting the tube few times 800 µL of isopropanol was added into the aqueous solution and mixed before incubating at -80°C for 2 hours. After the incubation, the aqueous solution was brought to spin at 12500 rpm at 4°C for 30 min. After centrifuged, supernatant was decanted, leaving just the DNA pellet. DNA pellet was then washed with 500 µL of 70% ethanol and brought to spin for 5 min at 12500 rpm at 4°C followed by decantation of supernatant and vacuum dry the pellet for 5 min. After the pellet has completely dried, 50 µL of autoclaved TE buffer (Appendix F) was used to resuspend the DNA pellet and store at -20 °C. DNA quality and quantity was determined by using spectrophotometer (E-Spect, Malcom, Japan). Ratio for A260/A230 and A260/A280 was recorded.

3.3.2 Quantification PCR of antibiotic resistance genes

Four selected ARGs: *tet*(M), *sul*1, *sul*2 and *sul*3 and 16S rRNA gene were quantified by quantitative PCR (qPCR) using CFX 96 Real-Time system (Biorad, Laboratories, Hercules, CA, USA) according to the method established by Suzuki et al (2013). The 16S rRNA gene was analyzed to quantify the total bacteria in the collected water samples. qPCR amplification was performed in a 20 µL reaction volume containing of 2X Sso Fast EvaGreen Supermix (Bio-Rad), 500 nM of each primer and 1 µL of 50 ng/µL DNA template. Each sample was measured in triplicates. The amplification condition and primer sequence used for detection of 16S rRNA, *sul*1, *sul*2, *sul*3 and *tet*(M) are shown in Table 3.2. Standard curve was generated using known quantities of plasmid DNA that carried the cloned target genes. Ten times serial dilution was performed and generated a five-point (16S rRNA) and six-point [*tet*(M), *sul* genes] standard curve in triplicate for each qPCR analysis. The linearity of the calibration curve was above 0.99, efficiency for each run was more than 80% and the value of slope was within -3.0 to -3.5. The ARGs were normalized to 16S rRNA (copies/16S) and used to report and discuss the results.

Table 3.2: qPCR primer sequences, target and conditions of reactions.

Target gene	Primers	Sequences	Conditions
16S rRNA[a]	*Bact1369F* *Bact1492R*	cggtgaatacgttcycgg ggwtaccttgttacgactt	95ºC for 30 s (1 cycle); 95ºC for 5 s and 50ºCfor 10 s (40 cycles)
tet(M)[b]	*tet*(M) *F* *tet*(M) *R*	gcaattctactgatttctgc ctgtttgattacaatttccgc	95ºC for 30 s (1 cycle); 95ºC for 10 s and 57ºC for 20 s (40 cycles)
*sul*1[c]	*qsul 1F* *qsul 1R*	ccgttggccttcctgtaaag ttgccgatcgcgtgaagt	95ºC for 30 s (1 cycle); 95ºC for 5 s and 51ºC for 10 s (40 cycles)
*sul*2[d]	*qsul 2F* *qsul 2R*	cggctgcgcttcgatt cgcgcgcagaaaggatt	
*sul*3[e]	*qsul 3F* *qsul 3R*	tccgttcagcgaattggtgcag ttcgttcacgccttacaccagc	95ºC for 30 s (1 cycle); 95ºC for 5 s and 60ºC for 20 s (40 cycles)

For all genes, melting curves was at 60ºC - 95ºC for 5 s/step
[a]. Suzuki et al. (2000); [b]. Tamminen et al. (2011); [c]. Heuer and Smalla (2007); [d]. Heuer et al. (2008); [e]. Pei et al. (2006)

3.4 Ecological risk assessment

In order to evaluate the potential ecological effect of detected antibiotic in the environment, a Risk Quotient (RQ) was calculated according to the European technical guidance document on risk assessment (European Commission, 2003). The RQ was calculated through the predicted environment concentration (PEC) or measured environmental concentration (MEC) and then divided by predicted no-effect concentration (PNEC). As stated by the European technical guidance document on risk assessment (European Commission, 2003), the value of assessment factor was selected based on the type of toxicity data EC50/LC50. In this study, the toxicity data of the selected antibiotic on non-target organisms were selected from other toxicological studies (Appendix G). The predicted no-effect concentration is the division of EC50/LC50 and assessment factor. According to Hernando et al. (2006), the RQ were classified into three risk level, high (RQ > 1), medium (0.1 < RQ < 1) and low (RQ < 0.1).

3.5 Statistical analysis

Correlation and linear regression analysis were conducted to analyses the effect between antibiotic residue concentration and ARGs detected in aquaculture farm. Cluster analysis was performed to identify the spatial distribution and the detected antibiotic residue in aquaculture farm using Past version 4.03 (Hammer et al., 2001).

CHAPTER 4: RESULTS

4.1 Antibiotic residues

Twenty-three antibiotics belonging to six classes were detected in Malaysian aquaculture farms, including seven sulfonamides (SPD, STZ, SMR, SMT, SMZ, SMX, SMA), five fluoroquinolones (CIP, ENRO, OFX, NOR, NAL), four tetracyclines (MNC, OTC, TC, DOX), five macrolides (AZM, TYL, ETM, CTM, RTM), TMP and LIN. The concentration of detected antibiotics ranging from < LOQ to 957 × 10^3 ng/L (Table 4.1). Tetracyclines had the highest detection frequencies (83%) followed by sulfonamides (72%) and fluoquinolones (69%).

For tetracycline compounds tested, OTC was the most frequently detected (41%) but the concentrations were less than LOQ. TC and MNC were detected in the range <LOQ – 73 ng/L and <LOQ – 245 ng/L, respectively. TC was detected in the farms from Johor (J6: 2.3 ng/L), Perak (P1: 2.0 ng/L and P6: 7.3 ng/L) and Pahang (PA2: 1.4 ng/L). MNC was detected in Johor (J1: < LOQ, J5: 5.1 ng/L), Malacca (M4: 2.4 ng/L), Pahang (PA2: < LOQ), Penang Island (P12: < LOQ), where the highest concentration was recorded in Perak (P6: 245 ng/L,). DOX was detected only in one farm located in Perak (P5: 234 ng/L) whereas CTC was not detected in any of the farms.

All the sulfonamide compound tested was detected (< LOQ to 282.4 ng/L) in all the states except for Selangor. SMR (41%) and STZ (21%) were the most frequently detected. The highest concentration of STZ (282.4 ng/L) and SPD (29 ng/L) were found in Pahang (PA2) and Perak (P6), respectively whereas other sulfonamides compounds concentrations in most of the farm (95%) were less than 6 ng/L. SMT was only detected in farms located in Pahang (PA2: 5.68 ng/L and PA4: 2.21 ng/L) and Penang (PI1, PI2, PI3: 0.72 ng/L, 1.14 ng/L and 2.98 ng/L) whereas SMZ was present in Pahang (PA3: 1.11 ng/L and 3.63 ng/L) and Perak (P5:4.79 ng/L and P7:0. 84 ng/L).

The five tested fluoroquinolone antibiotics were detected with concentrations ranging from <LOQ – 131 x 10^3 ng/L. ENRO was the most frequently detected (52%) followed by NAL (2 8%), OFX, (21%), CIP (14%) and NOR (14%). CIP, ENRO and NAL were found dominant in Perak while NOR and OFX were dominant in Kelantan and Pahang, respectively. The highest concentration of ENRO, CIP, NOR and NAL were detected in P6 in Perak with concentrations at 958 × 10^3 ng/L, 131 × 10^3 ng/L, 6.7 × 10^3 ng/L and 946 ng/L, respectively.

Macrolides (AZM, ETM, CTM, TYL and RTM) were found in notably low concentrations ranging from <LOQ – 6.9 ng/L and accounted for the lowest total concentration (20 ng/L). No macrolides were detected in Pahang. TMP was only detected in Pahang (PA4), Perak (P4) and Penang (PI2) at concentrations of 4.7 ng/L, 0.5 ng/L and 0.4 ng/L, respectively. LIN was found in all states (<LOQ – 74.7 ng/L) with the highest concentration detected in Pahang (PA6: 74.7 ng/L). CAR was not detected in all the water samples at all sites.

Table 4.1: The concentration of sulfonamides, macrolides, tetracyclines, fluoroquinolones, trimethoprim, lincomycin and carbadox in surface water of aquaculture farm.

Location		Antibiotic (ng/L)											
		Tetracyclines (83% [a])						Fluoroquinolones (69% [a])					
		MNC (24% [b])	OTC (41% [b])	TC (31% [b])	CTC	DOX (17% [b])	Mean	CIP (14% [b])	ENRO (52% [b])	OFX (21% [b])	NOR (14% [b])	NAL (28% [b])	Mean
Johor	J1	<LOQ	n.d.	n.d.	n.d.	n.d.	6.91	n.d.	3.08	2.75×10^{-1}	2.51×10^{1}	n.d.	9.47
	J2	n.d.	<LOQ	n.d.	n.d.	n.d.	7.10	n.d.	1.81	n.d.	n.d.	n.d.	1.81
	J3	n.d.	n.d.	<LOQ	n.d.	n.d.	4.70	n.d.	4.56	n.d.	n.d.	n.d.	4.56
	J4	n.d.	n.d.	n.d.	n.d.	n.d.	-	n.d.	n.d.	n.d.	n.d.	n.d.	-
	J5	5.05×10^{1}	<LOQ	n.d.	n.d.	n.d.	30.41	n.d.	n.d.	n.d.	n.d.	2.21×10^{1}	22.06
	J6	n.d.	n.d.	2.31×10^{1}	n.d.	n.d.	23.04	n.d.	n.d.	n.d.	n.d.	1.84×10^{1}	18.42
Malacca	M1	n.d.	n.d.	n.d.	n.d.	n.d.	-	n.d.	n.d.	n.d.	n.d.	3.12×10^{1}	31.20
	M2	n.d.	<LOQ	n.d.	n.d.	<LOQ	5.09	n.d.	n.d.	n.d.	n.d.	n.d.	-
	M3	n.d.	<LOQ	n.d.	n.d.	<LOQ	4.23	n.d.	n.d.	n.d.	n.d.	n.d.	-
	M4	2.43×10^{1}	n.d.	n.d.	n.d.	n.d.	24.26	n.d.	n.d.	n.d.	n.d.	n.d.	-
Selangor	S1	n.d.	<LOQ	<LOQ	n.d.	n.d.	4.61	n.d.	n.d.	n.d.	n.d.	n.d.	-
	S2	n.d.	<LOQ	n.d.	n.d.	n.d.	6.30	n.d.	<LOQ	n.d.	n.d.	n.d.	-
Kelantan	K1	n.d.	<LOQ	n.d.	n.d.	n.d.	3.88	1.46×10^{1}	3.73	<LOQ	<LOQ	n.d.	0.23
	K2	n.d.	<LOQ	n.d.	n.d.	n.d.	111.60	n.d.	4.93	n.d.	3.56×10^{1}	n.d.	5.67
	K3	n.d.	<LOQ	n.d.	n.d.	n.d.	26.39	n.d.	n.d.	n.d.	n.d.	n.d.	20.25
Perak	P1	n.d.	n.d.	2.00×10^{1}	n.d.	<LOQ	12.87	n.d.	5.55×10^{1}	5.45×10^{-1}	n.d.	n.d.	-
	P2	n.d.	<LOQ	n.d.	n.d.	n.d.	168.82	n.d.	1.64×10^{1}	n.d.	n.d.	n.d.	28.03
	P3	1.22×10^{1}	n.d.	n.d.	n.d.	n.d.	12.18	n.d.	n.d.	n.d.	n.d.	n.d.	16.43
	P4	n.d.	n.d.	<LOQ	n.d.	n.d.	0.99	n.d.	n.d.	n.d.	n.d.	1.62×10^{1}	-
	P5	n.d.	n.d.	n.d.	n.d.	2.34×10^{2}	234.23	n.d.	8.27×10^{-1}	n.d.	n.d.	1.03×10^{2}	16.17
	P6	2.45×10^{2}	<LOQ	7.30×10^{1}	n.d.	n.d.	180.62	1.31×10^{5}	9.58×10^{5}	n.d.	6.67×10^{3}	9.46×10^{2}	51.72
	P7	n.d.	n.d.	n.d.	n.d.	n.d.	-	8.61×10^{1}	2.13×10^{2}	n.d.	n.d.	<LOQ	274×10^{3}
Pahang	PA1	n.d.	n.d.	n.d.	n.d.	<LOQ	0.55	n.d.	n.d.	5.33×10^{-1}	n.d.	n.d.	0.53
	PA2	<LOQ	n.d.	1.35×10^{1}	n.d.	n.d.	20.42	n.d.	n.d.	n.d.	n.d.	n.d.	-
	PA3	n.d.	n.d.	<LOQ	n.d.	n.d.	0.49	n.d.	1.08	n.d.	n.d.	n.d.	1.08
	PA4	n.d.	n.d.	n.d.	n.d.	n.d.	-	1.58×10^{1}	3.55	<LOQ	n.d.	1.93×10^{1}	9.72
Penang	PI1	n.d.	n.d.	<LOQ	n.d.	n.d.	4.65	n.d.	<LOQ	2.94×10^{-1}	n.d.	n.d.	0.33
	PI2	<LOQ	<LOQ	n.d.	n.d.	n.d.	6.50	n.d.	7.46	n.d.	n.d.	n.d.	7.46
	PI3	n.d.	n.d.	n.d.	n.d.	n.d.	-	n.d.	n.d.	n.d.	n.d.	n.d.	-

Table 4.1: continued.

Location		Antibiotic (ng/L)																	
		Sulfonamides (72%[a])								Macrolides (55% [a])						Others			∑Antibiotic
		SPD (17%[b])	STZ (20% [b])	SMR (34% [b])	SMT (17% [b])	SMZ (13% [b])	SMX (17% [b])	SMA (10% [b])	Mean	AZM (17% [b])	TYL (7% [b])	ETM (44% [b])	CTM (13% [b])	RTM (7% [b])	Mean	TMP (10% [b])	LIN (55% [b])	CAR	
Johor	J1	n.d.	2.55	<LOQ	n.d.	n.d.	n.d.	1.40	1.32	n.d.	n.d.	<LOQ	<LOQ	n.d.	0.15	n.d.	n.d.	n.d.	3.96×10^{1}
	J2	n.d.	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	0.49	6.88×10^{-1}	n.d.	n.d.	n.d.	n.d.	0.69	n.d.	<LOQ	n.d.	1.04×10^{1}
	J3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-	1.54	n.d.	<LOQ	<LOQ	n.d.	0.63	n.d.	n.d.	n.d.	1.11×10^{1}
	J4	n.d.	n.d.	n.d.	n.d.	n.d.	3.35	n.d.	3.35	n.d.	n.d.	n.d.	n.d.	n.d.	-	n.d.	<LOQ	n.d.	3.77
	J5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-	n.d.	n.d.	n.d.	n.d.	n.d.	-	n.d.	<LOQ	n.d.	8.31×10^{1}
	J6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-	n.d.	n.d.	<LOQ	2.7×10^{-1}	n.d.	0.36	n.d.	7.47×10^{1}	n.d.	1.17×10^{2}
Malacca	M1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-	n.d.	n.d.	<LOQ	n.d.	n.d.	0.86	n.d.	n.d.	n.d.	3.21×10^{1}
	M2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-	<LOQ	n.d.	<LOQ	n.d.	<LOQ	0.33	n.d.	<LOQ	n.d.	1.12×10^{1}
	M3	n.d.	n.d.	1.57	n.d.	n.d.	n.d.	n.d.	1.57	n.d.	n.d.	n.d.	n.d.	n.d.	-	n.d.	n.d.	n.d.	1.00×10^{1}
	M4	n.d.	<LOQ	1.23	n.d.	n.d.	3.78	n.d.	1.87	n.d.	n.d.	n.d.	n.d.	n.d.	-	n.d.	n.d.	n.d.	2.99×10^{1}
Selangor	S1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-	n.d.	n.d.	<LOQ	n.d.	<LOQ	0.48	n.d.	<LOQ	n.d.	1.04×10^{1}
	S2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-	n.d.	n.d.	<LOQ	<LOQ	n.d.	0.85	n.d.	<LOQ	n.d.	8.28
	K1	n.d.	n.d.	n.d.	n.d.	n.d.	1.94	n.d.	1.94	n.d.	n.d.	<LOQ	n.d.	n.d.	0.28	n.d.	n.d.	n.d.	2.88×10^{1}
Kelantan	K2	<LOQ	n.d.	<LOQ	n.d.	n.d.	n.d.	n.d.	0.14	n.d.	2.28×10^{-1}	<LOQ	n.d.	n.d.	0.21	n.d.	3.77×10^{1}	n.d.	1.91×10^{2}
	K3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-	n.d.	n.d.	n.d.	n.d.	n.d.	-	n.d.	n.d.	n.d.	2.64×10^{1}
	P1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.03	n.d.	n.d.	n.d.	n.d.	n.d.	-	n.d.	n.d.	n.d.	8.58×10^{1}
	P2	1.67×10^{-1}	5.20×10^{-1}	4.71×10^{-1}	n.d.	n.d.	n.d.	n.d.	0.39	n.d.	n.d.	n.d.	n.d.	n.d.	-	n.d.	n.d.	n.d.	1.86×10^{2}
	P3	n.d.	n.d.	3.33×10^{-1}	n.d.	n.d.	n.d.	n.d.	0.33	<LOQ	n.d.	n.d.	n.d.	n.d.	0.34	n.d.	1.80×10^{-1}	n.d.	1.30×10^{1}
Perak	P4	n.d.	n.d.	1.18	n.d.	n.d.	n.d.	1.57	1.37	n.d.	n.d.	<LOQ	n.d.	n.d.	1.14	5.24×10^{-1}	1.60×10^{-1}	n.d.	2.17×10^{1}
	P5	1.20	n.d.	n.d.	n.d.	4.79	n.d.	n.d.	2.99	n.d.	n.d.	n.d.	n.d.	n.d.	-	n.d.	n.d.	n.d.	3.44×10^{2}
	P6	2.91×10^{1}	n.d.	4.20	n.d.	n.d.	n.d.	n.d.	16.66	6.95	n.d.	n.d.	n.d.	n.d.	6.95	n.d.	9.05×10^{-1}	n.d.	1.09×10^{6}
	P7	n.d.	n.d.	3.93×10^{-1}	n.d.	8.36×10^{-1}	n.d.	n.d.	0.61	n.d.	n.d.	<LOQ	n.d.	n.d.	1.11	n.d.	1.84	n.d.	3.03×10^{2}
	PA1	n.d.	n.d.	<LOQ	n.d.	n.d.	n.d.	n.d.	0.05	n.d.	n.d.	n.d.	n.d.	n.d.	-	n.d.	LOQ	n.d.	1.16
Pahang	PA2	n.d.	2.82×10^{2}	3.83	5.68	n.d.	n.d.	n.d.	97.30	n.d.	n.d.	n.d.	n.d.	n.d.	-	n.d.	5.40×10^{-1}	n.d.	3.33×10^{2}
	PA3	n.d.	n.d.	n.d.	n.d.	1.11	3.21	n.d.	2.16	n.d.	n.d.	n.d.	n.d.	n.d.	-	n.d.	n.d.	n.d.	5.89
	PA4	<LOQ	n.d.	n.d.	2.21	3.63	n.d.	n.d.	2.00	n.d.	n.d.	n.d.	n.d.	n.d.	-	4.68	n.d.	n.d.	4.95×10^{1}
	PI1	n.d.	<LOQ	<LOQ	7.21×10^{-1}	n.d.	1.97	n.d.	0.70	n.d.	n.d.	<LOQ	n.d.	n.d.	0.53	n.d.	<LOQ	n.d.	8.76
Penang	PI2	n.d.	n.d.	n.d.	1.14	n.d.	n.d.	8.54×10^{-1}	1.00	n.d.	<LOQ	<LOQ	n.d.	n.d.	0.46	3.47×10^{-1}	<LOQ	n.d.	2.42×10^{1}
	PI3	n.d.	n.d.	n.d.	2.98×10^{0}	n.d.	n.d.	n.d.	2.98	n.d.	n.d.	n.d.	n.d.	n.d.	-	n.d.	<LOQ	n.d.	3.23

LOQ = Limit of quantification, n.d. = non- detected, Mean and sum were calculated using the measured values if above the LOQ, the 1/2 MQL if < MQL and 0 if not detected
J=Johor, M = Malacca, S = Selangor, K= Kelantan, P = Perak, PA= Pahang, PI = Penang Island
[a] Detection frequency was calculated based on the number of farms were detected with the present of antibiotic in antibiotic class.
[b] Individual antibiotic detection frequency was calculated based on the number of farms were detected with the present of antibiotic

4.2 Antibiotic resistance genes

The *sul* genes detected among the aquaculture farms ranged from 10^{-7} – 10^{0} copies/16S (Figure 4.1). Among the targeted *sul* genes, *sul*2 was the most abundant (10^{-6} – 10^{0} copies/16S) followed by *sul*1 (10^{-5} – 10^{-1} copies/16S). The *sul*3 was the least abundant (10^{-7} – 10^{-4} copies/16S) or not detectable at most sites. The co-existence of *sul* genes were observed in which both *sul*1 and *sul*2 were predominantly found in 93% of the aquaculture farms with the exception of P1, Perak where only *sul*1 was present and PA1, Pahang where only *sul*2 was present. In contrast, the abundance of *tet*(M) (2.36×10^{-5} – 3.12×10^{0} copies/16S) was higher than *sul* genes. The highest abundance of *tet*(M) were detected in two farms located in Perak (P6: 2.42×10^{0} copies/16S and P7: 3.12×10^{0} copies/16S) (Figure 4.1).

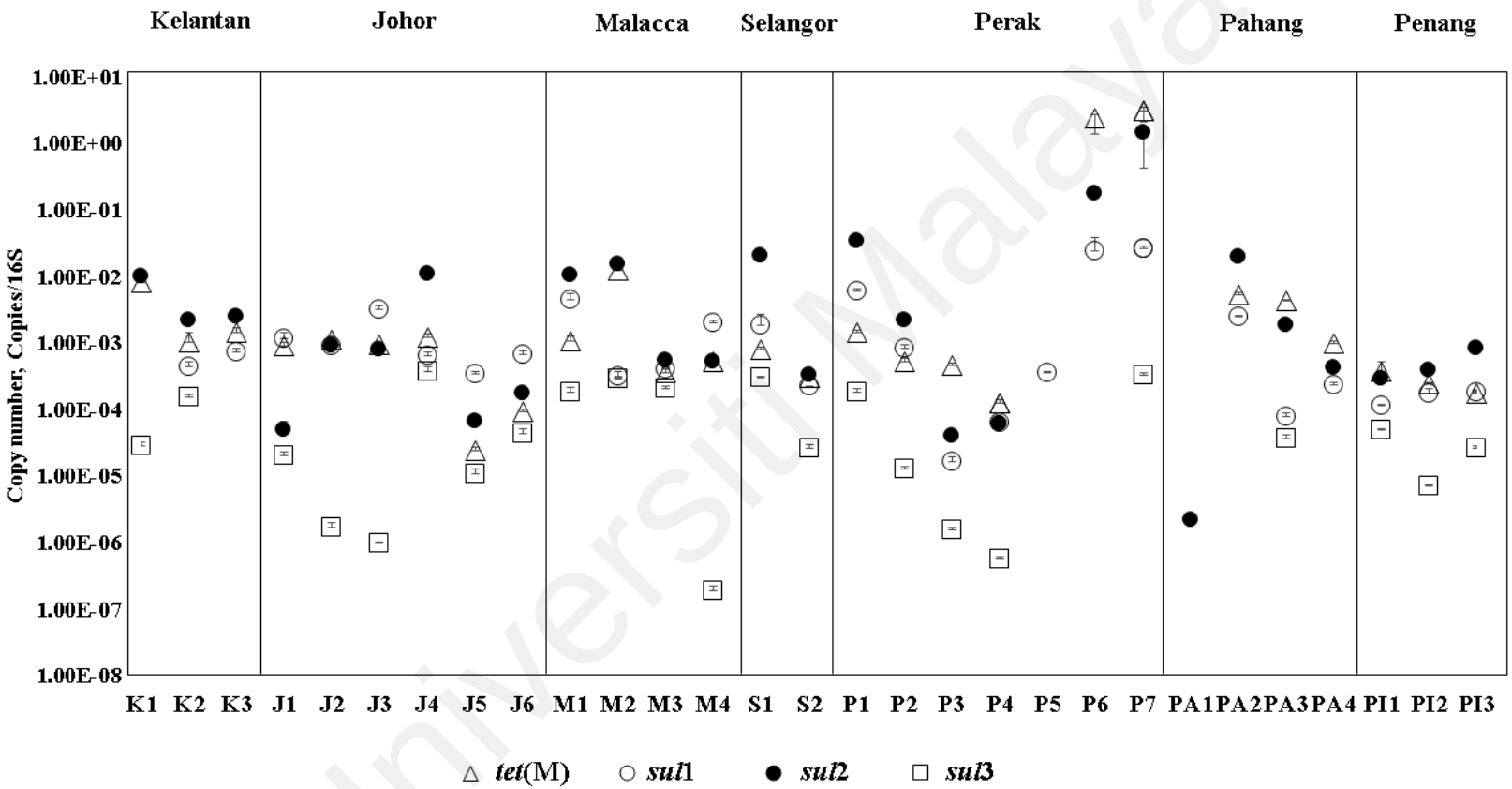

Figure 4.1: Relative abundance of selected ARGs, *tet*(M), *sul*1, *sul*2 and *sul*3 in seven main aquaculture production states. Vertical axis is copy number with normalized by 16S rRNA gene. (J=Johor, M = Malacca, S = Selangor, K= Kelantan, P = Perak, PA= Pahang, PI = Penang Island)

4.3 Environmental ecological risk

In this study, the detected antibiotics posed negligible risk to fish. However, for fluoroquinolones, in which CIP detected in P6 and P7, Perak, K1 in Kelantan and PA4 in Pahang, exhibited high risk to *Microcystis aeruginosa*. ENRO detected in Johor (J1, J2, J3), Kelantan (K1, K2), Penang (PI1, PI2), Perak (P2) and Pahang (PA4) posed medium risk to *Vibrio fischeri* whereas in Perak (P1, P6 and P7), ENRO posed high risk. NOR posed medium risk to *Vibrio fischeri* at Kelantan (K1) and Johor (J1) whereas at P6, Perak, high risk was detected (Table 4.2).

In contrast, TC only posed medium risk to *Microcystis aeruginosa* at Perak (P1, P6), Johor (J6) and Pahang (PA2) whereas DOX posed medium risk to *Synechoccus leopoliensis* at P5, Perak. Among the sulfonamides, only SMX detected at Johor (J4), Malacca, (M4), Kelantan (K1), Pahang (PA3) and Penang (PI1) posed medium risk to *Syneschococcus leopoliensis*. LIN and CTM posed a medium to high risk to *Pseudokirchneriella subcapitata* at Johor (J6) and Kelantan (K2), respectively. RQ was not calculated for NAL, MNC and SMZ due to the lack of toxicology data.

Table 4.2: The calculated RQs for the selected antibiotic detected in aquaculture farm.

Antibiotics	Johor						Melaka			Selangor			Kelantan			Perak							Pahang			Penang Island		
	J1	J2	J3	J4	J5	J6	M1	M2	M3	M4	S1	S2	K1	K2	K3	P1	P2	P3	P4	P5	P6	P7	PA 1	PA 2	PA3	PA 4	PI 1	PI 2	PI 3
Sulfapyridine																													
Sulfathiazole																													
Sulfamerazine																													
Sulfamethazine																													
Sulfamethizole *																													
Sulfamethoxazole																													
Sulfadimethoxine																													
Azithromycin																													
Tylosin																													
Clarithromycin																													
Trimethoprim																													
Lincomycin																													
Minocycline *																													
Tetracycline																													
Doxycycline																													
Ciprofloxacin																													
Enrofloxacin																													
Ofloxacin																													
Norfloxacin																													
Nalidixic acid *																													

Red = high risk, Yellow = medium risk, Green = low risk, Grey = no antibiotic was detected.
* no toxicology data available

CHAPTER 5: DISCUSSION

5.1 Antibiotic residues in aquaculture waters

In this study, 23 antibiotic residues belonging to six classes were identified (total concentration: 1.099×10^6 ng/L) in which tetracyclines, sulfonamides and fluoroquinolones were the most prevalent antibiotics detected suggesting the wide usage of these antibiotics in aquaculture farms in Malaysia (Table 4.1). Other studies in Asia have reported the frequent use of similar antibiotic compounds in aquaculture production (Rico et al., 2012; Lulijwa et al., 2019). Cluster analysis of the antibiotics identified among the states suggested three different practices of antibiotic use in the aquaculture farms. Antibiotic utilization in Malacca, Johor, Kelantan, and Pahang were more similar compared to Johor and Selangor. Perak was notably different from the other states as it was the state with the most frequently detected and the highest concentration of antibiotics (Figure5.1). The difference of concentration between Perak and other states, may be due to the farming practices of applying antibiotic to feed pellets without following the recommended guideline.

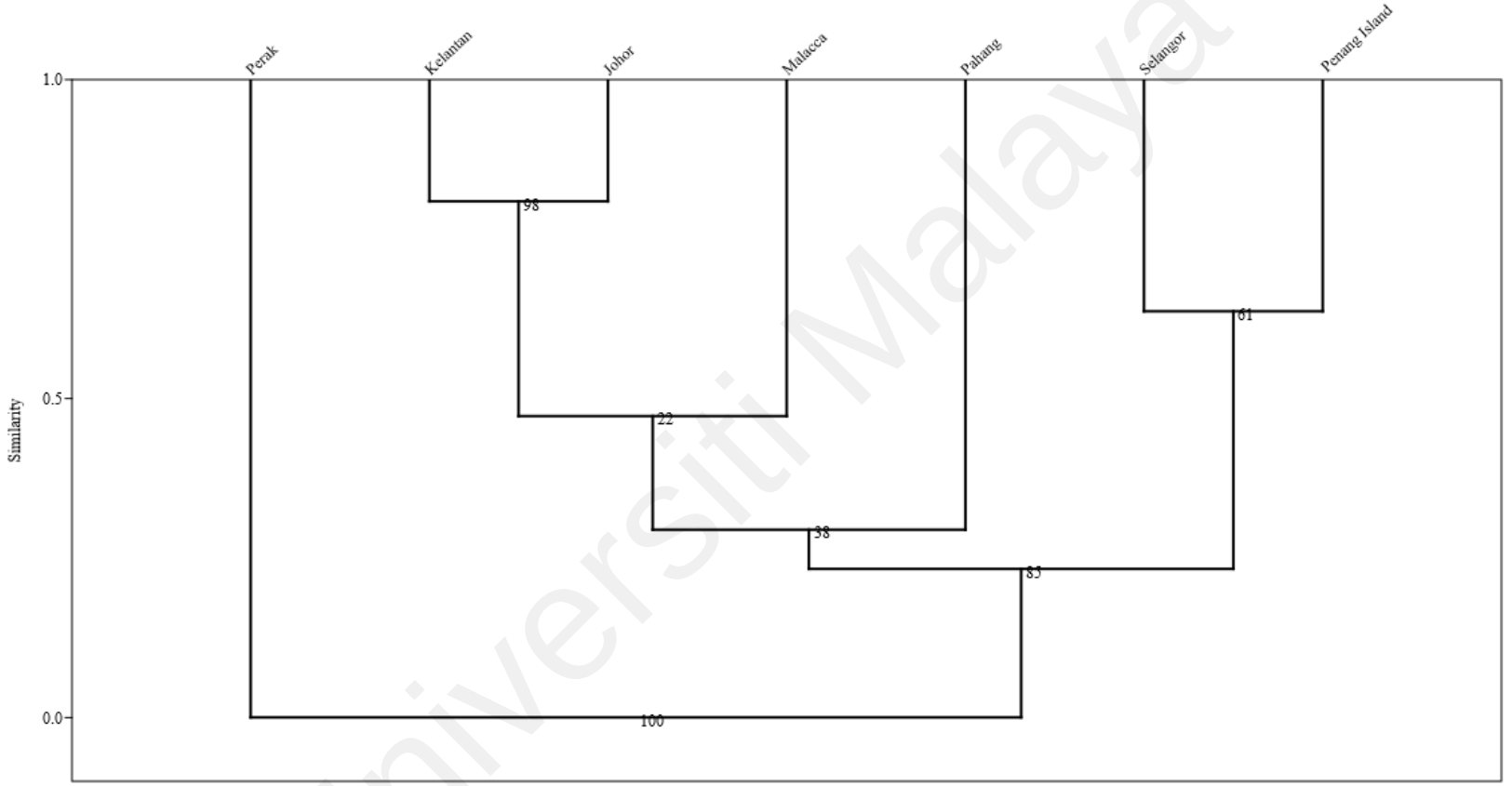

Figure 5.1: Cluster analysis of sampling sites according to the concentrations of four classes of antibiotics (tetracyclines, sulfonamides, fluoroquinolones, macrolides), trimethoprim and lincomycin in Malaysian aquaculture farm.

The variation in distribution, composition, detection frequency and concentration levels among the aquaculture farms may be attributed to the usage practices in aquaculture (e.g. disease, type of feed and feed additive containing antibiotic used by farmer), physicochemical reaction of antibiotic towards environmental parameters, and microbiological degradation of antibiotic by the aquatic or sediment bacteria (Hektoen et al., 1995; Le et al., 2005). In this study, low or negligible concentrations of macrolides were observed as most bacterial pathogens of fish are Gram negative bacteria (Haenen, 2017). Macrolides are broad spectrum antibiotics that are effective against most Gram-positive bacteria, and ETM is the only macrolide used in fish farming and shrimp hatcheries in Southeast Asia (Lavilla-Pitogo, 2017). Also, LIN was found in all the farms as it is commonly used in livestock and aquaculture infections (FAO, 2005).

Most of the farms (n=25, 86.2%) sampled in this study, used more than two antibiotics (average of five antibiotics). The highest total number of antibiotic compounds used in fish farms was nine, detected in four farms located at Kelantan (K2), Johor (J1) and Penang (PI1 and PI2), respectively. For shrimp farms, only one farm located in Perak (P6) used 11 antibiotic compounds. It is well known that there exists an extensive use of antibiotics in aquaculture around the globe (Tuševljak et al., 2013) with Asian countries more notable for their wider range of approved antibiotics (Serrano, 2005). The sampled farms in Malaysia used a relatively lower and less diverse number of antibiotic compounds than the top producing countries in Asia [Thailand, (13 antibiotics used), China (33) and Vietnam (39)] (Rico et al., 2012; Phu et al., 2016; Lulijwa et al., 2019). In contrast, Japan has significantly reduced antibiotic usage, and no antibiotic is reported in their aquaculture industry (Lulijwa et al., 2019).

The data obtained in this study has given a more insight information on the concentration and composition of antibiotic residues present in the marine aquaculture environment and understand the potential risk of antibiotics to the environment. As a first study on the occurrence and distribution of antibiotic in Malaysia aquaculture, this could help to fill up the gap of information on the usage of antibiotic, level of antibiotic pollution in aquaculture globally. With such databases, it could be able to use to identify and predict the global hotspots where antibiotic is discriminately used. Besides, the data may subsequently help in formulating sustainability guidelines to monitor the application of antibiotics and improve the quality and management in aquaculture locally. Eventually, improve food safety and quality and reduce the emergence and spread of antibiotic resistance genes. In addition, this study also suggests that the efficient of treating wastewater or effluent from aquaculture farm is a matter for further research.

5.1.1 Tetracyclines

Tetracyclines were the most prevalent antibiotic detected in this study. Tetracyclines are widely used in the aquaculture industry, animal husbandry and human therapy due to their low cost and high efficacy against a broad spectrum of bacteria, parasite, and fungi (Mo et al., 2017). In Malaysia, tetracyclines are the second highest antibiotic used and is recently reported to reach 73910 kg per year (Zakaria, 2017). These antibiotics are commonly administered in feeds or dissolved in water to be absorbed by the gills. Among tetracyclines, results showed that OTC, TC, MNC were the most commonly used tetracycline compounds among the farms, but no CTC was detected. Results were contrasted with previous findings that reported OTC, TC, CTC and DOX as the most used tetracycline for treatment and prevention of diseases in aquaculture (Shamsuzzaman & Biswas, 2012; Hazrat et al., 2016). However, this difference could be attributed to the different farms and the year of sampling. In Malaysia, OTC, TC and

CTC are permitted antibiotics used in aquaculture, and are registered under the National Pharmaceutical Regulatory Agency, Ministry of Health. OTC, CTC and DOX also fall under WHO's criteria of critically important antibiotics for human health and their usage are being restricted in veterinary and aquaculture sectors (Hassali et al., 2018).

Although the use of OTC as a growth promoter was banned by the EU in 2006 (Castanon, 2007), OTC remains the most commonly used antibiotic in animal production and aquaculture and are often detected in aquaculture water in different countries (Nonaka et al., 2007; Suzuki & Hoa, 2012). In Malaysia, pricing is one of the main reasons why OTC is more popular. In contrast, the usage of CTC and DOX is limited as they are more expensive (Treves-Brown, 2000). Moreover, OTC is poorly absorbed in fish, and a high dosage of OTC is often required. Therefore, excess OTC from aquaculture is eventually discharged to the environment (Lunestad & Goksøyr, 1990).

When OTC concentrations were not detected in this study (<LOQ), this might be attributed to its physiochemical properties, biodegradation and photodegradation. OTC half-life ranges from 21 – 25 mins in aquaculture water, 2 days in freshwater, 12 days in seawater to as high as 150 days in marine sediment depending on the environmental conditions (e.g. pH, temperature, salinity, light) (Choo, 1994; Brooks et al., 2008; Leal et al., 2016). The degradation rates of OTC in river sediment and wastewater sludge have been reported to be higher than TC (Chang & Ren, 2015; Yang et al., 2020).

Although tetracyclines remain one of the top three antibiotics used in the top producing countries in Asia, Lulijwa et al. (2019) reported a reduction from 92% to 73% in the usage of tetracyclines (Sapkota et al., 2008). In order to tackle the indiscriminate use of antibiotics, some Asian countries have banned the use of selected tetracyclines in aquaculture. TC was recently banned in Malaysia (The Sun Daily, 2020)

and is also not currently used in Vietnam and Singapore whereas CTC is not used in Indonesia, Singapore and Vietnam (ASEAN, 2013; Whitehead, 2016). Singapore is the only country that do not use OTC (ASEAN, 2013) whereas CTC and OTC are prohibited in China (Liu et al., 2017). In Thailand, OTC and TC are still authorized for use in food animal (Lulijwa et al., 2019).

MNC was also found in this study. MNC is a semisynthetic, second-generation tetracycline derivative which is typically used in humans to treat acne (Garrido-Mesa et al., 2013). To the best of my knowledge, no study has reported the presence of MNC residues in aquaculture water environments in worldwide. Moreover, this antibiotic is not authorized for use in aquaculture farms in Malaysia. Thus, further studies are needed to confirm this finding in Malaysian aquaculture farms.

5.1.2 Sulfonamides

After tetracyclines, sulfonamides were the second most prevalent antibiotic found in aquaculture farms in this study. Sulfonamides were found in all the farms with the exception of two farms located in Selangor. The presence of sulfonamides in farm waters concurred with other studies (Jayachandran et al., 2010). Sulfonamides are ubiquitous in the developing Asia aquatic ecosystem due to their low cost and more importantly, sulfonamides can be absorbed through gills. In addition, sulfonamides are highly soluble in water and highly mobile thus they can be easily transported and distributed in aquatic environments (Shi et al., 2012; Liu et a., 2017).

All the selected commonly used SAs antibiotics in animal treatment were detected in this study. SMR and STZ were the most commonly detected in Malaysia aquaculture farm. This was in contrast with other studies where SMT and SMX were commonly found in aquaculture or its adjacent environment (Giang et al., 2015; Hossain et al., 2017; Lai et al., 2018). Sulfonamides are commonly used alone or in combination with

TMP or ormetoprim for better efficacy to combat bacterial infection. However only three farms (Pahang:PA4, Perak: P4 and Penang Island: PI2) were detected in conjunction with low concentrations of TMP, suggesting that the usage of combination sulfonamides and TMP was less common in Malaysian aquaculture.

Similar to Vietnam, SMX, SMT, STZ and SMR were also detected in this study (Hoa et al., 2011; Giang et al., 2015; Harada, 2018; Thai et al., 2018). The sulfonamides composition detected here is less diverse relative to China [SMX, SMT, SPD, sulfadiazine, sulfametoxydiazine, sulfomonothoxine, sulfameter, sulfaquinoxaline, sulfachloropyridazine] and Taiwan [SMX, STZ, sulfadiazine sulfaguanidine, sulfathazine, sulfamonomethoxine and sulfadimethoxine] (Lin et al., 2008; Chen et al., 2017; Lai et al., 2018; Wang et al., 2018a; Zhong et al., 2018; Yuan et al., 2019).

5.1.3 Fluoroquinolones

For fluoroquinolones, ENRO, NAL and OFX were the most commonly used. The selected fluoroquinolones were detected among the farms with the highest total concentration of 1.097×10^6 ng/L. These antibiotics (ENRO, NAL and OFX) are widely administered in Asia aquaculture and have become more popular than oxytetracycline over the last two decades (Hektoen et al., 1995). They are stable in water and sediment (Kümmerer 2004; Le & Munekage 2004). Lulijwa et al. (2019) revealed that about 55% of the global major aquaculture producing countries applied ENRO whereas the usage of CIP and NOR were at a lower frequency. This is distinctly different from Thailand and Vietnam (Suzuki & Hoa, 2012) where 74% of Thailand shrimp farms primarily used NOR (Holmström et al., 2003). In shrimp pond areas in the mangroves of Vietnam, Le and Munekage (2004) reported that NOR is detected in all shrimp ponds and surrounding canals whereas in Taiwan aquaculture, OFX, CIP and flumequine were present (Lin et al., 2008; Lai et al., 2018). In recent years, ENRO has

been banned in Taiwan, Vietnam, Thailand but the ban in Malaysia only began from the year 2020 (MARDI, 2014; Tsai et al., 2019; The Sun Daily, 2020). This could explain why were still able to detect ENRO in the farms in Malaysia.

5.1.4 Regional comparison of antibiotic use

The composition of antibiotics varies between different countries showing the different practice of antibiotic administration in aquaculture. From the comparison of the published antibiotic concentrations available in aquaculture (Figure 5.2), the concentration of tetracyclines detected in this study were still lower than Thailand (2 - 500 ng/L) and China (0.32 – 15 × 103 ng/L) but higher than Taiwan (11 – 75 ng/L) and Korea (7.1 – 95.4 ng/L). The concentrations of sulfonamides were comparable to aquaculture water in Taiwan but relatively lower compared to mariculture and aquaculture farms in China (0.4 – 12429 ng/L), and Vietnam (0.08 – 2,390,000 ng/L).

For quinolones, the concentrations detected in Malaysia were higher than aquaculture farms in Thailand (13.2 – 490 ng/L), Taiwan (0.2 – 331 ng/L), Korea (0.88 – 54.5 ng/L) and Vietnam (0.2 - 60000 ng/L) (Figure 5.2). NOR and CIP are currently not used in Indonesia, Singapore, and Thailand (ASEAN, 2013). Although CIP has been banned in China and Vietnam, illegal use of this banned antibiotic is still being reported (Mo et al., 2017; MARDI, 2016; Chi et al., 2017; Tran et al., 2018). On the other hand, the LIN detected in this study was comparable to Vietnam (8 –10 ng/L, Shimizu et al., 2013) and Korea (<LOQ –14.8 ng/L, Kim et al., 2017) but lower than China and Taiwan (<LOQ – 1643 ng/L, Zhong et al., 2018).

A study by Segura et al. (2015), suggested that the level of a country's income has an effect on the occurrence of antibiotic in environment. However, results revealed that the type of antibiotic measured and used for comparison will also influence the former statement. Results (after excluding P6 result) showed that fluoroquinolone was two-fold

higher compared to the low-income group (Ghana, India, Indonesia, Kenya, Philippines, Vietnam, Mozambique, Pakistan) whereas tetracyclines and sulfonamides measured were in the category comparable with low-income group (sulfonamides: 15.5 – 112 ng/L tetracyclines: 29.3 – 289 ng/L). From the observations, results in this study were in contrast to the status of Malaysia as an upper middle-income country (World Bank, 2020).

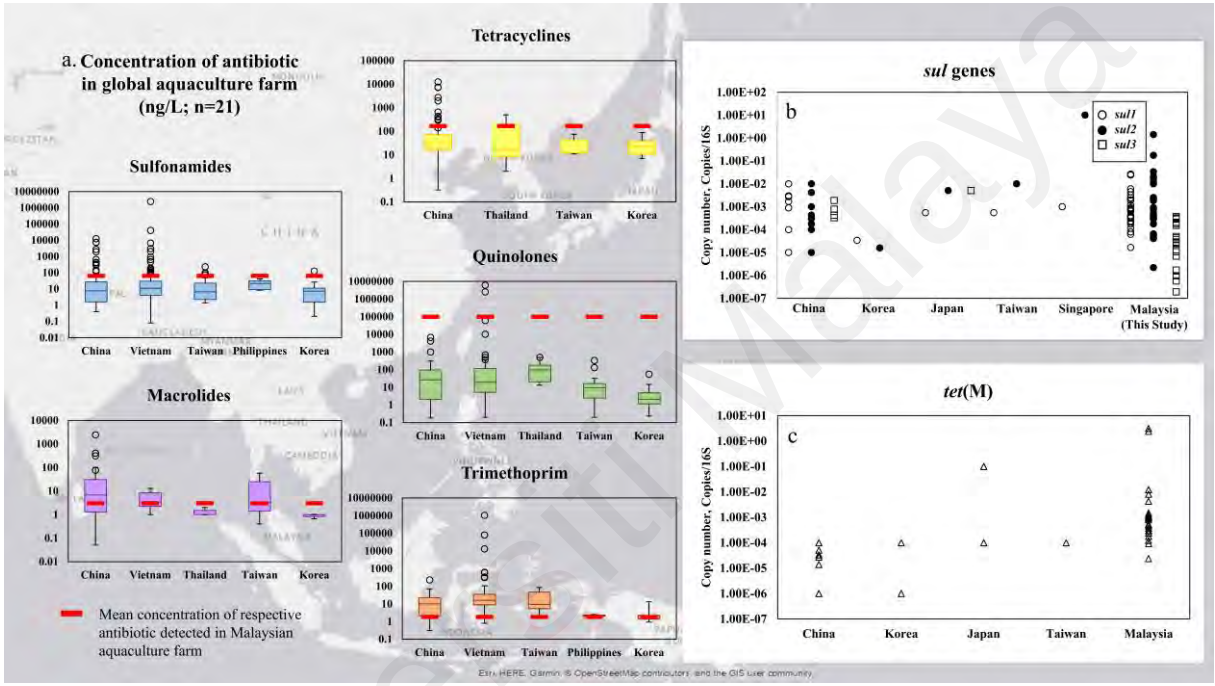

Figure 5.2: Comparison of antibiotic concentration and ARGs in aquaculture farm in East and Southeast Asia. (adapted from Le & Munekage, 2004; Lin et al., 2008; Hoa et al., 2011; Takasu et al., 2011;Gao et al., 2012b; He et al., 2012; Shimizu et al., 2013; Suzuki et al., 2013; Rico et al., 2014; Andrieu et al., 2015; Chen et al., 2015; Giang et al., 2015; Xiong et al., 2015; Song et al., 2016; Chen et al., 2017; Kim et al., 2017; Gao et al., 2018; Harada, 2018; Jang et al., 2018; Lai et al., 2018; Ng et al., 2018; Thai et al., 2018; Wang et al., 2018a; Suzuki et al., 2019; Yuan et al., 2019; Han et al., 2020)

5.2 Antibiotic resistance genes

The prevalence of *sul* genes in Malaysian aquaculture farm was in the following frequency: *sul*2>*sul*1>*sul*3. The results suggested that these genes were ubiquitous in aquaculture farms in Malaysia and was similar with marine mariculture in Japan (Suzuki et al., 2019) and marine mariculture in China (Chen et al., 2017). However, for pond aquaculture in China (Xiong et al., 2015; Su et al., 2017; Yuan et al., 2019) and effluent in Korea aquaculture farm (Jang et al., 2018), a different order of *sul*1>*sul*2>*sul*3 has been reported. The variation in gene distribution observed could be attributed to the difference in farming practices, bacterial population composition, types and antibiotic dosages used (Shimizu et al., 2013, Muñoz-Atienza et al., 2013; Rico et al., 2013). For instance, integrated fish farming practiced throughout Asia is often with a closed system aquaculture where pond water does not frequently exchange. This can result in ARGs accumulating in pond water and sediment through HGT (Watts et al., 2017).

The *sul*1 and *sul*2 values detected in this study were comparable with net-pen aquaculture in Japan (*sul*1:10^{-4} – 10^{-3} copies/16S and *sul*2: 10^{-4} – 10^{-2} copies/16S, Suzuki et al., 2019), Taiwan (*sul*1: 10^{-4} – 10^{-3} copies/16S, *sul*2:10^{-2} copies/16S, Suzuki et al., 2019) and higher than aquaculture farm in Tianji, China (*sul*1: 10^{-5} – 10^{-4} copies/16S, *sul*2: 10^{-4} – 10^{-3} copies/16S, Gao et al., 2012b), coastal aquaculture farm in South Korea (*sul*1: 10^{-6} – 10^{-5} copies/16S, *sul*2: 10^{-7} – 10^{-5} copies/16S, Jang et al., 2018) and aquaculture farm sediment (*sul*1 and *sul*2: 10^{-5}– 10^{-2} copies/16S, Gao et al., 2018). In comparison with the floating open cage aquaculture farm in Singapore (*sul*1: 10^{-4} copies/16S, *sul*2:10^{1} copies/16S) with this study the *sul*2 abundance was lower (Ng et al., 2018). For *sul*3, the abundance was generally lower than China (10^{-4} – 10^{-3} copies/16S, Xiong et al., 2015) and Japan (10^{-4} copies/16S, Suzuki et al., 2019) (Figure 5.2).

Other than *sul* genes, *tet*(M) also measured in this study. This study results are consistent with other findings that *tet*(M*)* was prevalent in aquaculture farms. The concentrations detected in this study were comparable with aquaculture farms in Taiwan (10^{-4} copies/16S, Suzuki et al., 2019), China (10^{-5} – 10^{-3} copies/16S, Gao at al., 2012b; Xiong et al., 2015; Niu et al., 2016; Yan et al., 2018), South Korea (10^{-6} – 10^{-4} copies/16S, Jang et al., 2018) and Japan (10^{-4} – 10^{-1} copies/16S; Suzuki et al., 2019).

In this study, no statistically significant ($p>0.05$) correlation was found between concentrations of antibiotic and resistance genes. The targeted genes are historically "older" ones, which are already widely dispersed even as antibiotic contamination is reduced or non-prevalent. Similar uncoupling of ARGs and antibiotics have been reported in other areas (Takasu et al., 2011; Suzuki et al., 2015). Studies have shown that exposure to low concentrations of antibiotics for a long period can exert selective pressure and their transformation products also contribute in the development and dissemination of resistant bacteria and ARGs (Gullberg et al., 2011).

The presence and prevalence of ARGs in this study may be due to long term application of antibiotics in feed and water which may result in the accumulation of antibiotic residues at aquaculture farm suggesting aquaculture may serve as reservoirs to antibiotic resistance genes. The farm then becomes a resistance hotspot to promote the growth of ARB by exchanging resistance genes and thus altering the microbial community in water and sediment (Mohamed et al., 2000). The leaching of free-antibiotic, unconsumed antibiotic-feed or undegraded antibiotic-faeces from aquaculture may also reach the groundwater and ocean through water circulation. This eventually end up in humans that consume antibiotic-contaminated drinks and food which pose a risk to public health. Therefore, further study is needed to access the occurrence of antibiotic in aquatic in order to monitors antibiotic use and to mitigate the development

and spread of multi- antibiotic resistance among environment, food and human as the aquatic environment often constitute final receiver of both anthropogenic and agriculture waste.

5.3 Environmental ecological risk

For the ecological risk analysis, it was found that farms located in Kelantan, Perak and Pahang were detected with the highest RQs. Three fluoroquinolones (ENRO, NOR, CIP) and LIN were found to have the potential to pose high risk to *M. aeruginosa*, *S. leopoliensis*, and *P. subcapitata* in aquaculture farms in Malaysia. Results of this study were concurred with the findings from South Yellow Sea and aquaculture pond water around Lake Honghu in China where these antibiotics could pose high risk to cyanobacteria and algae (Du et al., 2017; Wang et al., 2017). In this study, SMX, CTM and TC were found to pose medium risk to cyanobacteria and algae. Several studies have also reported that SMX and CTM posed medium risk to various primary producers in rivers and pond waters where there are aquaculture activities (Zheng et al., 2012, Wang et al., 2017). In contrast, recent reports revealed that SMX and TC posed high risk to algae in Pearl River and Yellow Sea, China (Xu et al., 2013; Du et al., 2017; Wang et al., 2017).

Studies have shown that the coexistence of mixed antibiotics would pose a direct or indirect threat to the environment (González-Pleiter et al., 2013; Liu et al., 2014; Wang et al., 2018b). However, the risk caused by mixed antibiotics to the environment was not evaluated in this study as a single-compound approach was only employed. In the future, environmental toxicity risk employing multi antibiotic approach is needed as the coexistence of mixed antibiotics can cause more significant adverse impact. The toxicity effect to eukaryotic organisms might be paid attention as well.

CHAPTER 6: CONCLUSION

The present study reported on the antibiotic residues, ARGs and its associated potential ecology in the seven-main aquaculture production state in Peninsular Malaysia. Our study detected 23 antibiotics with the total concentration 1.099 x 106 ng/L in which tetracyclines (83%), sulfonamides (72%) and quinolones (69%) had the highest detection frequency, indicating a wide distribution of antibiotics in aquaculture farms in Malaysia. Oxytetracycline, tetracycline, minocycline, sulfamerazine, sulfathiazole, enrofloxacin, nalidixic acid, and ofloxacin were the most abundant antibiotics. The minocycline was detected for the first time in aquaculture farms. The antibiotic residues detected were at a low or moderate level compared with Asian aquaculture farms except for quinolones. Overall, the relative abundance of resistance gene decreased according to the following frequencies: sul2>tet(M)>sul1>sul3 and no significant correlation was observed between antibiotic residue and resistance genes. Ciprofloxacin, enrofloxacin, norfloxacin and lincomycin were found to have high ecological risk to cyanobacteria and algae in Kelantan, Perak and Pahang.

In overall, this study intensifies our understanding on antibiotic profile and bacterial resistome in aquaculture wastewater, as well as their potential impacts to organisms in environment. More importantly, the result of this study has called the urgent strengthening of surveillance for the usage of antibiotic and enhanced public awareness and understanding on the risk of antibiotic resistance and residue on aquatic animal, environment and human. Prevention with proper environmental management should be conducted for aquaculture wastewater to mitigate the risks of antibiotic resistance to environment and public health through food chain. Besides, investment in research, international collaboration, coordination of policies, regulation and MRLs are needed in order to promote the antibiotic stewardship. In term of research, it is urgently needed to

further understand the interaction of pathogenic-host, and the relationship between the concentration of antibiotic residue and antibiotic resistance development, as well as to establish a model to assess the ecological and human risk of antibiotic resistance associated with multiple antibiotic residues. Moreover, education and media are the best strategies to deliver the important and prudent use of antibiotic and raise awareness on antibiotic resistance to farmer and public. As part of the stakeholder, farmers have the responsibility to improve the farm management practices to reduce the outbreak of disease and thus reduce the use of antibiotic, and improve the knowledge on antibiotic and the prudent use of antibiotic by receiving training from relevant department or organization. Together with the relevant government regulatory authorities who play important role in establish or stricter enforcement of regulations, policies and guidelines on monitoring and prudent use of antibiotic in aquaculture, the global issue – antibiotic resistance may be able to mitigate in no time.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

PUBLICATION

Thiang, E. L., Lee, C. W., Takada, H., Seki, K., Takei, A, Suzuki, S., Wang, A, and Bong, C. W. (2021). Antibiotic residues from aquaculture farms and their ecological risks in Southeast Asia: A case study from Malaysia. *Ecosystem Health and Sustainability*, 7 (1). doi: 10.1080/20964129.2021.1926337

PAPER PRESENTED

Thiang, E. L., Lee, C. W., Chai, L. C., Zhang, R. J., Zhang, G., and Bong, C. W. (2017). *Detection of selected veterinary antibiotics in Peninsular Malaysian aquaculture waters*. Paper presented at the 10th International Scientific Conference, 17 – 20 April 2017, Qingdao, China

Thiang, E. L., Lee, C. W., Chai, L. C., Zhang, R. J., Zhang, G., Suzuki, S., and Bong, C. W. (2017). *Prevalence and Ecological Risk of Antibiotics in Peninsular Malaysian Aquaculture.* Paper presented at Asia Oceania Geosciences Society (AOGS), 6 – 11 August 2017. Singapore

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Thiang, E. L., Bong, C. W., Lee, C. W., Takada, H., Suzuki, S., Wang, A, and Chai, L. C. (2018). *Occurrence of Antibiotics and Antibiotic Resistance Gene in Peninsular Malaysian Aquaculture and an Associated Ecological Risk Assessment.* Paper presented at The ASEAN Emerging Researchers Conference, 3 – 4 December 2018, Kuala Lumpur, Malaysia.