Sains Malaysiana 51(2)(2022): 345-357
http://doi.org/10.17576/jsm-2022-5102-02

Tetracycline Resistance and Prevalence of Tetracycline Resistance Genes in Bacteria from Marine Aquaculture Farms in Peninsular Malaysia

(Rintangan Tetrasiklin dan Kelaziman Gen Rintangan Tetrasiklin dalam Bakteria daripada Ladang Akuakultur Marin di Semenanjung Malaysia)

Ee Lean Thiang, Choon Yip Stanley Chai, Choon Weng Lee, Hideshige Takada, Ai Jun Wang, Lay Ching Chai & Chui Wei Bong*

ABSTRACT

The indiscriminate use of antibiotic in aquaculture and leaching of antibiotic from aquaculture to the environment may led to the emergence and spread of antibiotic resistant bacteria and their resistant genes which is a public concern. Five tetracycline antibiotics (minocycline, doxycycline, chlorotetracycline, oxytetracycline and tetracycline) and 14 types of tetracycline genes, tet*[(A), (B), (C), (D), (E), (G), (K), (L), (M), (O), (S), (P), (Q) and (X)], were investigated in waters from five marine aquaculture farms in Peninsular Malaysia. Tetracycline was detected in low concentrations from <LOQ to 25.6 ng/L. A total of 93 isolates of bacteria were isolated whereby* Vibrio *(n=29) and* Pseudoalteromonas *(n=7) were the predominant bacteria. Forty-eight of the isolates carried* tet *genes with 22.9% encoded multiple* tet *genes and 72.9% encoded a single* tet *gene.* tet*(A) (n = 20, 42%) was the most prevalent gene followed by* tet*(B) (n = 14, 29%) and* tet*(K) (n = 13, 27%). A few common* tet *carriers* (Enterobacter, Vibrio, Photobacterium, *and* Pseudoalteromona*s*) *carrying* tet *genes that have not been reported were identified. The values of the risk quotients (RQs) of tetracycline in Matang was 0.28 which posed a medium ecological risk to algae. Thus, the antibiotic residues in the aquaculture farm in Matang need to be monitored closely.*

Keywords: Aquaculture; risk assessment; tetracycline; tetracycline resistance gene

ABSTRAK

Penggunaan antibiotik secara sembarangan dalam akuakultur dan pengaliran antibiotik daripada akuakultur ke alam sekitar menyebabkan kemunculan dan penyebaran bakteria rintangan antibiotik dan gen rintangan, ini merupakan isu dibimbangkan oleh masyarakat. Lima antibiotik tetrasiklin (minosiklin, doksisiklin, klorotetrasiklin, oksitetrasiklin dan tetrasiklin) dan 14 jenis gen tetrasiklin, tet*[(A), (B), (C), (D), (E), (G), (K), (L), (M), (O), (S), (P), (Q) dan (X)], dikaji di perairan daripada lima ladang akuakultur laut di Semenanjung Malaysia. Tetrasiklin dikesan dalam kepekatan dari <LOQ hingga 25.6 ng/L. Sebanyak 93 isolat bakteria telah dipencilkan,* Vibrio *(n= 29) dan* Pseudoalteromonas *(n = 7) merupakan bakteria yang dominan. Empat puluh lapan isolat didapati membawa gen* tet *dengan 22.9% membawa pelbagai gen* tet *dan 72.9% membawa satu gen* tet*.* tet*(A) (n = 20, 42%) merupakan gen yang paling lazim diperoleh diikuti* tet*(B) (n = 14, 29%) dan* tet*(K) (n = 13, 27%). Sebilangan besar pembawa* tet *biasa* (Enterobacter, Vibrio, Photobacterium *dan* Pseudoalteromonas) *didapati membawa gen* tet *yang belum dilaporkan dan telah dikenal pasti dalam kajian ini. Nilai kuota risiko (RQ) tetrasiklin di Matang didapati 0.28, ia akan menimbulkan risiko ekologi yang sederhana terhadap alga. Oleh itu, sisa antibiotik di ladang akuakultur Matang perlu dipantau dengan teliti.*

Kata kunci: Akuakultur; penilaian risiko; tetrasiklin; tetrasiklin gen rintangan

Introduction

With a growing population, wild captured fishes are no longer sufficient to fulfil the need for human consumption of seafood. Aquaculture industry has become an important sector with the production of global aquaculture in 2016 at 80 million tonnes with an estimated value of USD231.6 billion (FAO 2018). Malaysia was ranked 15th top aquaculture producer globally in 2014

(FAO 2016). However, intensive aquaculture often encounters problems with infection that greatly affects the production and development of aquaculture. Vibriosis, a disease caused by *Vibrio* spp. and *Photobacterium* spp. often causes disease outbreaks in aquaculture farms, and each outbreak incurs huge losses (Lee & Wee 2014). In Malaysia, *Vibrio parahaemolyticus* is responsible for the outbreak of early mortality syndrome (EMS) which is a new emerging disease affecting the shrimp culture in Southeast Asia. Production losses from EMS were estimated at USD 100 million in 2011 (FAO 2013). In order to maximize profit, farmers indiscriminately misuse antibiotics as growth promoter and prophylaxis treatment, a common practice in aquaculture to combat bacterial infections.

As most of the antibiotics used in aquaculture end up in the environment, aquaculture is a potential source of antibiotic pollution in the environment (Cabello et al. 2013). Eutrophication in aquaculture waters also allows the promotion of growth and emergence of antibiotic resistant bacteria in the water and seafood gut that subsequently alter the microbial community in the aquatic environment (Watts et al. 2017). Antibiotics in the waters can also exert selective pressure on bacteria to acquire resistance genes by horizontal gene transfer (HGT) (Tamminen et al. 2011).

Tetracyclines (TCs) are a common antibiotic class used on humans and animal husbandry, mainly as it is inexpensive and effective in combating infections caused by a wide variety of Gram-positive and Gram-negative bacteria. In Malaysia, TCs was the second highest antibiotic used for prophylaxis and treatment of animals (Zakaria 2017). Among the TCs, tetracycline, oxytetracycline, minocycline, doxycycline, chlorotetracycline and demeclocycline are commonly used in aquaculture, and their concentration in aquatic environments has been reported at a range from 1 to 100000 ng L^{-1} (Liyanage & Manage 2019; Yuan et al. 2019). To date, 60 tetracycline (*tet*) resistance determinants have been determined and grouped into three types of resistance mechanisms: active efflux pump (33), ribosomal protection (13), enzyme inactivation (13) and one unknown (Roberts 2019).

The distribution of *tet* resistance determinant has been extensively studied in environmental, clinical and food settings (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 (Martins et al. 2019; Nguyen et al. 2017). The presence of these *tet* resistance genes is due to broad-host-range conjugative plasmids and transposons that may play a significant role in dissemination of resistance among environmental and clinically important species (Han et al. 2015). Most of the studies to date were mainly focused on the most frequently detected *tet* resistance determinants which are *tet*[(A), (B), (D), (E) and (M)] (Piotrowska et al. 2017; Shen et al. 2019). As the distribution of TCs and *tet* resistance determinants differ geographically, little is known about antibiotic residues, resistance genes and their environmental risks in aquaculture farms of developing countries. Hence, this study aims to examine the tetracyclines residues, tetracycline resistance bacteria and their resistant genes in selected aquaculture farms in Malaysia. We also accessed the potential ecological risks of the detected antibiotics based on the risk quotients (RQs).

Materials and Methods

Sampling

Three aquaculture farms at Matang, state of Perak and two aquaculture farms at Pulau Ketam, state of Selangor, located along the west coast of Peninsular Malaysia were visited (Figure 1). The aquaculture farms at Matang practised a closed-system culture in which the pond is built inland whereas aquaculture farms at Pulau Ketam practised an open system culture (net or pen) offshore. A stainless-steel bucket was used to collect water samples. Prior to sample collection, the bucket was rinsed three times with the water sample. After collection, the bucket was immediately cleaned and rinsed thoroughly with distilled water. The collected water sample was then passed through a 20 µm mesh net and stored into a clean 2 L amber glass bottle for antibiotic residue quantification. For bacterial isolation, a sterilized glass bottle was used to collect the water sample. All the collected samples were kept in an ice box with ice and brought back to laboratory for further processing.

Antibiotic Extraction and Quantification of Antibiotic Residue

Five compounds (minocycline (MIN), doxycycline (DC), chlorotetracycline (CTC), oxytetracycline (OTC) and tetracycline (TC)) belonging to the tetracyclines (TCs) class were analysed using liquid chromatograph equipped with a Quantum Access tandem mass spectrometer (LC-MS/MS) (Thermo Fisher Scientific, Yokohama, Japan) and electrospray (ESI) ionization in positive mode after extraction using Solid Phase Cartridge (500 mg, Oasis HLB, Waters, UK). The method and analytical

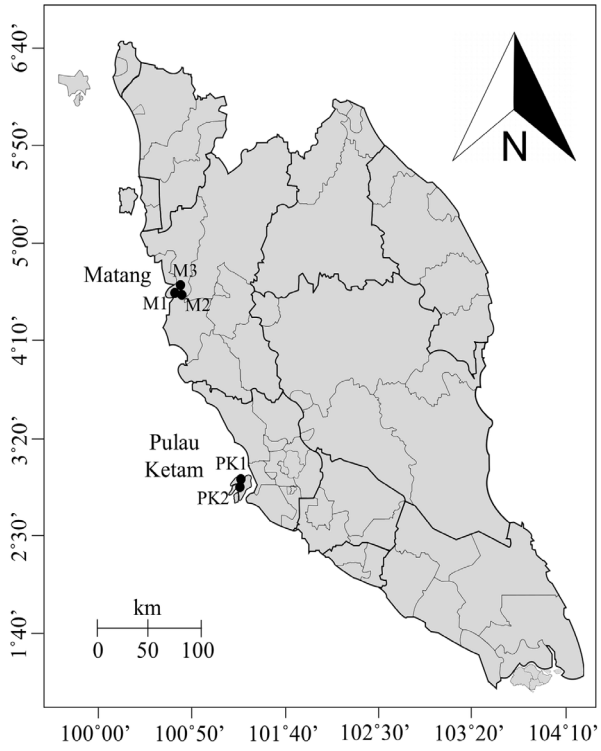

FIGURE 1. Five sampling sites in Peninsular Malaysia

performance for antibiotic quantification were adapted with modification from Shimizu et al. (2013). Additional details are shown in supplementary material (Tables S1 & S2).

TOTAL CELL COUNT, ABUNDANCE OF CULTURABLE, AND TETRACYCLINE RESISTANT BACTERIA

Total bacteria were determined by direct count using DAPI staining method (Kepner & Pratt 1994) whereas abundance of culturable bacteria was determined by spread plating method using marine agar. The enumeration of tetracycline resistant bacteria (TC^r) was carried out on marine agar (BD, Difco, UK) supplemented with 60 µg/mL TC (Amresco, USA). Prior to plating, a serial ten-fold dilution of sample was performed. All plate counts were performed in triplicates and incubation was carried at 30 °C for 18 h. Random selection of TC^r isolates were performed followed by purification on nutrient agar for further analysis.

PCR AMPLIFICATION

For *tet* gene detection, DNA of selected isolate was extracted using heat boil method. PCR reaction mixtures contained 0.5 µg template DNA, 1 × PCR buffer, 2.5 U *Taq* DNA polymerase (iNtRON Biotechnology, Korea), 300 µM of deoxynucleotides and sterile ultra-pure water in a total volume of 50 µL. Five multiplex groups were used to screen the 14 *tet* genes (Table S3) (Ng et al. 2001). The concentrations of $MgCl_2$ and primers were optimized for each multiplex group. Amplification of DNA was carried out using the condition adapted from Ng et al. (2001). PCR product was electrophoresed on a 1.5% (w/v; 0.5× TBE buffer) agarose gel and pre-stained with Redsafe. UV transillumination was used to visualise the DNA band. The size of the PCR product was determined by comparing it with a 100 bp ladder (New England BioLabs Inc, USA). Isolates with the presence of tetracycline resistant genes were then identified using 16S rRNA followed by sequence analysis using the Basic Local Alignment Search Tool (BLAST). PCR was performed by using a volume of 25 µL reaction mixture consisting 1 µL of template DNA, sterile ultra-pure water and a final concentration of 1× PCR buffer with $MgCl_2$, 1.4 U DNA *Taq* polymerase, 0.2 mM deoxynucleotides and 0.4 µM for each universal primer (20F and 1500R). The condition was set at 94 °C for 2 min of initial

denaturation followed by 35 cycles: 30 s denaturation at 94 °C, 40 s annealing at 60 °C and 90 s elongation at 72 °C.

ECOLOGICAL RISK ASSESSMENT

To evaluate the potential ecological effect of detected antibiotic in Matang and Pulau Ketam, a Risk Quotient (RQ) was calculated according to the European technical guidance document on risk assessment (European Commission 2003). RQ was calculated using the formula, RQ = MEC/PNEC, where MEC stands for measured environmental concentration while PNEC stands for predicted no-effect concentration. PNEC is the division of EC50/LC50 and assessment factor. As stated by the European technical guidance document on risk assessment (European Commission 2003), the value of the assessment factor was selected based on the type of toxicology data (EC50/LC50). The toxicology data of each selected antibiotic on four types of aquatic organisms (algae, bacteria, fish and invertebrate) were used from the literature review of toxicological studies (Table S4). RQ were classified into three levels of risk; high risk when RQ >1, medium when RQ fell between 0.1 and 1 and low risk when RQ < 0.1.

STATISTICAL ANALYSIS

The principal component analysis, multivariate cluster analysis and Tukey test were used to compare and analyse the antibiotic residue composition at different aquaculture sites.

RESULTS AND DISCUSSION

ANTIBIOTIC RESIDUES AND ENVIRONMENTAL RISK ASSESSMENT

Of the five tetracycline compounds measured in this study, only TC was detected in M1 and M2 at 25.6 and 14.4 ng/L, respectively (Table 1). OTC (M1 and M2) and DC (M3, PK1 and PK2) were below LOQ whereas MIN and CTC were not detected in any of the farms. Our results suggest the prevalent use of TC in Malaysian aquaculture farms. The concentration of TC detected in this study was comparable to aquaculture, mariculture and Yangtze River in China (Chen et al. 2017; Yan et al. 2018; Yuan et al. 2019) and Cache la Poudre River in United States (Kim & Carlson 2007) but one order magnitude higher than Dou River, China (Zou et al. 2011) and Thailand (Shimizu et al. 2013) and two to three orders of magnitude lower than the pond aquaculture in Sri Lanka (Liyanage & Manage 2019), Hailing Island, China (Chen et al. 2015), livestock farm in Vietnam (Shimizu et al. 2013) and coastal water from Hong Kong (Gulkowska et al. 2007) (Table S5). In this study, concentration of OTC was below LOQ, and this is contrary to other studies that reported OTC as commonly detected e.g. in pond or cage aquaculture farms in Asian countries and Portugal (Jia et al. 2018; Lai et al. 2018; Pereira et al. 2015; Rico et al. 2014; Yan et al. 2018; Yuan et al. 2019). This may be due to the different practices and regional differences in dosage applied. Besides the quality of the water, physicochemical reactions of antibiotics towards environmental parameters in certain regions can also affect the fate of antibiotics in the environment (Le et al. 2005).

TABLE 1. The concentration of tetracyclines and calculated risk quotient (RQs) for detected tetracyclines in aquaculture farms

Location		Antibiotic ng/L (RQs)				
MIN		OTC	TC	CTC	DC	
LOQ		12	583	3	6	4.3
Matang, Perak	M1	n.d.	n.d.	25.61(0.28)	n.d.	<LOQ (<0.01)
	M2	n.d.	n.d.	14.43 (0.16)	n.d.	<LOQ (<0.01)
	M3	n.d.	<LOQ (<0.01)	n.d.	n.d.	n.d.
LOQ		6	317	25	8	31
Pulau Ketam, Selangor	PK1	n.d.	<LOQ (<0.01)	<LOQ (<0.01)	n.d.	n.d.
	PK2	n.d.	<LOQ (<0.01)	n.d.	n.d.	n.d.

LOQ = Limit of quantification, n.d. = non- detected

The concentration of antibiotics from aquaculture farms in this study and the PNECs of each antibiotic for the four types of aquatic organisms (bacteria, algae, invertebrate, and fish) were used to evaluate the potential ecological risk and calculate the RQs. Results showed that the risk to fish and invertebrate was negligible. All the detected antibiotic with concentration below LOQ had a RQ value < 0.01, suggesting these antibiotics were not likely to cause toxic effects on algae. In contrast, the detected TC in M1 and M2 posed medium risk to *Microcystis aeruginosa* in this area.

Although the concentration of tetracycline was low in aquaculture, their highly hydrophilic character and low volatility have resulted in their persistence in aquatic environments which can be toxic to aquatic organisms. Moreover, the usage of tetracyclines in Malaysian aquaculture farms remained unclear. Thus, this is important to understand the potential environmental risk posed by these antibiotics in the aquatic environment. Moreover, studies have shown that antibiotics at sub-inhibitory concentration may accelerate the emergence and dissemination of antibiotic resistance genes and antibiotic resistant bacteria in the environment and pose high risk to humans and ecosystem (Gullberg et al. 2011). Studies have also demonstrated the existence of multiple antibiotics that might pose potential threat to the aquatic microorganisms and environment. As the detailed antibiotic usage history at the study sites is unavailable, further study is needed to understand the distribution and occurrence of other antibiotic classes in order to evaluate the effects of multiple antibiotics toward aquatic organisms (Wang et al. 2017).

BACTERIA ABUNDANCE AND *tet* GENES AMONG THE CULTURABLE ISOLATES

Total bacteria varied within a relatively narrow range between the sites (1.39 - 5.13 × 10^6 cell/mL). The total culturable count in Matang (4.60 × 10^3 - 1.21 × 10^4 CFU/mL) was one order of magnitude higher than the total culturable count in Pulau Ketam (1.01 - 1.04 × 10^3 CFU/mL) (Figure 2). For TC^r the abundance was higher at Matang in which M1 was detected with the highest count of TC^r (2.27 × 10^3 CFU/mL) followed by M2 (9.17 × 10^2 CFU/mL) and M3 (3.43 × 10^2 CFU/mL). In contrast, TC^r at Pulau Ketam was lower (PK1: 1.30 × 10^2 CFU/mL; PK2: 6.3 × 10^1 CFU/mL). The contribution of TC^r over total culturable count was 3 - 20% in Matang and 6 - 13% in Pulau Ketam (Figure 2). The abundance of TC^r in this study was comparable to Drwęca River in Poland (~ >10^2 CFU/mL; Harnisz et al. 2015) but lower than the abundance of TC^r at lagoon in Illinois (>10^5 CFU/mL; Chee-Sanford et al. 2001), salmon farm in Chile (~ >10^3 CFU/mL; Miranda & Zemelman 2002) and mariculture in China (10^4 CFU/mL; Dang et al. 2007). The abundance of TC^r in this study was, however, two orders of magnitude higher than Zimny Potok River in Poland (1 - 12 CFU/mL) (Makowska et al. 2016).

In this study, a total of 93 isolates were isolated from the aquaculture waters. Forty-eight antibiotic resistant bacterial strains were isolated, identified and classified into 10 different genera except two isolates that were not identified. Forty-five isolates (98%) belonged to the phylum *Proteobacteria* and only one isolate belonged to *Firmicutes* with a similarity 95 - 100% (Table 2). From the identified TC^r bacteria, *Vibrio* spp. (63%) was the predominant genus, followed by *Pseudoalteromonas* (15%) and *Photobabacterium* (7%). The other seven bacterial genera present were rare (2%): *Bacillus*, *Bacterioplanes*, *Shewanella*, *Salinimonas*, *Thalassomonas*, *Alteromonas,* and *Enterobacter*.

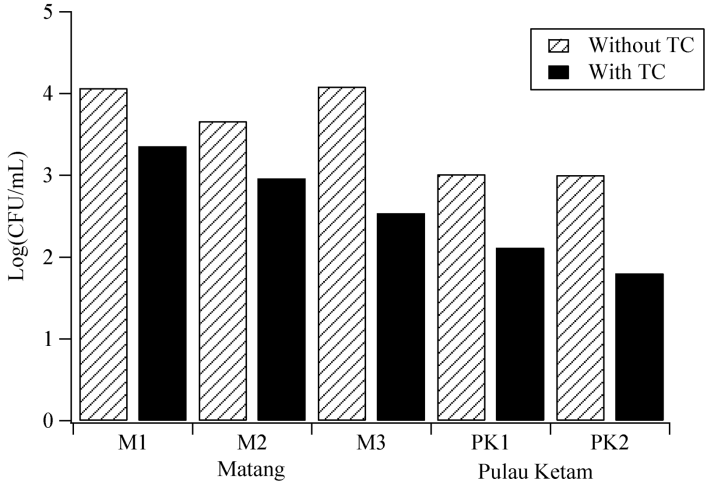

FIGURE 2. Bacterial abundance of culturable bacteria with tetracycline and without tetracycline

TABLE 2. Bacterial species identified with their *tet* gene profile

Location		Isolate	*tet* genes harboured	Species based on 16S rRNA (Similarity, %)
Matang, Perak	M1	MT 1	A, K	Unidentified
		MT 2	A, K, L	*Vibrio parahaemolyticus* (99%)
		MT3	B	*Vibrio diazotrophicus* (99%)
		MT 4	A	Unidentified
		MT 5	K	*Pseudoalteromonas nigrifaciens* (97%)
		MT 6	A	*Vbrio vulnificus* (98%)
		MT 7	O	*Bacillus subtilis* (99%)
		MT 8	X	*Pseudoalteromonas piscicida* (100%)
		MT 9	B, K	*Photobacterium damselae* (95%)
		MT 10	K	*Photobacterium damselae* (96%)
		MT 11	B	*Vibrio rotiferianus* (99%)
	M2	MT 12	A	*Vibrio campbellii* (99%)
		MT 13	A	*Vibrio harveyi* (99%)
		MT 14	B, K	*Vibrio owensii* (99%)
		MT 15	A	*Vibrio alginolyticus* (99%)
		MT 16	A	*Vibrio parahaemolyticus* (100%)
		MT 17	B, E, M	*Shewanella corallii* (99%)
		MT 18	C	*Salinimonas lutimaris* (95%)
		MT 19	O	*Vibrio alginolyticus* (99%)
		MT 20	K	*Vibrio campbellii* (99%)
		MT 21	A, C	*Vibrio harveyi* (99%)
		MT 22	K, M, X	*Pseudoalteromonas piscicida* (100%)
		MT 23	A, K	*Vibrio campbellii* (99%)
		MT 24	A	*Vibrio harveyi* (99%)
		MT 25	E	*Thalassomonas loyana* (97%)
	M3	MT 26	B	*Vibrio alginolyticus* (95%)
		MT 27	A	*Vibrio parahaemolyticus* (99%)
		MT 28	A, B	*Vibrio brasiliensis* (99%)
		MT 29	B	*Vibrio parahaemolyticus* (99%)
		MT 30	B	*Vibrio parahaemolyticus* (100%)
		MT 31	O	*Vibrio rhizosphaerae* (99%)
		MT 32	B	*Vibrio coralliilyticus* (99%)
		MT 33	B	*Vibrio parahaemolyticus* (100%)
		MT 34	B, D, K	*Photobacterium damselae* (100%)
		MT 35	B, K	*Vibrio parahaemolyticus* (100%)
		MT 36	A	*Vibrio parahaemolyticus* (100%)
Pulau Ketam	PK1	CI 1	S	*Alteromonas macleodii* (99%)
		CI 2	O	*Enterobacter ludwigii* (99%)
		CI 3	B	*Pseudoalteromonas xiamenensis* (99%)
		CI 4	C	*Pseudoalteromonas piscicida* (100%)
		CI 5	K	*Pseudoalteromonas piscicida* (100%)
	PK2	CI 6	M	*Pseudoalteromonas piscicida* (100%)
		CI 7	A	*Vibrio campbellii* (99%)
		CI 8	A, K, L	*Vibrio campbellii* (99%)
		CI 9	A	*Vibrio campbellii* (99%)
		CI 10	A	*Bacterioplanes sanyensis* (99%)
		CI 11	A	*Vibrio campbellii* (99%)
		CI 12	A	*Vibrio campbellii* (100%)

The genes detected were *tet*((A), (B), (C), (D), (E), (K), (L), (M), (O), (S) and (X)). The highest detection frequency was *tet*(A) (42%) followed by *tet*(B) (29%) and *tet*(K) (27%). None of *tet*((G), (P) and (Q)) were detected in any of the aquaculture farms. As the practice of aquaculture is different among different geographical regions, the different distribution and diversities of *tet* genes among the sites reflected the regional variation of gene diversity. *tet*(A) and *tet*(B) were the most prevalent genes found in *Vibrio* isolates isolated in this study. Six isolates of *Vibrio* spp. (*V. campbellii* (n = 3), *V. owensii* (n = 1), *V. parahaemolyticus* (n = 2)) were found to carry *tet*(K) whereas *tet*(L) was detected in *V. campbellii* (n = 1) and *V. parahaemolyticus* (n=1). The results were consistent with previous findings that reported active efflux via membrane associated proteins is most common mechanism of tetracycline resistance in *Vibrio* spp. (Dang et al. 2009). We also detected the presence of ribosomal protection protein *tet*(O) in *V. alginolyticus* and *V. rhizosphaerae* (n = 2). This the first study to show the presence of *tet*(O) in *Vibrio* spp. which was previously considered as Gram positive *tet* gene. Further studies are needed to confirm this finding in *Vibrio* spp. from aquaculture farms in different geographical locations.

In this study, 24% of the *Vibrio* spp. were found to carry more than one *tet* genes whereas two isolates isolated from M1 and P2 were found to carry three *tet* genes. A similar trend was observed in China, Japan, Korea, and Australia marine aquaculture farms where *Vibrio* spp. was the most prevalent TC^r in aquaculture farms (Dang et al. 2007; Kim et al. 2004; Nonaka et al. 2007). *Photobacterium damselae,* a marine pathogen of the family *Vibrionaceae* that commonly causes infections in both marine animals and humans, was also detected in this study (n = 3) with the presence of *tet*(K), *tet*(B), *tet*(D). As these bacteria occur naturally in the marine environment, they act as a good reservoir and host for the dissemination of resistance genes (You et al. 2016). In this study, the high detection rates of *Vibrio* spp. included important marine pathogens (*V. alginolyticus*, *V. campbellii*, *V. harveyi*, *V. parahaemolyticus,* and *P. damselae*) in aquaculture waters may pose risks to food security and human health (Akram et al. 2015).

All *Pseudoalteromonas* were detected with the presence of one *tet* gene except for one strain isolated from M2 that carried three *tet* genes (*tet*(K), (M) and (X)). *Shewanella corallii* isolated from M2 had *tet*((B), (E), and (M)) whereas *Salinimonas lutimaris*, *Thalassomonas loyana*, *Alteromonas macleodii,* and *Bacterioplanes sanyensis* were detected with only one single *tet* gene (*tet*(C), (E), (S), and (A), respectively). *Bacillus subtilis* and *Enterobacter ludwigii* were detected with only the presence of *tet*(O).

In this study, the predominant genes detected *tet*((A), (B) and (K)) belonged to the active efflux resistant mechanism. The predominance of these genes could reflect the lower concentrations of tetracycline in the environment. Wang et al. (2019) reported that *tet*(A) gene works better in lower tetracycline concentrations. The *tet*(A) and *tet*(B) have been shown to have a wide host range carrying in different environmental (wastewater, aquaculture and river) genera. Moreover, efflux pump genes (*tet*(A), *tet*(B), *tet*(C), *tet*(K)) are always the predominant tetracycline resistance genes and spread earlier and quicker than other genes (Wang et al. 2019). The Gram-negative and Gram-positive bacteria that carry the active efflux pump are facilitated by highly mobile genetic elements (e.g. plasmids and transposons) that are readily transferable (Chopra & Roberts 2001). *S. lutimaris*, *T. loyana* and *B. sanyensis*, which are rare environmental bacteria, were also found to carry the active efflux pump resistance mechanism (*tet*(C), (E) and (A)). This is the first description of *tet* gene present in these genera which contrasted with other studies that reported these bacteria to be susceptible to TCs compounds (Thompson et al. 2006; Wang et al. 2014; Yoon et al. 2012). Further tests are probably required to confirm the emergence and dissemination of the *tet* genes in these genera in the aquatic environment.

The *tet*(K) gene is commonly found on small transmissible plasmids of both Gram-positive and Gram-negative bacteria that on occasions are integrated into the bacterial chromosome. However, the presence of bacteria with *tet*(K) gene is lower in aquaculture farms (< 21%; Lee et al. 2017) relative to clinical, food, livestock and wastewater (> 50%; Milanović et al. 2016). Our study found a significant correlation between the abundance of TC^r and TC concentrations ($R^2 = 0.920$, $p < 0.01$). This finding was comparable with other studies (Gao et al. 2018) that reported the presence of TC antibiotics in aquaculture waters is of great importance on the selection of *tet* gene. However, among the *tet* genes, only *tet*(K) showed significant correlation with TC concentration ($p < 0.05$, $R^2 = 0.864$).

The occurrence of ribosomal protection genes (*tet*(M), (O) and (S)) in this study was lower (n = 8, < 5%). This was in contrast with other studies that reported higher frequency of these ribosomal protection genes in aquaculture farms (Suzuki et al. 2019). The variation in *tet* gene occurrence among aquaculture farms in

various geographical regions may be due to farming systems, practices, antibiotic dosages used and bacterial community composition at different densities. *tet*(M) has been reported in a broad variety of Gram-positive and Gram-negative bacteria, and usually associated with broad-host-range conjugative transposons (CTns). Ammor et al. (2008) found that the expression of *tet*(M) may be induced only at high tetracycline concentrations. Therefore, the low prevalence of this gene in this study could be attributed to the low concentration of tetracycline.

In the present study, we observed that some of the strains isolated carried multiple genes with different resistance mechanisms. Although identification and characterization of plasmid content were not determined in this study, numerous studies have shown that one plasmid may harbour different *tet* genes or an isolate may carry different plasmids with different *tet* genes or a plasmid may harbour certain *tet* genes and some *tet* genes can found in the chromosome (Roberts 2012). The coexistence of different resistance genes is common in the aquatic environment for providing bacteria a more efficient and higher-level of resistance (Dang et al. 2009; Machado et al. 2015).

The occurrence of tetracycline and prevalence of *tet* genes in aquaculture farm were determined in this study. Of the five tetracycline compounds measured, TC was the most prevalent in these aquaculture farms. The ecological risk assessment showed that the detected TC could pose a medium risk to *Microcystis aeruginosa*. The tetracycline efflux genes (*tet*((A), (B), (C), (D), (E), (K) and (L)) were more prevalent in the isolates compared to ribosomal protection genes (*tet*(M), (O) and (S)). We isolated a few common *tet* carriers (*Enterobacter*, *Vibrio,* and *Photobacterium*) containing *tet* genes that have not been reported in these genera. We also report for the first time, three rare environmental species (*Salinimonas lutimaris*, *Thalassomonas loyana,* and *Bacterioplanes sanyensis*) that harboured active efflux genes. Although further work is needed to confirm this finding and the bacterial host range for *tet* genes in aquatic farms, this present study showed that aquaculture farms could serve as a potential reservoir for the development and dissemination of resistance genes.

ACKNOWLEDGEMENTS

This work was supported by research grants from the Ministry of Higher Education (IOES-2014D, FP048-2013A, and SF022-2013) and University of Malaya (RU009D-2015, PG309-2016A).

REFERENCES

Akram, A., Stevens, R.P. & Konecny, P. 2015. *Photobacterium damselae* and *Vibrio harveyi* hand infection from marine exposure. *The Medical Journal of Australia* 203(5): 224-225.

Amador, P., Fernandes, R., Prudêncio, C. & Duarte, I. 2019. Prevalence of antibiotic resistance genes in multidrug-resistant Enterobacteriaceae on Portuguese livestock manure. *Antibiotics* 8(1): 23.

Ammor, M.S., Gueimonde, M., Danielsen, M., Zagorec, M., van Hoek, A.H., de los Reyes-Gavilán, C., Mayo, B. & Margolles, A. 2008. Two different tetracycline resistance mechanisms, plasmid-carried *tet*(L) and chromosomally located transposon-associated *tet* (M), coexist in *Lactobacillus sakei* Rits 9. *Applied and Environmental Microbiology* 74(5): 1394-1401.

Cabello, F.C., Godfrey, H.P., Tomova, A., Ivanova, L., Dölz, H., Millanao, A. & Buschmann, A.H. 2013. Antimicrobial use in aquaculture re-examined: Its relevance to antimicrobial resistance and to animal and human health. *Environmental Microbiology* 15(7): 1917-1942.

Chee-Sanford, J.C., Aminov, R.I., Krapac, I.J., Garrigues-Jeanjean, N. & Mackiem, R.I. 2001. Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Applied and Environmental Microbiology* 67(4): 1494-1502.

Chen, C.Q., Zheng, L., Zhou, J.L. & Zhao, H. 2017. Persistence and risk of antibiotic residues and antibiotic resistance genes in major mariculture sites in Southeast China. *Science of the Total Environment* 580: 1175-1184.

Chen, H., Liu, S., Xu, X.R., Liu, S.S., Zhou, G.J., Sun, K.F., Zhao, J.L. & Ying, G.G. 2015. Antibiotics in typical marine aquaculture farms surrounding Hailing Island, South China: Occurrence, bioaccumulation and human dietary exposure. *Marine Pollution Bulletin* 90(1-2): 181-187.

Chopra, I. & Roberts, M. 2001. Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and Molecular Biology Review* 65(2): 232-260.

Dang, H., Zhao, J., Song, L., Chen, M. & Chang, Y. 2009. Molecular characterizations of chloramphenicol-and oxytetracycline-resistant bacteria and resistance genes in mariculture waters of China. *Marine Pollution Bulletin* 58(7): 987-994.

Dang, H., Zhang, X., Song, L., Chang, Y. & Yang, G. 2007. Molecular determination of oxytetracycline-resistant bacteria and their resistance genes from mariculture environments of China. *Journal of Applied Microbiology* 103(6): 2580-2592.

Deng, W., Li, N., Zheng, H. & Lin, H. 2016. Occurrence and risk assessment of antibiotics in river water in Hong Kong. *Ecotoxicology and Environmental Safety* 125: 121-127.

European Commission. 2003. *Technical Guidance Document on Risk Assessment in Support of Commission Directive 93/67/EEC on Risk Assessment for New Notified Substances, Commission Regulation (EC) No 1488/94 On Risk*

Assessment For Existing Substances and Directive 98/8/EC of the European Parliament and of the Council Concerning the Placing of Biocidal Products on the Market. Ispra: European Chemicals Bureau.

FAO. 2018. *The State of World Fisheries and Aquaculture. Meeting the Sustainable Development Goal.* Rome: Food and Agriculture Organization.

FAO. 2016. *The State of World Fisheries and Aquaculture 2016. Contributing to Food Security and Nutrition for All*. Rome: Food and Agriculture Organization.

FAO. 2013. *Report of the FAO/MARD Technical Workshop on Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis Syndrome (AHPNS) of Cultured Shrimp (under TCP/VIE/3304)*. Hanoi: FAO Fisheries and Aquaculture.

Gao, Q., Li, Y., Qi, Z., Yue, Y., Min, M., Peng, S., Shi, Z. & Gao, Y. 2018. Diverse and abundant antibiotic resistance genes from mariculture sites of China's coastline. *Science of the Total Environment* 630: 117-125.

Gulkowska, A., He, Y., So, M.K., Yeung, L.W., Leung, H.W., Giesy, J.P., Lam, P.K.S., Martin, M. & Richardson, B.J. 2007. The occurrence of selected antibiotics in Hong Kong coastal waters. *Marine Pollution Bulletin* 54(8): 1287-1293.

Gullberg, E., Cao, S., Berg, O.G., Ilbäck, C., Sandegren, L., Hughes, D. & Andersson, D.I. 2011. Selection of resistant bacteria at very low antibiotic concentrations. *PLoS Pathogens* 7(7): e1002158.

Halling-Sørensen, B. 2000. Algal toxicity of antibacterial agents used in intensive farming. *Chemosphere* 40(7): 731-739.

Han, J.E., Mohney, L.L., Tang, K.F., Pantoja, C.R. & Lightner, D.V. 2015. Plasmid mediated tetracycline resistance of *Vibrio parahaemolyticus* associated with acute hepatopancreatic necrosis disease (AHPND) in shrimps. *Aquaculture Reports* 2: 17-21.

Harnisz, M., Korzeniewska, E. & Gołaś, I. 2015. The impact of a freshwater fish farm on the community of tetracycline-resistant bacteria and the structure of tetracycline resistance genes in river water. *Chemosphere* 128: 134-141.

Jia, J., Guan, Y., Cheng, M., Chen, H., He, J., Wang, S. & Wang, Z. 2018. Occurrence and distribution of antibiotics and antibiotic resistance genes in Ba River, China. *Science of the Total Environment* 642: 1136-1144.

Kang, S.H., Chang, J.G., Ka, S.K., Kim, H.Y., Kim, S.D. & Lee, M. 2012. A study on the aquatic eco-risk assessment of antibiotics treated by radiation. *Journal of Korean Society of Water and Wastewater* 26(3): 373-381.

Kepner, R.L. & Pratt, J.R. 1994. Use of fluorochromes for direct enumeration of total bacteria in environmental samples: Past and present. *Microbiology and Molecular Biology Review* 58(4): 603-615.

Kim, H.Y., Lee, I.S. & Oh, J.E. 2017. Human and veterinary pharmaceuticals in the marine environment including fish farms in Korea. *Science of the Total Environment* 579: 940-949.

Kim, S.C. & Carlson, K. 2007. Temporal and spatial trends in the occurrence of human and veterinary antibiotics in aqueous and river sediment matrices. *Environmental Science and Technology* 41(1): 50-57.

Kim, S.R., Nonaka, L. & Suzuki, S. 2004. Occurrence of tetracycline resistance genes *tet* (M) and *tet* (S) in bacteria from marine aquaculture sites. *Journal of the Federation of European Microbiological Societies Microbiology Letters* 237(1): 147-156.

Lai, W.W.P., Lin, Y.C., Wang, Y.H., Guo, Y.L. & Lin, A.Y.C. 2018. Occurrence of emerging contaminants in aquaculture waters: Cross-contamination between aquaculture systems and surrounding waters. *Water, Air, and Soil Pollution* 229(8): 249.

Le, T.X., Munekage, Y. & Kato, S.I. 2005. Antibiotic resistance in bacteria from shrimp farming in mangrove areas. *Science of the Total Environment* 349(1): 95-105.

Lee, D.W., Jun, L.J. & Jeong, J.B. 2017. Distribution of tetracycline resistance genes in pathogenic bacteria isolated from cultured olive flounder (*Paralichthys olivaceus*) in Jeju in 2016. *Journal of Fisheries and Marine Sciences Education* 29(3): 834-846.

Lee, S.W. & Wee, W. 2014. Disease in aquaculture. *Research Journal of Animal and Veterinary Sciences* 7(1): 1-6.

Liyanage, G. & Manage, P. 2019. Occurrence and distribution of tetracycline resistance determinants and their pollution profile in the aquaculture environment in Sri Lanka. *Journal of the National Science Foundation of Sri Lanka* 47(4): 455-465.

Lützhøft, H.C.H., Halling-Sørensen, B. & Jørgensen, S.E. 1999. Algal toxicity of antibacterial agents applied in Danish fish farming. *Archives of Environmental Contamination and Toxicology* 36(1): 1-6.

Machado, H., Sonnenschein, E.C., Melchiorsen, J. & Gram, L. 2015. Genome mining reveals unlocked bioactive potential of marine Gram-negative bacteria. *BioMed Central Genomics* 16(1): 158.

Makowska, N., Koczura, R. & Mokracka, J. 2016. Class 1 integrase, sulfonamide and tetracycline resistance genes in wastewater treatment plant and surface water. *Chemosphere* 144: 1665-1673.

Marking, L.L., Howe, G.E. & Crowther, J.R. 1988. Toxicity of erythromycin, oxytetracycline, and tetracycline administered to lake trout in water baths, by injection, or by feeding. *The Progressive Fish Culturist* 50(4): 197-201.

Martins, A.F.M., Pinheiro, T.L., Imperatori, A., Freire, S.M., Sá-Freire, L., Moreira, B.M. & Bonelli, R.R. 2019. *Plesiomonas shigelloides*: A notable carrier of acquired antimicrobial resistance in small aquaculture farms. *Aquacultur*e 500: 514-520.

Milanović, V., Osimani, A., Pasquini, M., Aquilanti, L., Garofalo, C., Taccari, M., Cardinali, F., Riolo, P. & Clementi, F. 2016. Getting insight into the prevalence of antibiotic resistance genes in specimens of marketed edible insects. *International Journal of Food Microbiology* 227: 22-28.

Miranda, C.D. & Zemelman, R. 2002. Bacterial resistance to oxytetracycline in Chilean salmon farming. *Aquaculture* 212(1-4): 31-47.

Nonaka, L., Ikeno, K. & Suzuki, S. 2007. Distribution of tetracycline resistance gene, *tet* (M), in gram-positive and gram-negative bacteria isolated from sediment and seawater at a coastal aquaculture site in Japan. *Microbes and Environments* 22(4): 355-364.

Ng, L.K., Martin, I., Alfa, M. & Mulvey, M. 2001. Multiplex PCR for the detection of tetracycline resistant genes. *Molecular and Cellular Probes* 15(4): 209-215.

Nguyen, T.T.T., Nguyen, H.T., Tsai, M.A., Byadgi, O., Wang, P.C., Yoshida, T. & Chen, S.C. 2017. Genetic diversity, virulence genes, and antimicrobial resistance of *Streptococcus dysgalactiae* isolates from different aquatic animal sources. *Aquaculture* 479: 256-264.

Park, S. & Choi, K. 2008. Hazard assessment of commonly used agricultural antibiotics on aquatic ecosystems. *Ecotoxicology* 17(6): 526-538.

Pereira, A.M., Silva, L.J., Meisel, L.M. & Pena, A. 2015. Fluoroquinolones and tetracycline antibiotics in a Portuguese aquaculture system and aquatic surroundings: Occurrence and environmental impact. *Journal of Toxicology and Environmental Health, Part A* 78(15): 959-975.

Piotrowska, M., Rzeczycka, M., Ostrowski, R. & Popowska, M. 2017. Diversity of antibiotic resistance among bacteria isolated from sediments and water of carp farms located in a Polish nature reserve. *Polish Journal of Environmental Studies* 26(1): 239-252.

Rico, A., Oliveira, R., McDonough, S., Matser, A., Khatikarn, J., Satapornvanit, K., Nogueira, A.J.A., Soares, A.M.V.M., Domingues, I. & van den Brink, P.J. 2014. Use, fate and ecological risks of antibiotics applied in tilapia cage farming in Thailand. *Environmental Pollution* 192: 8-16.

Roberts, M.C. 2012. Mechanisms of bacterial antibiotic resistance and lessons learned from environmental tetracycline resistant bacteria. In *Antimicrobial Resistance in the Environment.* Hoboken, United States: Wiley-Blackwell. pp. 93-121.

Roberts, M.C. 2019. Tetracyclines: Mode of action and their bacterial mechanisms of resistance. In *Bacterial Resistance to Antibiotics - from Molecules to Man.* Hoboken, United States: Wiley-Blackwell. pp. 101-124.

Shen, Y., Lv, Z., Yang, L., Liu, D., Ou, Y., Xu, C., Liu, W., Yuan, D., Hao, Y., He, J., Li, X., Zhou, Y., Walsh, T.R., Shen, J., Xia, J., Ke, Y. & Wang, Y. 2019. Integrated aquaculture contributes to the transfer of *mcr*-1 between animals and humans via the aquaculture supply chain. *Environment International* 130: 104708.

Shimizu, A., Takada, H., Koike, T., Takeshita, A., Saha, M., Nakada, N., Murata, A., Suzuki, T., Suzuki, S., Chiem, N.H., Tuyen, B.C., Viet, P.H., Siringan, M.A., Kwan, C., Zakaria, M.P. & Reungsang, A. 2013. Ubiquitous occurrence of sulfonamides in tropical Asian waters. *Science of the Total Environment* 452-453: 108-115.

Suzuki, S., Nakanishi, S., Tamminen, M., Yokokawa, T., Sato-Takabe, Y., Ohta, K., Chou, H., Muziasari, W.I. & Virta, M. 2019. Occurrence of *sul* and *tet* (M) genes in bacterial community in Japanese marine aquaculture environment throughout the year: Profile comparison with Taiwanese and Finnish aquaculture waters. *Science of the Total Environment* 669: 649-656.

Tamminen, M., Karkman, A., Lohmus, A., Muziasari, W.I., Takasu, H., Wada, S., Suzuki, S. & Virta, M. 2011. Tetracycline resistance genes persist at aquaculture farms in the absence of selection pressure. *Environmental Science and Technology* 45(2): 386-391.

Thompson, F.L., Barash, Y., Sawabe, T., Sharon, G., Swings, J. & Rosenberg, E. 2006. *Thalassomonas loyana* sp. nov., a causative agent of the white plague-like disease of corals on the Eilat coral reef. *International Journal of Systematic and Evolutionary Microbiology* 56(2): 365-368.

VMD. 2015. *Publicly Available Assessment Report for a Veterinary Medical Product.* United Kingdom: Veterinary Medicines Directorate.

Wang, G., Jia, Q., Li, T., Dai, S., Wu, H., He, H., Fan, J., Xiang, W. & Li, X. 2014. *Bacterioplanes sanyensis* gen. nov., sp. nov., a PHB-accumulating bacterium isolated from a pool of *Spirulina platensis* cultivation. *Archives of Microbiology* 196(10): 739-744.

Wang, Q., Li, X., Yang, Q., Chen, Y. & Du, B. 2019. Evolution of microbial community and drug resistance during enrichment of tetracycline-degrading bacteria. *Ecotoxicology and Environmental Safety* 171: 746-752.

Wang, Z., Du, Y., Yang, C., Liu, X., Zhang, J., Li, E., Zhang, Q. & Wang, X. 2017. Occurrence and ecological hazard assessment of selected antibiotics in the surface waters in and around Lake Honghu, China. *Science of the Total Environment* 609: 1423-1432.

Watts, J., Schreier, H., Lanska, L. & Hale, M.S. 2017. The rising tide of antimicrobial resistance in aquaculture: Sources, sinks and solutions. *Marine Drugs* 15(6): 158.

Wollenberger, L., Halling-Sørensen, B. & Kusk, K.O. 2000. Acute and chronic toxicity of veterinary antibiotics to *Daphnia magna*. *Chemosphere* 40(7): 723-730.

Xiong, W., Sun, Y., Zhang, T., Ding, X., Li, Y., Wang, M. & Zeng, Z. 2015. Antibiotics, antibiotic resistance genes, and bacterial community composition in fresh water aquaculture environment in China. *Microbial Ecology* 70(2): 425-432.

Yan, M., Xu, C., Huang, Y., Nie, H. & Wang, J. 2018. Tetracyclines, sulfonamides and quinolones and their corresponding resistance genes in the Three Gorges Reservoir, China. *Science of the Total Environment* 631-632: 840-848.

Yoon, J.H., Kang, S.J. & Lee, S.Y. 2012. *Salinimonas lutimaris* sp. nov., a polysaccharide-degrading bacterium isolated from a tidal flat. *Antonie Van Leeuwenhoek* 101(4): 803-810.

You, K.G., Bong, C.W. & Lee, C.W. 2016. Antibiotic resistance and plasmid profiling of *Vibrio* spp. in tropical waters of Peninsular Malaysia. *Environmental Monitoring and Assessment* 188(3): 171.

Yuan, J., Ni, M., Liu, M., Zheng, Y. & Gu, Z. 2019. Occurrence of antibiotics and antibiotic resistance genes in a typical

estuary aquaculture region of Hangzhou Bay, China. *Marine Pollution Bulletin* 138: 376-384.

Zakaria, M. 2017. Use of antimicrobial agents in veterinary medicine in Malaysia. *2nd OIE Information Seminar for Practicing Veterinarians: Combating AMR.* Kuala Lumpur, Malaysia.

Zou, S., Xu, W., Zhang, R., Tang, J., Chen, Y. & Zhang, G. 2011. Occurrence and distribution of antibiotics in coastal water of the Bohai Bay, China: Impacts of river discharge and aquaculture activities. *Environmental Pollution* 159(10): 2913-2920.

Zounková, R., Klimešová, Z., Nepejchalová, L., Hilscherová, K. & Bláha, L. 2011. Complex evaluation of ecotoxicity and genotoxicity of antimicrobials oxytetracycline and flumequine used in aquaculture. *Environmental Toxicology and Chemistry* 30(5): 1184-1189.

Ee Lean Thiang, Choon Yip Stanley Chai, Choon Weng Lee, Lay Ching Chai & Chui Wei Bong*
Institute of Ocean and Earth Sciences
University of Malaya
50603 Kuala Lumpur, Federal Territory
Malaysia

Ee Lean Thiang & Choon Yip Stanley Chai
Institute of Graduate Studies
University of Malaya
50603 Kuala Lumpur, Federal Territory
Malaysia

Choon Weng Lee, Lay Ching Chai & Chui Wei Bong*
Institute of Biological Sciences
University of Malaya
50603 Kuala Lumpur, Federal Territory
Malaysia

Hideshige Takada
Laboratory of Organic Geochemistry
Tokyo University of Agriculture and Technology
Fuchu, Tokyo 18308509
Japan

Ai Jun Wang
Third Institute of Oceanography
State Oceanic Administration
Xiamen 361005
China

*Corresponding author; email: cwbong@um.edu.my

Received: 6 July 2020
Accepted: 11 July 2021

TABLE S1. Optimized MS/MS parameter for tetracyclines and surrogate

Category	Compound name	Acronym	Acronym (LOG)	Parent mass (m/z)	Product ions (m/z)		Collision energy (V)		Surrogate applied
Tetracycline	Tetracycline	TC	TC	445.3	410.0	154.0	26	36	d-OTC
Tetracycline	Doxycycline	DOX	DOX	445.3	428.0	320.9	23	37	d-OTC
Tetracycline	Oxytetracycline	OTC	OTC	461.3	426.0	336.8	26	36	d-OTC
Tetracycline	Chlortetracycline	CTC	CTC	479.1	443.9	462.2	22	17	d-OTC
Tetracycline	Minocycline	MNC	MNC	458.3	441.1	352.0	27	37	d-OTC
Surrogate Standard									
Tetracycline, labelled	Oxytetracycline-13C,d3	d-OTC		465.3	430.1	283.0	26	46	

TABLE S2. Reproducibility and recovery of target antibiotics

Antibiotic	Blank (ng/L)	non-spiked*		spiked**		
		averaged concentration (ng/L)	RSD (%)	averaged concentration (ng/L)	RSD (%)	Recovery (%)
MC	23	12	111	10108	5	78
OTC	19	136	25	11816	1	91
TC	4	7	173	11319	1	88
CTC	1	4	173	13359	2	103
DC	39	52	53	14368	3	110

*1L of sewage effluent; n=3, **1L of the sewage effluent spiked with 12650 ng tetracycline, n=3
n.d. = not detected, gray cells: Below LOQ; LOQ = Blank × 10

TABLE S3. PCR primers for 14 tetracycline resistance gene (Ng et al. 2001)

Primer	Primer sequence (5'-3')	Product size (bp)
tet (A)-F	GCT ACA TCC TGC TTG CCT TC	210
tet (A)-R	CAT AGA TCG CCG TGA AGA GG	
tet (B)-F	TTG GTT AGG GGC AAG TTT TG	659
tet (B)-R	GTA ATG GGC CAA TAA CAC CG	
tet (C)-F	CTT GAG AGC CTT CAA CCC AG	418
tet (C)-R	ATG GTC GTC ATC TAC CTG CC	
tet (D)-F	AAA CCA TTA CGG CAT TCT GC	787
tet (D)-R	GAC CGG ATA CAC CAT CCA TC	
tet (E)-F	AAA CCA CAT CCT CCA TAC GC	278
tet (E)-R	AAA TAG GCC ACA ACC GTC AG	
tet (G)-F	GCT CGG TGG TAT CTC TGC TC	468
tet (G)-R	AGC AAC AGA ATC GGG AAC AC	
tet (K)-F	TCG ATA GGA ACA GCA GTA	169
tet (K)-R	CAG CAG ATC CTA CTC CTT	
tet (L)-F	TCG TTA GCG TGC TGT CAT TC	267
tet (L)-R	GTA TCC CAC CAA TGT AGC CG	
tet (M)-F	GTG GAC AAA GGT ACA ACG AG	406
tet (M)-R	CGG TAA AGT TCG TCA CAC AC	
tet (O)-F	AAC TTA GGC ATT CTG GCT CAC	515
tet (O)-R	TCC CAC TGT TCC ATA TCG TCA	
tet (S)-F	CAT AGA CAA GCC GTT GAC C	667
tet (S)-R	ATG TTT TTG GAA CGC CAG AG	
tetA (P)-F	CTT GGA TTG CGG AAG AAG AG	676
tetA (P)-R	ATA TGC CCA TTT AAC CAC GC	
tet (Q)-F	TTA TAC TTC CTC CGG CAT CG	904
tet (Q)-R	ATC GGT TCG AGA ATG TCC AC	
tet (X)-F	CAA TAA TTG GTG GTG GAC CC	468
tet (X)-R	TTC TTA CCT TGGACA TCC CG	

TABLE S4. Ecotoxicity for fish, invertebrate, algae and bacteria and PNEC value for detected antibiotics

Analytes	Taxonomic group	Species	LC/EC50s (mg L^{-1})	PNEC (ng L^{-1})	References
Oxytetracycline	Bacteria	*Vibrio fischeri*	EC50=21	21000	(Zounková et al. 2011)
	Algae	*Microcystis aeruginosa*	EC50 = 0.207	**207**	(Lützhøft et al. 1999)
	Plankton	*Moina macrocopa*	EC50=126.7	126,700	(Park & Choi 2008)
	Fish	*Oryzias latipes*	EC50-=110.1	110,100	(Park & Choi 2008)
Tetracycline	Bacteria	*Vibrio fischeri*	EC50=35.99	35990	(Kang et al. 2012)
	Algae	*Microcystis aeruginosa*	EC50 = 0.09	**90**	(Halling-Sørensen 2000)
	Invertebrate	*Daphnia magna*	NOEC[b] =342	342,000	(Wollenberger et al. 2000)
	Algae	*Salvelinus namaycush*	LC50=220	220,000	(Marking et al. 1988)
Doxycycline	Algae	*Syneschococcus leopoliensis*	EC50=0.316	**316**	(Deng et al. 2016)
	Invertebrate	*Daphnia* sp.	EC50=>140	140,000	(VMD 2015)
	Fish	-	LC50=>84.7	84,700	(VMD 2015)

NOEC[b]= no observable effect concentration, Number in bold indicate lowest value

TABLE S5. The concentration of TC and OTC detected in different water sources in different countries

Country	Type of water	Concentration of TC ng/L	Concentration of OTC ng/L	Reference
Malaysia	Aquaculture	25.6	<LOQ	This study
	Aquaculture	14.4	<LOQ	This study
Thailand	Aquaculture	<LOQ	-	(Shimizu et al. 2013)
	Aquaculture	-	1760-3050	(Rico et al. 2014)
Vietnam	Livestock	275	-	(Shimizu et al. 2013)
China	River	3	-	(Zou et al. 2011)
	River	22.28 – 26.29	6.14 – 15.62	(Yan et al. 2018)
	River	4.38 - 118	9.44 - 164	(Jia et al. 2018)
	Aquaculture	9.89 – 22.12	12.35 -38.33	(Yuan et al. 2019)
	Aquaculture	2305	-	(Chen et al. 2015)
	Aquaculture	-	22.5 – 43.5	(Xiong et al. 2015)
	Mariculture	n.d. - 54.7	n.d. - 158.6	(Chen et al. 2017)
Korea	Aquaculture effluent	-	7.05 – 95.39	(Kim et al. 2017)
Hong Kong	Coastal water	<LOQ - 122	-	(Gulkowska et al. 2007)
Portugal	Aquaculture	-	3	(Pereira et al. 2015)
Taiwan	Aquaculture	-	75	(Lai et al. 2018)
Sri Lanka	Aquaculture	1000- 112000	-	(Liyanage & Manage 2019)
United States	River	10 - 30	n.d.	(Kim & Carlson 2007)