

A multi-pronged approach to assessing antimicrobial resistance risks in coastal waters and aquaculture systems

Shin Giek Goh^a, Luhua You^a, Charmaine Ng^a, Xuneng Tong^a, Sanjeeb Mohapatra^a, Wei Ching Khor^b, Hong Ming Glendon Ong^b, Kyaw Thu Aung^{b,c,d}, Karina Yew-Hoong Gin^{a,e,*}

^a NUS Environmental Research Institute, National University of Singapore, 1 Create way, Create Tower, #15-02, Singapore 138602, Singapore

^b National Centre for Food Science, Singapore Food Agency, 7 International Business Park, Singapore 609919, Singapore

^c School of Biological Sciences, Nanyang Technological University, Singapore 637551, Singapore

^d Department of Food Science and Technology, National University of Singapore, Singapore 117543, Singapore

^e Department of Civil & Environmental Engineering, National University of Singapore, 1 Engineering Drive 2, Singapore 117576, Singapore

ARTICLE INFO

Keywords:

Antimicrobial resistance
Antibiotic-resistant bacteria
Antibiotic resistance genes
Antibiotics
Risk
Aquaculture
Open cage farming
Recirculating aquaculture system

ABSTRACT

Antimicrobial resistance (AMR) is a global challenge that has impacted aquaculture and surrounding marine environments. In this study, a year-long monitoring program was implemented to evaluate AMR in two different aquaculture settings (i.e., open cage farming, recirculating aquaculture system (RAS)) and surrounding marine environment within a tropical coastal region. The objectives of this study are to (i) investigate the prevalence and co-occurrence of antibiotic-resistant bacteria (ARB), antibiotic resistance genes (ARGs), antibiotics (AB) and various associated chemical compounds at these study sites; (ii) explore the contributing factors to development and propagation of AMR in the coastal environment; and (iii) assess the AMR risks from different perspectives based on the three AMR determinants (i.e., ARB, ARGs and AB). Key findings revealed a distinct pattern of AMR across the different aquaculture settings, notably a higher prevalence of antibiotic-resistant *Vibrio* at RAS outfalls, suggesting a potential accumulation of microorganisms within the treatment system. Despite the relative uniform distribution of ARGs across marine sites, specific genes such as *qepA*, *bla_{CTX-M}* and *bacA*, were found to be abundant in fish samples, especially from the RAS. Variations in chemical contaminant prevalence across sites highlighted possible anthropogenic impacts. Moreover, environmental and seasonal variations were found to significantly influence the distribution of ARGs and chemical compounds in the coastal waters. Hierarchical cluster analysis that was based on ARGs, chemical compounds and environmental data, categorized the sites into three distinct clusters which reflected strong association with location, seasonality and aquaculture activities. The observed weak correlations between ARGs and chemical compounds imply that low environmental concentrations may be insufficient for resistance selection. A comprehensive risk assessment using methodologies such as the multiple antibiotic resistance (MAR) index, comparative AMR risk index (CAMRI) and Risk quotient (RQ) underscored the complexity of AMR risks. This research significantly contributes to the understanding of AMR dynamics in natural aquatic systems and provides valuable insights for managing and mitigating AMR risks in coastal environments.

1. Introduction

Antibiotic resistance has been well recognized as one of the major global challenges, with antibiotic resistance-associated diseases causing >700,000 death annually (O'Neill, 2014). This number is predicted to rise sharply if immediate actions are not taken to address this issue. A recent study conducted by Murray et al. (2022) that collected data from 204 countries and territories estimated >1.2 million deaths from

antibiotic-resistant infections in 2019. Studies have documented the presence and impact of antibiotics (AB) and their transformation products in diverse environmental matrices. The presence of these antibiotics in the environment has intensified concerns regarding their potential role in promoting the selection and spread of antibiotic-resistant bacteria (ARB) and associated antibiotic resistance genes (ARGs) (Ajayi et al., 2024; Larsson and Flach, 2022). Coastal ecosystems, serving as interfaces between terrestrial and marine biomes, are vulnerable to

* Corresponding author.

E-mail address: ceeginyh@nus.edu.sg (K.Y.-H. Gin).

anthropogenic influences, including those from aquaculture practices and marine exploitation.

The increasing incorporation of aquatic animals into the human diet marks a notable dietary shift. Concurrently, capture fisheries production has plateaued since the early 1990s, promoting a shift towards aquaculture to meet rising global demand (Anon., FAO, 2022). This shift has manifested in a significant expansion of aquaculture practices. In the realm of intensive farming, specifically tailored to meet the escalating food demands, there has been a notable increase in fish densities. This escalation correlates with an increased incidence of disease, thereby necessitating augmented antibiotic intervention for both disease treatment and prophylactic purposes (Ellis et al., 2002; Shoemaker et al., 2000). It is projected that the global consumption of antibiotics in food animals will increase by 67 % from 2010 to 2030, if there is no regulatory action on antibiotics usage being exercised (Van Boeckel et al., 2015). Dosage of antibiotics in aquaculture is often higher than in livestock, causing about 70–80 % of antibiotics fed to fish to leak into the surrounding environment, and potentially promoting horizontal transfer of resistance genes (Watts et al., 2017). High dosage of antibiotics also causes higher antibiotic residues (as high as 52 %) to remain in fish products (Wang et al., 2017). It is concerning that 76 % of antibiotics used in livestock and aquaculture are from the same classes as those used in human medicines (Done et al., 2015).

The selective pressure exerted by antibiotics is a critical factor in the development of resistance. For instance, the use of broad-spectrum antibiotics in aquaculture can lead to the selection of multi-drug resistant bacterial strains (Higuera-Llantén et al., 2018; Zhang et al., 2022b). These strains can then disseminate ARGs through mechanisms such as horizontal gene transfer (HGT), a process where genetic material is exchanged between different bacterial species. This exchange can occur in various environmental contexts, particularly in aquatic ecosystems, where mobile genetic elements like plasmids, transposons, and integrons play a pivotal role in the transfer of ARGs between different bacterial populations (An et al., 2023).

Moreover, the selective pressure for antimicrobial resistance is not limited to antibiotics alone. Chemical compounds such as heavy metals, pesticides, disinfectants, insecticides and personal care products (PCPs) also play a significant role in this context. For example, the presence of heavy metals like copper and zinc, often used in agricultural practices, has been associated with the co-selection of antibiotic-resistant strains in various environments (Baker-Austin et al., 2006; Chenia and Jacobs, 2017; Pal et al., 2017; Poole, 2017). Similarly, the widespread use of biocides and disinfectants, particularly in healthcare and agricultural settings, can select for bacteria with cross-resistance or co-resistance to antibiotics (Pal et al., 2015; Paul et al., 2019). This phenomenon is exemplified by the increase in resistance to quaternary ammonium compounds (QACs), a common class of disinfectants, with increasing usage particularly during the Covid-19 pandemic. QACs have been linked to an increased resistance to certain antibiotics (Hora et al., 2020; Jia et al., 2022). The use of certain pesticides/insecticides has also been linked to the development of resistance in pest/insect populations, which could have downstream effects on the spread of resistance genes in other microorganisms (Qiu et al., 2022; Rangasamy et al., 2017). Similarly, components found in some personal care products, such as triclosan, have been implicated in the development of resistance mechanisms in bacteria (Carey and McNamara, 2015; Lu et al., 2020). These examples highlight the complex interplay between various chemical compounds and the development and spread of AMR in different ecosystems.

With increasing evidence of the risks associated with AMR, recent studies have explored different approaches for evaluating AMR risks. These risks are assessed based on three key AMR determinants: ARB, ARG and antibiotics. The threat posed by ARB is well-documented, particularly in clinical settings, where numerous studies have linked ARB related infections to increased mortality, prolonged hospital stays and escalated medical costs (Dadgostar, 2019; Peters et al., 2019;

Wozniak et al., 2022). However, due to the grave risk of untreatable infections from ARB, conducting clinical trials to understand the dose-response relationships between ARB and infection is challenging. One common approach for assessing ARB risks is the multiple antibiotic resistance (MAR) index. The MAR index offers the advantage of providing a straightforward and comparative measure of resistance across different bacterial strains, but it is limited by its inability to reflect the complexity of resistance mechanisms and the potential for cross-resistance. An integrated quantitative microbial risk assessment (QMRA)- disability adjusted life years (DALY) approach has been introduced to quantify the additional health burden caused by ARB-related infections compared to infections from antibiotic-susceptible bacteria (Goh et al., 2023). With advancement in metagenomic analysis, several frameworks have been developed to assess ARG risks. Notable examples include the Resistance Readiness Condition (RESCon) by Martínez et al. (2015), the ARG Risk Ranker by Zhang et al. (2021a), and the MetaCompare by Oh et al. (2018), as well as the latest version of MetaCompare that estimates the human health resistome risk and ecological resistome risk by Monjura Afrin et al. (2024). A comprehensive framework that integrates the burden of AMR from both ARB and ARGs has also been applied to identify AMR hotspots (Goh et al., 2022). Antibiotic residues in the environment, a primary driver of bacterial resistance, also pose ecotoxicity risks to aquatic life. The Predicted No-Effect Concentration (PNEC) is a critical tool in environmental risk assessment, particularly for regulating chemical and antibiotic concentrations to safeguard ecosystems (Vestel et al., 2022). However, this method faces challenges due to the absence of standardized thresholds and limited data for some chemicals or antibiotics.

While extensive research has documented the prevalence and mechanisms of AMR in various ecosystems, there is a critical gap in understanding how different aquaculture practices influence the distribution and dynamics of ARB and ARGs. Two common aquaculture settings, open cage farm and recirculating aquaculture system (RAS), vary significantly in operational practices, water exchange rates, and exposure to external contaminants. Such differences can influence the prevalence and spread of AMR in distinct ways. Given the complex issues influencing the transmission of AMR in environmental waters, this study aims to establish a thorough and comprehensive understanding of the overall trends of AMR determinants in tropical coastal waters, including various aquaculture settings, of a city-island state such as Singapore. The specific objectives of this study are to: (i) determine the occurrence and prevalence of AB, ARG, ARB, and co-occurring chemical compounds in the diverse marine waters, including aquaculture sites operating with open cage and RAS, surrounding Singapore island over a 1-year period. This dataset allows the examination of the spatial and temporal distribution of water quality and their correlation with AMR determinants; (ii) provide a comprehensive understanding of the various factors which may contribute to development and propagation of AMR in the coastal ecosystems, with a particular focus on the correlations and co-occurrence of ARGs, antibiotics and chemical compounds; and (iii) assess the AMR risks from three key determinants (ARB, ARGs and AB) and thus, identifying hotspot for dissemination of AMR in the environment.

2. Methodology

2.1. Sampling sites

Field monitoring and samples collection were conducted monthly at 12 sampling sites around the coastal waters of Singapore (Fig. 1) over a 1-year period from January 2022 to January 2023. Two of these sites (site 2 and site 10), marked with green dots in Fig. 1, are aquaculture farms. Site 10 features an open-cage system on the west side of the Johor Straits; while site 2 adopted a recirculating aquaculture system (RAS) on the east side of the Johor Straits. A total of 146 water samples were collected from the 12 sampling sites. At each site, 20 L of surface water

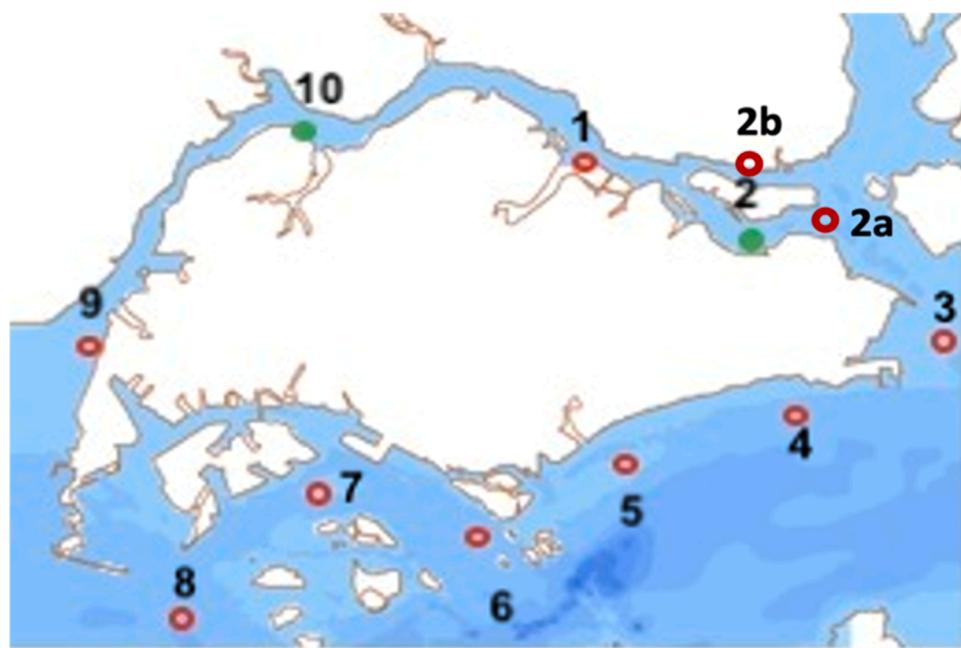


Fig. 1. Proposed sampling sites for collection of data. Red open circles indicates sampling sites in open coastal area, while green closed circles represent sampling sites at aquaculture farms.

(0.5 m below surface) was collected through grab sampling. For aquaculture farms, 4 sediment samples (500 g) and 20 fish samples (market-size) were also collected on quarterly basis for testing. Water and sediment samples were collected from the outfall of Site 2 (RAS) and within the premise of Site 10 (open cage farm).

2.2. Environmental variables and water quality measurement

Environmental field data including pH, conductivity, temperature, dissolved oxygen, turbidity and total dissolved solids were measured *in-situ* using an EXO2 sensor probe (YSI, Inc). The concentrations of faecal indicator bacteria were determined using IDEXX commercial kits which specifically designed to enumerate *E. coli* (ColilertTM) and *Enterococcus* spp. (EnterolertTM) in aquatic samples. These measurements provide a quantitative assessment of the microbial load indicative of the water quality at each sampling point.

2.3. Quantification of antibiotic resistant bacteria

The water samples, fish samples and sediment samples collected from aquaculture farms were analyzed to determine the prevalence and concentration of antibiotic resistant strains of *Vibrio* spp. Briefly, water samples were filtered through a 0.45 µm pore size membrane to concentrate microorganisms on the membrane filter. The filter was then transferred onto Thiosulfate-citrate-bile-salts-sucrose (TCBS) agar for the growth of *Vibrio* spp. 25 g of fish flesh was dissected and resuspended in 225 mL of 1x phosphate buffer saline (PBS) in a homogenizer sample bag. The sample was homogenized in a stomacher for 120 s and fish slurry was then serially diluted in 1x PBS before plating on to TCBS agar. 1 g of sediment sample was resuspended in 10 mL of 1x PBS through vigorous vortexing. After mixing, the sample was inoculated on to TCBS agar. The TCBS agar plates were incubated at 35 °C for 24 h. Following the incubation period, the number of colonies on the membrane filter were enumerated. A representative subset of colonies that grew on this selective agar media was picked and suspended in 50 µL of nuclease-free water, then heat lysed at 95 °C for 10 min. The heat lysed sample was then subjected to gene amplifications of the conserved region for *Vibrio* species (Table S1) (Thompson et al., 2004; Vezzulli et al., 2009). PCR was performed in a reagent mixture containing 1x of GoTaq Green

Mastermix (Promega), forward and reverse primers at final concentration of 0.5 µM each, 2 µL of heat lysed sample and topped up nuclease-free water to a final volume of 50 µL. The thermal conditions for PCR were as follows: an initial step of 95 °C for 10 min, followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, and a final extension of 72 °C for 7 min. The PCR products were loaded onto a 1.5 % agarose gel stained with GelRed®Nucleic Acid Gel Stain (Biotium) and subjected to electrophoresis in 1x TAE buffer at 80 VV for 1 hr. Samples showing a clear band at around 113 bp under UV light visualization were further analysed with 16S rRNA Sanger sequencing for species confirmation using 16S rRNA universal primers (Table S1) (Lane, 1991; Turner et al., 1999). PCR was carried out using 25 µL of 2x GoTaq Green Mastermix (Promega), with both forward and reverse primers added to a final concentration of 0.5 µM each and DNA template of 2 µL. The mixture was then brought to a final volume of 50 µL with nuclease-free water. The thermal cycling conditions for PCR included an initial denaturation of 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 52 °C for 45 s, 72 °C for 90 s, and final extension of 72 °C for 10 min. The PCR products were purified with Wizard® SV Gel and PCR Clean-Up System (Promega) before subjected to Sanger Sequencing (by BioBasic Asia Pacific, Singapore). The isolates confirmed to be *Vibrio* spp. were analysed for their antimicrobial susceptibility profiling using a Vitek® 2 Compact system (bioMérieux). The GN-79 AST card was selected due to its comprehensive panel of antibiotics, which is well-suited for assessing the antimicrobial susceptibility profiles of Gram-negative organisms, including *Vibrio* spp.. The GN-79 AST card includes a wide range of clinically relevant antibiotics from penicillin, cephalosporin and carbapenem classes. Specifically, it assesses susceptibility against a panel of 16 antibiotics: Ampicillin (AMP), Ampicillin/Sulbactam (SAM), Piperacillin/Tazobactam (TZP), Cefazolin (CFZ), Cefoxitin (FOX), Ceftazidime (CAZ), Ceftriaxone (CRO), Cefepime (FEP), Ertapenem (ETP), Meropenem (MEM), Amikacin (AMK), Gentamicin (GEN), Tobramycin (TOB), Ciprofloxacin (CIP), Nitrofurantoin (NIT), Trimethoprim/Sulfamethoxazole (SXT). The results obtained from the GN-79 AST card were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines, specifically the breakpoint outlined in the CLSI M100 document (2015).

2.4. Quantification of antibiotic resistant genes

40-L of water sample was concentrated in situ through a hollow fiber ultrafiltration cartridge (Hemoflow Fresenius, HF 80S). Upon reaching the laboratory, the biomass trapped in the cartridge was eluted by circulating 400 mL of elution buffer (0.1 g of Sodium Pyrophosphate, 5 mL of Tween 80, and 10 µL of Antiform in 1 L of nanopure water) for 30 min. The eluted biomass was filtered till saturation (max volume = 200 mL; equivalent to 20-L of raw sample) through a 0.45 µm cellulose nitrate membrane to concentrate the biomass. DNA was extracted from the biomass on the membranes, using the DNeasy PowerSoil Pro Kit (Qiagen, Netherlands) according to the manufacturer's recommendations. Subsequently, the DNA concentrations were determined using a Qubit spectrophotometer and analysed with high throughput qPCR (HT-qPCR) for a comprehensive profiling of ARG. In this study, out of 600 validated primer sets (www.resistomap.com), 20 ARGs representing major classes of ARGs were screened. The panel included ARGs for beta-lactam genes (*bla_{CTX-M}*, *bla_{NDM}*, *bla_{SHV}*, *bla_{OXA-48}* and *bla_{KPC}*), aminoglycoside (*aac (6')-Ib*), quinolone genes (*qepA* and *qnrS2*), phenicol gene (*floR*), sulfonamide gene (*sul1*), and macrolide-lincosamide-streptogramin B (MLSB) (*mphA* and *mefA*). The panel also encompassed genes for multidrug resistance (MDR) (*arsA*, *copA* and *czcA*), integron (*intI1*) and bacitracin resistance (*bacA*). In addition, genes like *mcr-1*, *fosB* and *qac*, which represent colistin, fosfomycin and quaternary ammonium compound resistance, respectively, were included. This comprehensive selection aimed to cover a broad spectrum of ARGs in the environmental samples.

2.5. Quantification of antibiotics and chemical compounds

The analytical methodologies for antibiotics and other chemical contaminants (Table S2) were primarily based on established protocols (Tran et al., 2016, 2019). Each 500 mL water sample was filtered through a 0.45 µm nylon membrane (Agilent, Singapore) and adjusted to pH 2 using 37 % HCl solution. The acidified samples were added with 0.5 g of ethylenediaminetetraacetic acid tetrasodium salt (Na₄EDTA) and then spiked with 50 ng of internal standards (IS) mixture solution to account for analyte losses during sample preparation and matrix effects due to organic matter during LC-MS analysis. After equilibrium overnight at 4 °C, samples were subjected to solid phase extraction (SPE) following the previous methods (Tran et al., 2016, 2019) with minor modifications. Briefly, Waters Oasis HLB (200 mg, 6 mL) cartridges were conditioned with 5 mL methanol, followed by 5 mL of acidified water (pH 2). Treated seawater was passed through the preconditioned cartridges at 5 mL/minute. The cartridges were then rinsed with 5 mL of acidified water (pH 2) and vacuum dried for 1 hour. Target analytes were eluted with 5 mL of methanol and 5 mL of methanol: acetone (50:50). The eluent was evaporated under nitrogen at 35 °C, and reconstituted with 1 mL of methanol: water (50:50). The final aliquots were stored at -20 °C before further analysis using high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS).

The HPLC-MS/MS used was an Agilent 1260 Infinity Quaternary LC, coupled with an Agilent 6490 Triple Quadrupole mass spectrometry featuring electrospray ionization. An Agilent Poroshell 120 EC18 reverse phase column (100 × 4.6 mm i.d.; 2.7 µm particle size) was used for the separation. The analytes were quantified using a calibration curve based on the peak area response ratio to corresponding labeled internal standard. The linearity was evaluated by least-squares regression for 15 concentrations (0.01 ng/mL to 400 ng/mL) with regression coefficients exceeding 98%. Field and procedural blanks were analyzed to check for contamination, with all analytes below method detection limits (MDLs). Methanol blank and calibration verification standards were injected every 10 samples to monitor instrument background and the calibration validity. The multiple-reaction monitoring (MRM) transition and method validation data, including method recoveries, method detection limits (MDLs) are shown in Table S2.

For measurement of heavy metal, 1 mL of filtered sample was first diluted with 9 mL ultrapure water to meet the salinity requirements of the instrument (<1 ppt) and acidified to a pH of 1.5–2 with nitric acid (HNO₃). The pretreated samples were then analyzed for arsenic (As), chromium (Cr), cadmium (Cd), copper (Cu), nickel (Ni), lead (Pb) and zinc (Zn) through inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7700 series, USA).

2.6. Risk assessment

The AMR risk assessment were conducted separately for each of the three determinants: ARB, ARG and antibiotics. This approach aims to identify specific sources and pathways of resistance, thereby facilitating more targeted and effective mitigation strategies.

2.6.1. Multiple antibiotic resistance (MAR) index

To evaluate the extent of bacterial resistance to multiple antibiotics, the multiple antibiotic resistance (MAR) index was utilized (Eq. (1)). In this study, the MAR index in *Vibrio* spp. isolates collected from the fish farms (including water, sediment and fish samples) was determined.

$$MAR = \frac{\text{number of antibiotics to which the isolate is resistant}}{\text{total number of antibiotics tested against the isolate}} \quad (1)$$

2.6.2. Comparative AMR risk index (CAMRI) model

A comparative AMR risk index (CAMRI) model, developed in our previous study (Goh et al., 2022), was used to understand the relative AMR risk across different samples, for identification of potential AMR hotspots. The evaluation of risks associated with ARGs to human health was based on priority rankings extracted from the arg_ranker v2.0 database (https://github.com/caozhichongchong/arg_ranker). This database assesses the risk of ARGs based on their potential to contribute to multidrug resistance in pathogens, considering three key criteria: enrichment in human-associated environments, gene mobility and presence in ESKAPE pathogens. ARGs are categorized into four risk levels: Rank I (highest risk – current threats), Rank II (future threats), Rank III (human-associated, non-mobile ARGs) and Rank IV (lowest risk) (Zhang et al., 2021a).

To assess the CAMRI, ARGs data obtained from qPCR from marine water samples were analyzed. Following protocols described in Goh et al. (2022), the abundance of ARGs was normalized among the sampling sites using min-max normalization. Each ARG was assigned a risk coefficient, which is the inverse value of the rank level based on the arg_ranker database. The ARG risk index was then calculated based on the likelihood (abundance of ARG) and severity (risk coefficient) of the associated resistance. Finally, the ARG risk index for all ARGs were summed and normalized.

2.6.3. Risk quotient (RQ)

Data from antibiotics were also used to evaluate the AMR risks. The risk quotient (RQ) approach, which involves comparing the levels of detected antibiotics against the threshold of the predicted no effect concentrations (PNEC), was used to examine the potential risk to aquatic organisms and the broader implications for antimicrobial resistance (Eq. (2)). The PNEC values were extracted from the AMR Industry Alliance and Vestel et al. (2022) study.

$$RQ_{AMR} = \frac{MEC_{max}}{PNEC_{AMR}} \quad (2)$$

3. Results

3.1. Spatial and temporal variability of environmental parameters

The statistics of the environmental parameters, including temperature, pH, salinity, DO and total dissolved solids (TDS) with breakdown

by site are presented in Table S3, whereas Table S4 shows the statistics of the environmental parameters with breakdown by time (month). From the Shapiro-Wilk Test, most parameters exhibited a non-normal distribution. As such, the Kruskal-Wallis test was applied to evaluate the statistical significance of difference in the environmental parameters between the sites and months. Table 1 displays the resultant p-values, which provide a comparative analysis of each parameter across the different sites and time periods. The Kruskal-Wallis test revealed that there were statistically significant differences ($P < 0.05$) in salinity and TDS between the sites, while all parameters demonstrated statistically significant differences across different months.

3.2. Abundance and distribution of Faecal indicator bacteria

Fig. 2 presents the distribution of *E. coli* and *Enterococcus* spp. in the marine sites around Singapore over a year. The field data showed that overall average concentration of *Enterococcus* spp. (142.6 MPN/100 mL) was higher than that of *E. coli* (16.7 MPN/100 mL) across the studied sites. Sampling sites located in the Singapore Straits (Site 3, 4, 5, 6, 7 and 8) generally had lower counts of *Enterococcus* spp. detected, with mean concentrations ranging from 50.8 MPN/100 mL to 115.1 MPN/100 mL, compared to the Johor Straits (Site 9, 10, 1, 2, 2a and 2b) where the mean concentrations ranged between 81.5 MPN/100 mL and 538.7 MPN/100 mL. A dashed line in Fig. 2B illustrates the threshold concentration of 200 MPN/100 mL, as per the recreational water quality guidelines (Anon., WHO, 2021). The geometric means at most of the sites were below this threshold level, except at Sites 9, 10, 2a and 2b. The Kruskal-Wallis test revealed no statistically significant difference in *E. coli* concentrations across the studied coastal sites. In contrast, significant differences in *Enterococcus* spp. concentrations were observed between certain sites. Pairwise Mann-Whitney U tests with Bonferroni correction showed a significant difference in *Enterococcus* spp. concentration between Site 8 (mean = 50.8 MPN/100 mL) and Site 2b (mean = 538.7 MPN/100 mL) ($p\text{-value} = 0.000240$), and between Site 8 (mean = 50.8 MPN/100 mL) and Site 10 (mean = 487.7 MPN/100 mL) ($p\text{-value} = 0.000711$).

Fig. 3 illustrates the monthly variations in concentrations of *E. coli* and *Enterococcus* spp. Both *E. coli* and *Enterococcus* spp. counts were high during the month of May, with average concentration of *E. coli* at 1079.9 MPN/100 mL and *Enterococcus* spp. at 371.8 MPN/100 mL. The Kruskal-Wallis test revealed statistically significant differences in the concentrations of both *E. coli* ($p\text{-value} < 0.001$) and *Enterococcus* spp. ($p\text{-value} = 0.047$) across various months of sampling. Pairwise Mann-Whitney U tests with Bonferroni correction showed significant differences in *E. coli* concentration across months (Table S5). However, pairwise comparison did not show significant difference in *Enterococcus* spp. concentration between any two months when considering the stricter criteria of multiple comparisons. This indicates that temporal variation is more significant in *E. coli* than in *Enterococcus* spp.. In the *Enterococcus* spp.

Table 1

Comparative analysis of water quality parameters across different sites and months.

Parameter	Comparison across sites		Comparison across months	
	P-Value	Significant Difference	P-Value	Significant Difference
Water Temperature (°C)	0.0633	No	4.4447E-16	Yes
pH	0.0599	No	2.7573E-06	Yes
Salinity (ppt)	4.5231E-12	Yes	2.5864E-05	Yes
DO (mg/L)	0.4048	No	0.0195	Yes
TDS (mg/L)	3.1072E-11	Yes	1.7226E-05	Yes

boxplot, a threshold line at 200 MPN/100 mL highlights May 2022, Oct 2022, Dec 2022 and Jan 2023 as the months with median concentrations above this level. This underscores potential health or environmental concerns during those months.

3.3. Prevalence of ARB

In this study, the abundances of *Vibrio* spp. in water, fish and sediment samples from two aquaculture sites, namely RAS (Site 2) and open cage farm (Site 10) were monitored. In general, higher abundance of *Vibrio* spp. was observed in water, fish and sediment samples at Site 2 compared to Site 10 (Fig. 4). Nevertheless, statistical analysis using the Mann-Whitney U test revealed no significant difference in the abundance of *Vibrio* spp. between these two sites. A total of 70 *Vibrio* spp. isolates from Site 2, and 53 *Vibrio* spp. isolates from Site 10 were randomly selected and screened for their species. The 16S rRNA Sanger sequencing analysis showed that *V. harveyi* (41 %) and *V. vulnificus* (19 %) were the two dominant *Vibrio* species isolated from Site 2 (Figure S1), while the dominant species of *Vibrio* at Site 10 were *V. alginolyticus* (40 %) and *V. vulnificus* (13 %) (Figure S2).

A total of 123 *Vibrio* spp. isolates were also analysed for their antibiotic susceptibility profiles. The levels of antibiotic resistance in the isolates from different sample type (water, fish and sediment) obtained from the different sites (RAS – Site 2 and open cage farm – Site 1) were investigated (Fig. 5). Isolates from water samples at Site 2 generally showed higher percentage of resistance to antibiotics tested in this study compared to isolates from water samples at Site 10, except for CFZ. For isolates from fish samples, those from Site 10 exhibited higher resistance rates for CFZ and AMP compared to those from Site 2. Isolates from the sediment samples from Site 2 demonstrated higher percentage resistance to AMP, CFZ, SAM, FOX, CAZ and FEP compared to Site 10. This could be due to varying microbial profiles in different environmental matrices. These findings highlight the varied pattern of antibiotics resistance across different aquaculture systems and sample types, where the resistance dynamics may be shaped by a complex interplay of aquaculture practice and environmental factors.

3.4. Abundance and distribution of genes and ARGs

Relative gene abundances in both water and fish samples were calculated from HT-qPCR analysis. Fig. 6A illustrates the average abundance of genes and ARGs in water samples collected from various aquatic sites. The highest total relative gene abundance was observed in water samples from Site 1 (1.73 GC/16S rRNA GC), followed by Site 9 (1.65 GC/16S rRNA GC), 2b (1.57 GC/16S rRNA GC), 2a (1.38 GC/16S rRNA GC) and 3 (1.34 GC/16S rRNA GC). These sites are located along the Johor Straits. The total relative gene abundance in water samples collected from fish farms at Site 2 and Site 10 were relatively low. Specifically, Site 2 had a relative gene abundance of 1.18 GC/16S rRNA GC, and Site 10 had a relative gene abundance of 1.16 GC/16S rRNA GC. These values were lower compared to other sites at the Johor Straits.

The distribution of gene abundance exhibited temporal variability, with the overall trend of gene abundance varying from month to month (Fig. 6B). Specifically, the relative abundance of the genes demonstrated a pronounced peak in the month of Aug 2022 (1.71 GC/16S rRNA GC) and Nov 2022 (1.98 GC/16S rRNA GC). Conversely, a lower abundance of genes was observed in Apr 2022 (0.99 GC/16S rRNA GC), Dec 2022 (0.99 GC/16S rRNA GC) and Jan 2023 (1.08 GC/16S rRNA GC) samples. The *qepA* gene, which is associated with resistance to quinolone, was found to be highly prevalent in the local marine environment (overall average concentration = 0.54 GC/16S rRNA GC). This was followed by the *bacA* gene and the *intl1* gene, with an overall average concentration of 0.24 and 0.23 GC/16S rRNA GC, respectively.

In contrast, the fish samples collected from Site 2 (RAS) had notably higher relative abundance of the integron-integrase gene (*intl1*) (13.3 GC/16S rRNA GC). The fish samples from both Site 2 (RAS) and Site 10

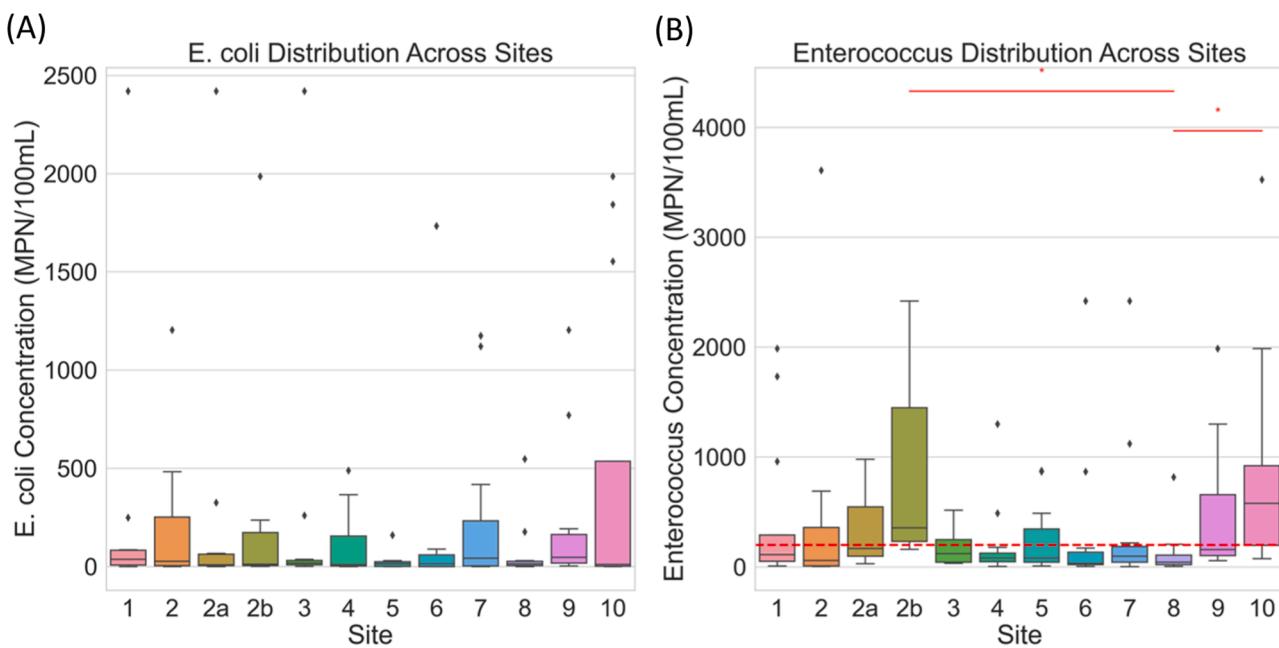


Fig. 2. Spatial distribution patterns of (A) *E. coli* (MPN/100 mL) and (B) *Enterococcus* spp. (MPN/100 mL) in water samples collected from the various coastal sites of Singapore. Sites with significant p-values are indicated with red asterisks (*). Error bars represent the standard deviation.

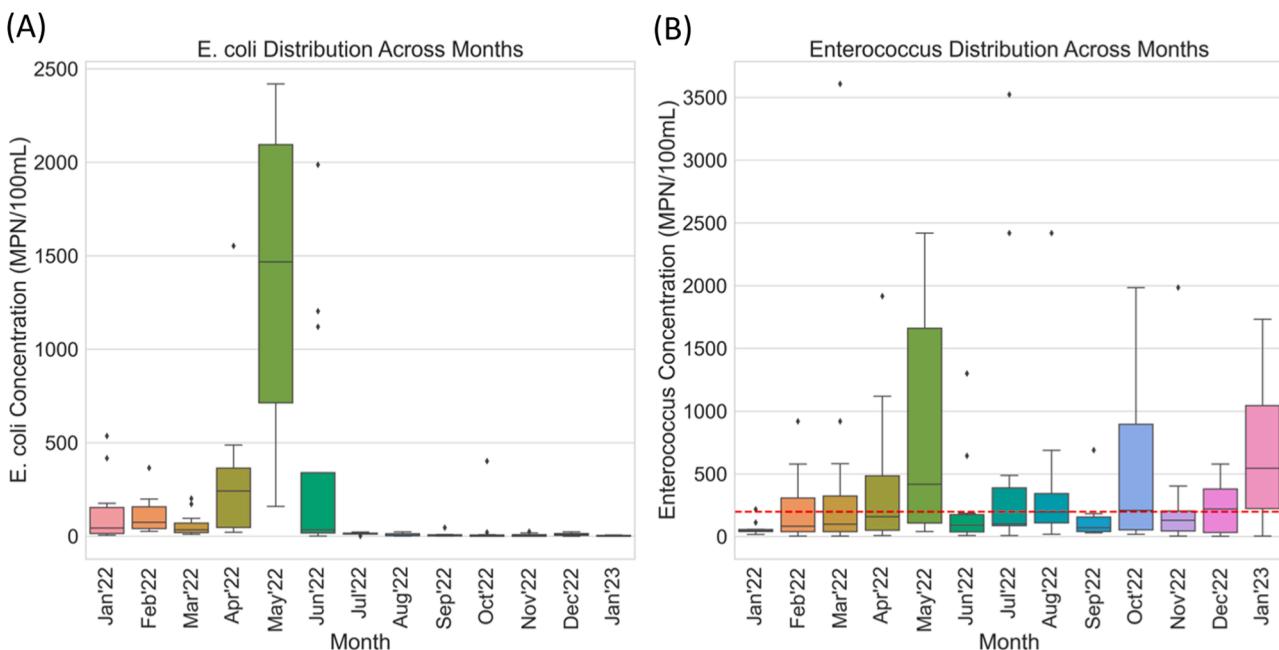


Fig. 3. Temporal variation of (A) *E. coli* (MPN/100 mL) and (B) *Enterococcus* spp. (MPN/100 mL) in monthly assessments. Months with significant difference in *E. coli* concentration are listed in Table S5. Error bars represent the standard deviation. The red dashed line indicates the recreational water quality threshold value at 200 MPN/100 mL.

(open cage farming area) also exhibited a pronounced relative abundance of the quinolone resistance gene (*qepA*) (1.99 and 2.15 GC/16S rRNA GC), the beta-lactamase gene (*bla_{CTX-M}*) (3.23 and 2.82 GC/16S rRNA GC), the antibiotic efflux gene (*bacA*) (1.98 and 1.45 GC/16S rRNA GC), and the macrolide phosphotransferase gene (*mphA*) (0.64 and 0.39 GC/16S rRNA GC).

3.5. Spatial and temporal distributions of emerging chemical contaminants

Fig. 7A shows the average concentrations of key chemical contaminants detected in water samples from different sampling sites. Benzophenone-3 (BP-3), a chemical extensively utilized in sunscreen formulations and in plastic products for UV protection, had the highest average concentration at Site 7 (Singapore Strait) (2634.4 ng/L), followed by Site 8 (Singapore Strait) (1050.6 ng/L) and 10 (West Johor Strait) (801.6 ng/L). Salicyclic acid (SA), widely used in

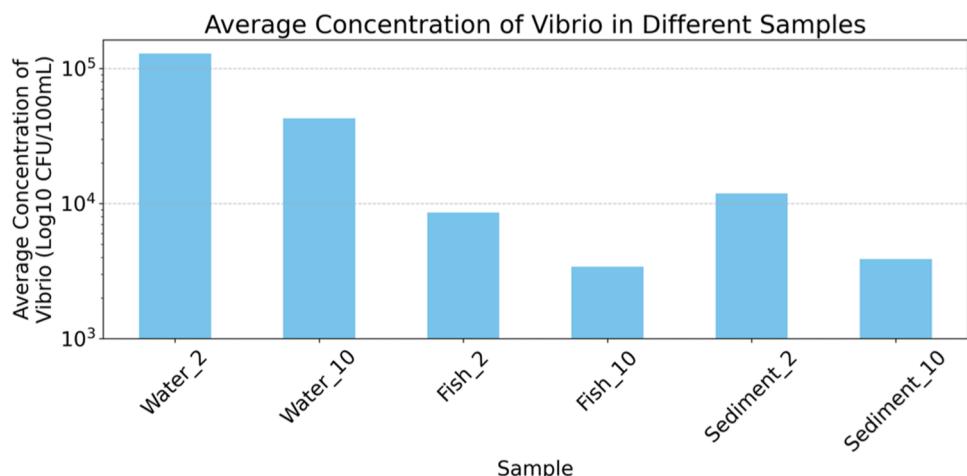


Fig. 4. Average concentration of Vibrio (\log_{10} CFU/100 mL) in water, fish and sediment samples collected from RAS (Site 2) and open cage farm (Site 10).

pharmaceuticals and personal care products, was also detected at high concentrations at Site 7 (1081 ng/L). Meanwhile, Erythromycin-H₂O (ERY-H₂O), an antibiotic commonly used in medical and veterinary practices, was found to have significantly higher concentrations at Site 10 (1170.3 ng/L) compared to the other sites. Caffeine (CF) was consistently found in all sampling sites, with Site 10 having the highest average concentration (952.2 ng/L). Cu emerged as the most prevalent heavy metal, with the highest mean (11.5 µg/L) and median (7.5 µg/L) concentrations at Site 2 (Fig. 8). Concentrations of Zn were also higher at Site 2, with the mean and median values of 5.8 µg/L and 0.7 µg/L, respectively.

In terms of temporal variation, the highest total chemical concentrations were generally observed in samples collected in Dec 2022 during the Northeast Monsoon (6153.5 ng/L) (Fig. 7B). During this period, BP-3 (5407.4 ng/L) was the predominant chemical contaminant detected in the coastal samples. Samples collected in Aug 2022 exhibited elevated concentrations of SA (1345.5 ng/L) and ERY-H₂O (854.3 ng/L) while Caffeine (CF) was consistently found at all sampling sites, with the highest prevalence in Jul 2022 (1594.4 ng/L) during the Southwest Monsoon. Unlike most of the chemical contaminants, Cu and Zn were more prevalent during the drier season, between March to May (Fig. 8).

3.6. Cluster analysis

The hierarchical cluster analysis, based on a distance matrix constructed using Ward's method, revealed three distinct clusters among the sampling sites when considering the combined microbial, chemical and environmental data (Fig. 9; Figure S3). Cluster 1 includes Sites 5, 6, 7 and 8 in the Singapore Straits, suggesting a shared environmental influence in this area. Cluster 2, which groups Site 1, 2a and 2b from the eastern Johor Straits, displays a seasonal pattern, with data spanning from February to September. Cluster 3, consisting of Site 2 (RAS), site 10 (open cage farm) and nearby sites, likely reflects the impact of aquaculture practices. These clusters indicate the significant role of both location and time in shaping the environmental characteristics of these sites.

The PCoA biplot shows that PCoA1 and PCoA2 explain 29.35 % and 22.93 % of the total variance, respectively (Fig. 10). Cluster 1, which comprises samples collected from the Singapore Straits, had similar salinity and TDS profiles, which were distinct from those of samples in Clusters 2 and 3. ARGs such as *bla_{CTX-M}*, *bla_{SHV-1}*, *mefA*, *bla_{OXA-48}*, *mphA*, *mcr-1*, *czcA* and *bacA* were directed towards Cluster 2. This suggests that the ARGs in the samples collected from the eastern Johor Straits during the months of February to September can be differentiated from other locations. Chemical contaminants such as insecticides (i.e. FIP, FIP-Sulfone, FIP_desulfinyl, FIP_Sulfide), the artificial sweetener Saccharin

(SAC) and gemfibrozil (Lopid), as well as temperature, were associated with Cluster 3 (mostly samples from fish farms). This association suggests that these variables are influential in differentiating Cluster 3 from the other clusters.

3.7. Correlation analysis

The heatmap (Fig. 11) illustrates a Spearman correlation analysis among ARGs, chemical compounds and environmental parameters. The correlation analysis showed a clear pattern within two distinct groups: ARGs and chemical compounds. Strong correlations were observed among beta lactamase genes (*bla_{CTX-M}*, *bla_{OXA-48}*, *bla_{SHV-1}*, *bla_{KPC}*) with Spearman's rho values ranging from 0.721 to 0.832 (p-value < 0.001), suggesting a possible genetic linkage or shared selective pressures. The antiseptic resistant gene, *qacE*, was strongly correlated with the sulfonamide resistant gene, *sul1* (Spearman's rho = 0.826, p-value < 0.001), indicating their co-selection on mobile genetic elements (MGEs) (Liu et al., 2022). In addition, genes such as *fosB*, *mphA* and *mefA*, showed correlations with beta lactamase genes (Spearman's rho > 0.7, p-value < 0.001), suggesting potential multi-drug resistance. In term of chemical contaminants, carbamazepine (CBZ) showed significant correlations (Spearman's rho > 0.7, p-value < 0.001) with various insecticide-related compounds (e.g. FIP, FIP_desulfinyl, FIP_Sulfide, FIP_Sulfone), highlighting their co-occurrence in the environment and possible anthropogenic influences. Environmental factors such as salinity and total dissolved solid (TDS) were inversely correlated with concentration of fipronil (FIP), its metabolite FIP_desulfinyl and saccharin (SAC), with Spearman's rho ranging from -0.702 to -0.779 (p-value < 0.001). This inverse relationship could be explained by the dilution effect of salinity from drainage outfalls, which could potentially carry these chemical contaminants, particularly during rainfall and increased storm runoff.

3.8. Risk assessment

In this study, three distinct risk assessments were conducted to elucidate the multifaceted risks associated with AMR: i) the MAR index, which utilized data from ARB, was applied to understand the prevalence of multidrug resistance and the potential implications for human and animal health; ii) the CAMRI drawing upon ARG data, was conducted to identify the hotspots of AMR; and iii) the RQ, utilizing antibiotics data, was used to assess the potential ecological harm posed to aquatic organisms, and the emergent risk of developing antibiotic resistance.

3.8.1. Multiple Antibiotic Risk (MAR) index

Given the high abundance of *Vibrio* spp. in marine environments,

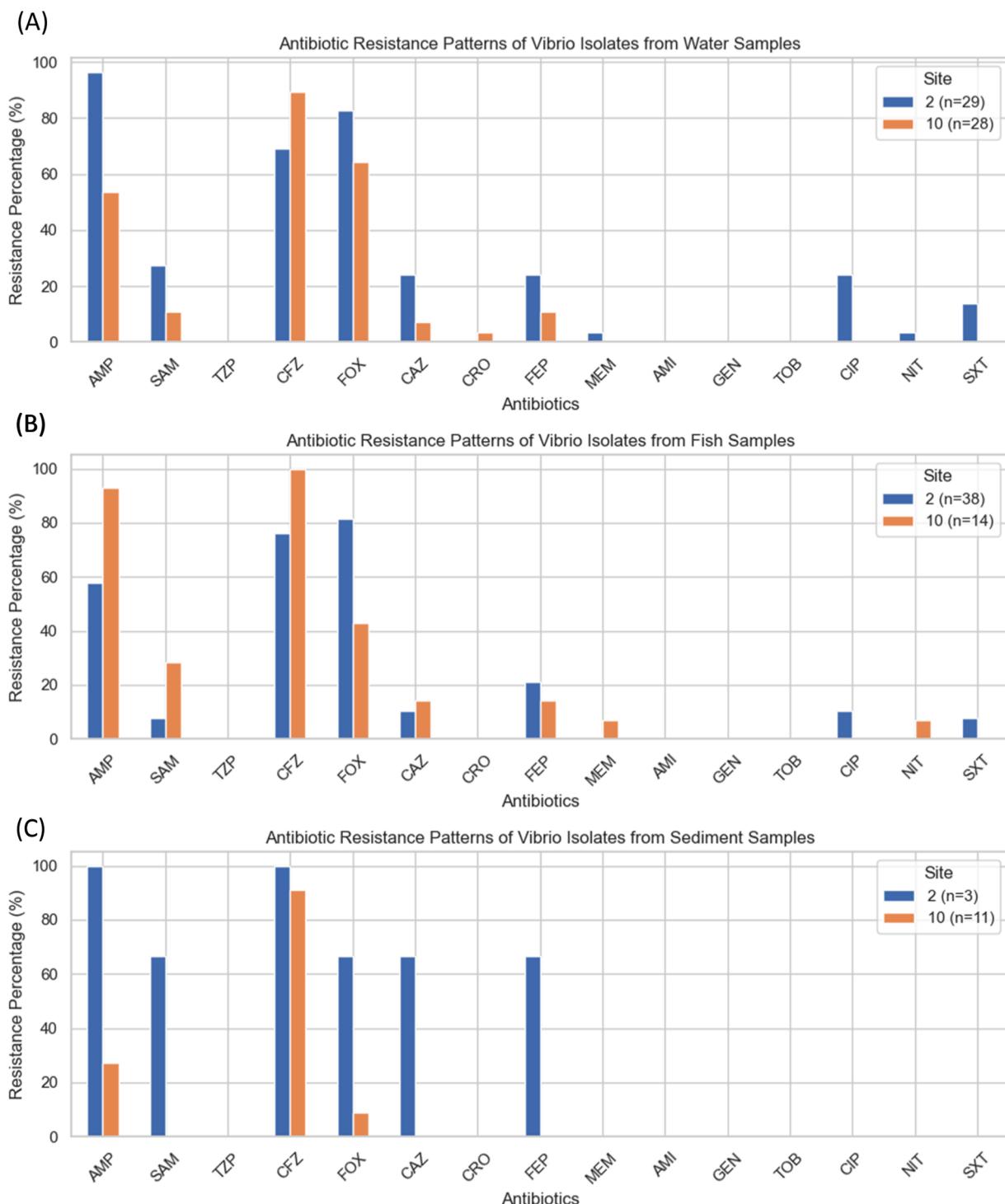
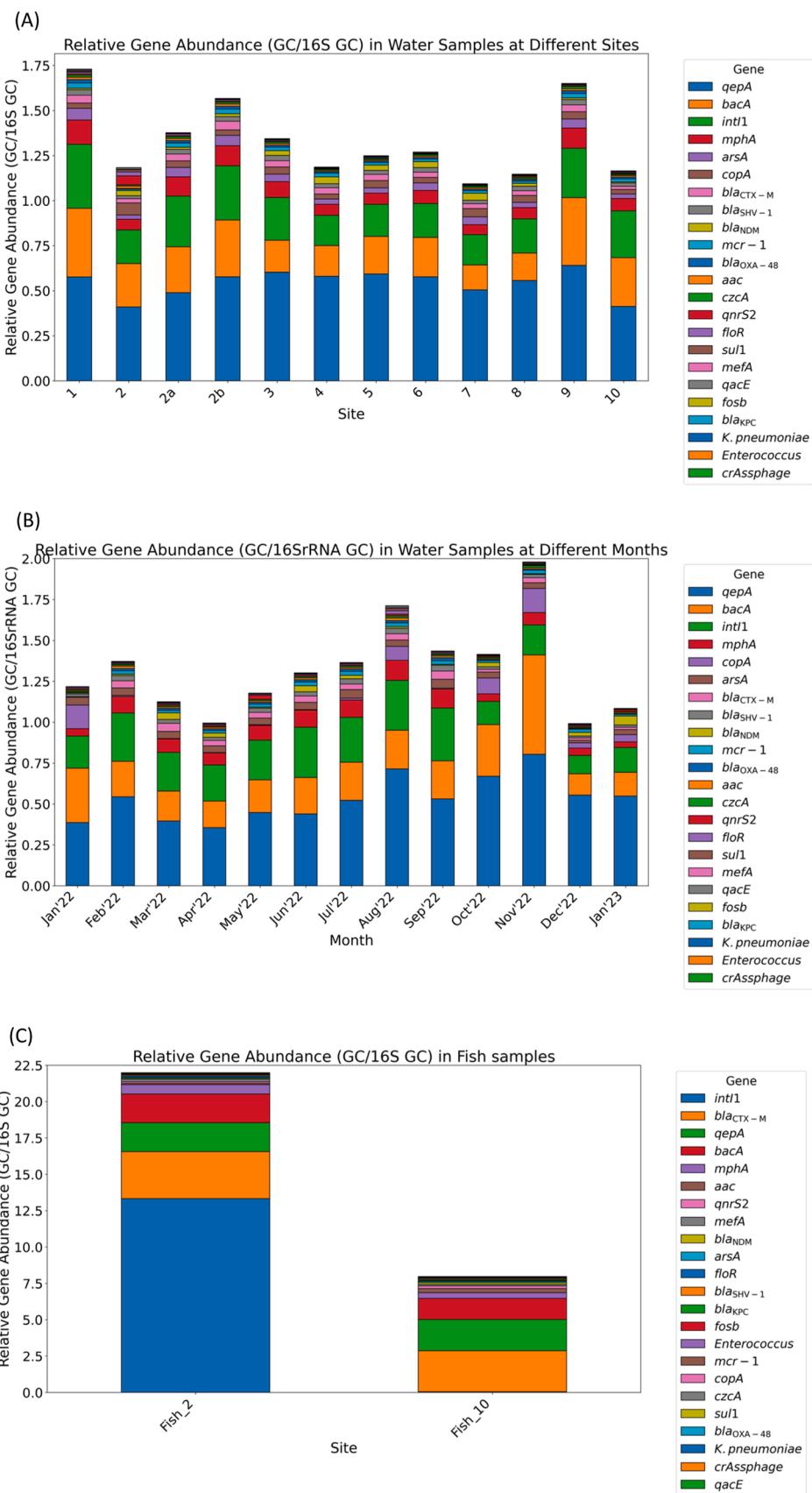


Fig. 5. Percentage of antibiotic resistance patterns of Vibrio isolates from (A) water, (B) fish and (C) sediment samples collected from Site 2 and 10. Antibiotics tested include ampicillin (AMP), ampicillin/sulbactam (SAM), piperacillin/tazobactam (TZP), cefazolin (CFZ), cefotaxime (FOX), ceftazidime (CAZ), ceftriaxone (CRO), cefepime (FEP), meropenem (MEM), amikacin (AMK), gentamicin (GEN), tobramycin (TOB), ciprofloxacin (CIP), nitrofurantoin (NIT), trimethoprim/sulfamethoxazole (SXT).

including a number of species recognized as pathogens to fish (e.g. *V. harveyi*, *V. alginolyticus*) and humans (e.g. *V. cholerae*, *V. vulnificus*), this study assessed the MAR index of isolated *Vibrio* spp. *Vibrio* spp. were isolated from water, fish and sediment samples from the two aquaculture farms (Sites 2 and 10), and subjected to antimicrobial susceptibility tests against 15 antibiotics (Table 2). A higher MAR index reflects increased multidrug resistance. *Vibrio* spp. from sediment at Site 2 (RAS) exhibited the highest MAR index (MAR index = 0.311). This was

followed by *Vibrio* spp. isolates from water samples at the same site (MAR index = 0.246). In contrast, the MAR index values for *Vibrio* spp. from both water and sediment samples at Site 10 (open-water cages) were lower than those at Site 2, implying a more pronounced AMR risk at the outfall of Site 2. However, the MAR index for *Vibrio* isolates from fish samples was marginally higher at Site 10 (MAR index = 0.205) than at Site 2 (MAR index = 0.182).



(caption on next page)

Fig. 6. Average relative abundance of ARGs (GC/16S rRNA GC) in (A) water samples at different sites, (B) water samples across different months, and (C) fish samples. The stacked bar charts show the distribution of various genes, including beta-lactam genes (bla_{CTX-M} , bla_{NDM} , bla_{SHV-1} , bla_{OXA-48} and bla_{KPC}), aminoglycoside gene ($aac(6')-Ib$), quinolone genes ($qepA$ and $qnrS2$), phenicol gene ($floR$), sulfonamide gene ($sul1$), macrolide-lincosamide-streptogramin B (MLSb) genes ($mphA$ and $mefA$), multidrug resistance (MDR) genes ($arsA$, $copA$ and $czcA$), integron-integrase gene ($intI1$), bacitracin resistance gene ($bacA$), colistin resistance gene ($mcr-1$), fosfomycin resistance gene ($fossB$), quaternary ammonium compound resistance (qac), fecal indicator bacteria (Enterococci), human-related microbial marker ($crAssphage$ - $crAss64$), and pathogen (*K. pneumoniae*).

3.8.2. Comparative AMR risk index (CAMRI)

In this study, 20 genes were analysed using high-throughput qPCR. Of these, 11 ARGs identified in Zhang et al. (2021a) database were selected to evaluate the relative ARG risk. Fig. 12 shows the resulting normalized total risk index for water samples from each location, along with fish samples collected from Sites 2 and 10. Fish samples from Site 2 exhibited a higher median value of relative ARG risk (0.194) compared to fish samples from Site 10 (0.140). Among the water samples, the analysis of relative ARG risk revealed that Sites 1 (0.170), 2b (0.156) and 9 (0.156) had higher median risk index compared to the other sites.

3.8.3. Risk quotient (RQ)

RQ analysis shows that the antibiotic levels in coastal waters were generally low and fell below an RQ of 1, except for ciprofloxacin (CIPX) and erythromycin (ERY-H2O) (Fig. 13 and 14). Elevated RQ were occasionally observed for CIPX at Site 2 (aquaculture farm deploying RAS), raising possible AMR risks to this aquatic ecosystem. ERY-H2O has been identified as a significant antibiotic contaminant in marine waters, and particularly posed risks at Site 10, an open cage farm situated in the Johor Straits. However, other antibiotics, including meropenem, amoxicillin, ampicillin, cefixime, chloramphenicol, ofloxacin, tylosin, sulfamethazine, sulfamethoxazole, trimethoprim, vancomycin, chlor-tetracycline, tetracycline and oxytetracycline, were found to be below detection limits, suggesting they were not likely to pose AMR risks to the ecosystem.

4. Discussion

Investigations into AMR in the coastal regions of Southeast Asia has been rising with expanding interest in aquaculture (Dewi et al., 2022; Faja et al., 2019; Makkaew et al., 2021; Mohamad et al., 2019; Ong et al., 2023; Schar et al., 2021; Siri et al., 2023; Suyamud et al., 2024; Thiang et al., 2021; Thongsamer et al., 2021; Yern et al., 2022; You et al., 2016). Nevertheless, there has been a significant gap in comprehensive research that encompasses the three critical determinants of AMR, notably, ARB, ARG and antibiotics. This study presents a holistic analysis of AMR, in conjunction with emerging chemical contaminants such as heavy metals, pharmaceuticals and personal care products, which could act as selective pressures for AMR. The distribution of AMR in coastal waters was examined both spatially and temporally, covering a dataset spanning monthly observations over a year. The spatial analysis included a comparison of different aquaculture settings, specifically contrasting an open cage farm (Site 10) with an RAS (Site 2), to understand their respective impacts on AMR distribution.

ERY-H2O, a derivative of ERY resulting from the loss of one water molecule, was detected in substantial concentrations of up to 5948 ng/L. Concentrations of CIPX, ENFLX, AZT and CLAR in this study were comparable to those reported in South China Sea (Wei et al., 2024), East China Sea (Li et al., 2020), and Laizhou Bay (Lu et al., 2022), while mean concentration of ERY-H2O was at the same magnitude as that in mariculture ponds in Beihu Gulf, China (Wei et al., 2024). The concentrations of CF, TCS and BPA observed in the present study exceeded previously reported levels (Bayen et al., 2013), indicating a potentially escalating trend in contamination levels. The prevalence of dehydrated ERY (ERY-H2O) across all sampling locations indicates its widespread dissemination throughout the coastal environment. For heavy metals, in comparison with previous data collected in 2007 in Singapore (Cuong et al., 2008), there has been a significant increase in the current levels,

with concentrations being approximately 5–10 times higher. However, our findings were considerably lower by a factor of 5–60 than those reported in the Red River coastal zone (Vietnam) (Da Le et al., 2022), the South Australian coastline (Chakraborty and Owens, 2014), and Black Sea in Turkey (Polat and Akkan, 2016). These regions are heavily impacted by anthropogenic activities such as industrial operations, agricultural runoff, shipping, and sewage disposal, which contribute to elevated heavy metal concentrations.

The results from this study revealed a discernible contrast in AMR determinants between the RAS and open cage farm. Water and sediment samples from RAS were specifically collected from the farm's outfall, reflecting the end point of the enclosed recirculation system to examine the impact of RAS effluent to the environment. A high prevalence of *Vibrio* spp. was observed in the water, sediment and fish samples from the RAS compared to the open cage farm. This could be due to its closed-loop environment which encourages nutrient and organic matter buildup, limited water exchange leading to concentration of these microorganisms, as well as the tendency for biofilm formation on the system surfaces, providing a stable habitat for the growth of microorganisms such as *Vibrio* spp. (King, 2001). When examining the antibiotic resistant profile of the *Vibrio* spp. from water, sediment and fish samples of these two farms, it was found that water and sediment samples from the RAS (MAR index = 0.246 in water samples and 0.311 in sediment samples) exhibited a higher percentage of antibiotics resistance compared to the open cage farm (MAR index = 0.16 in water samples and 0.085 in sediment samples). However, *Vibrio* spp. from fish samples in the RAS (MAR index = 0.182) showed a lower percentage of antibiotics resistance compared to those in fish samples from the open cage farm (MAR index = 0.205). Overall, the MAR indexes obtained in this study from water, sediment and fish samples were lower than the median value of approximately 0.3 reported from aquaculture farms in Southeast Asia (Suyamud et al., 2024). Antibiotic-resistant *Vibrio* primarily accumulated within the treatment system rather than the fish tanks. The formation of biofilms and the accumulation of bacteria within the treatment system may create a stronger selective pressure for antibiotic-resistant organisms, facilitating the spread and maintenance of resistant strains (Li et al., 2017; Watts et al., 2017).

A higher relative abundance of ARGs was observed in fish samples compared to water samples, particularly in fish samples from the RAS. This difference could be attributed to variations in microbial communities between water and fish, as well as the role of bioaccumulation and HGT. The microbial communities in fish often exhibit distinctive populations compared to those in the water column. The difference arises from the diverse environmental exposures and diets of the fish, which influence the composition and abundance of ARGs within their microbiomes (Mills et al., 2022; Zhang et al., 2022a). In addition, fish can bioaccumulate contaminants such as antibiotics and heavy metals from their environment and diets. This bioaccumulation can exert selective pressure for ARB within their microbiome, particularly in areas such as the gills and gut, leading to a higher ARG abundance in fish samples (Rajeshkumar and Li, 2018; Zhang et al., 2021b). Fish microbiomes can be hotspots for HGT, facilitating the transfer of ARGs between bacteria. This process amplifies the presence of ARGs in the fish microbiome to a greater extent than in the surrounding water (Fu et al., 2017). Furthermore, the fish themselves, their faeces and pellet feed meals could be potential sources of ARG introduction. For instance, a study in China found the propagation of ARG in an RAS that had no recorded historical antibiotic usage (Liu et al., 2020). Similarly, without the

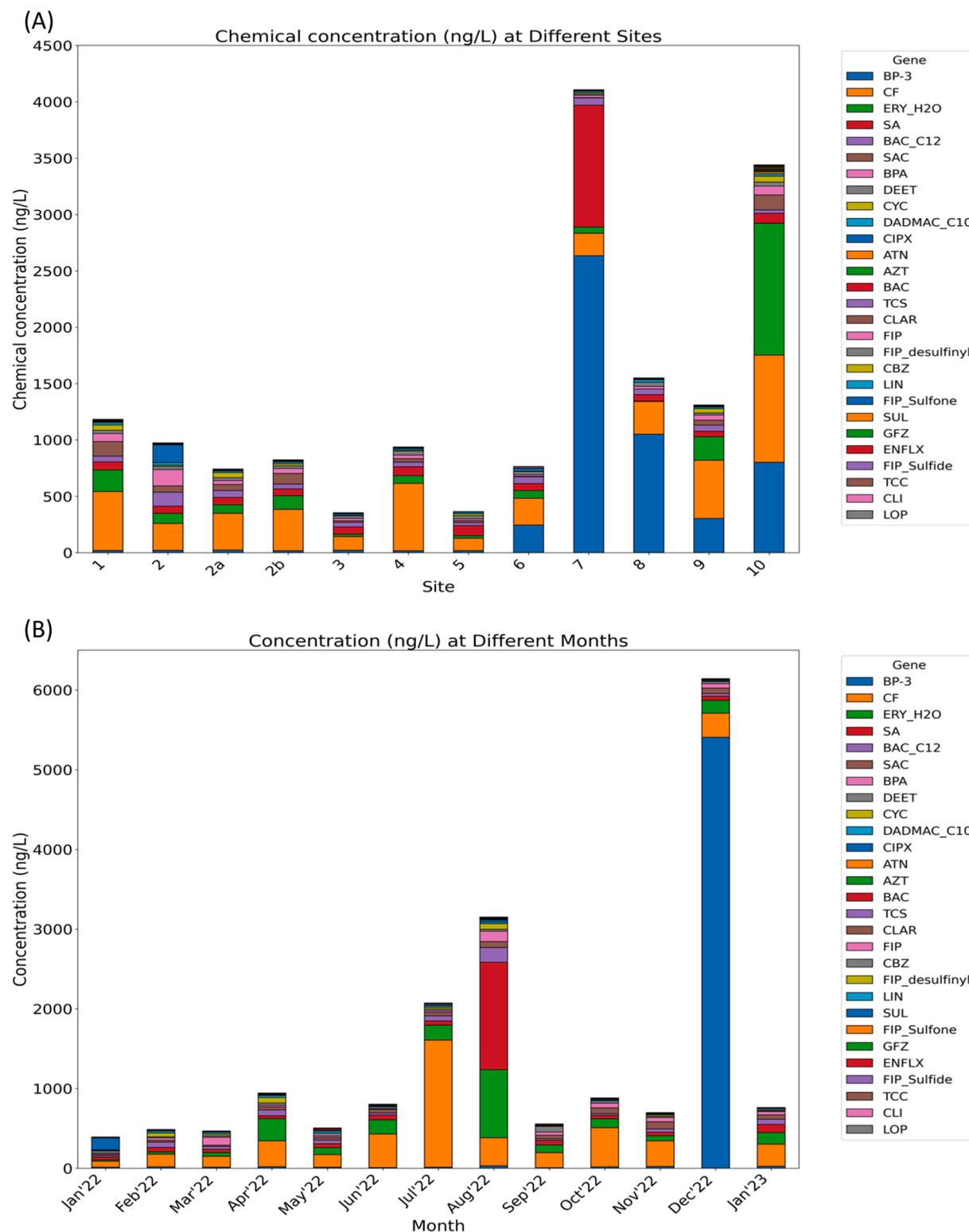


Fig. 7. Average concentration of various chemical compounds (ng/L) measured across (A) different sites and (B) different months. The stacked bar charts represent the distribution of chemical compounds including antibiotics (LIN, CLI, CIPX, ENFLX, ERY-H2O, AZT, CLAR), disinfectants (BAC_C12, BCI, DADMAC_C10, TCC, TCS), personal care products (ATN, SUL, CF, CBZ, SA, LOP, GFZ, BP-3, BPA), artificial sweeteners (SAC, CYC), and insecticides (DEET, FIP, FIP_desulfinyl, FIP_Sulfone, FIP_Sulfide).

application of antibiotics, a high but constant relative abundance of ARGs (*sul1*gene = 2.0 ± 1.5 log₁₀ copies/16S rRNA) was reported in a RAS in Germany (Kampouris et al., 2022).

On the other hand, the open cage farm (Site 10) demonstrated a higher abundance of chemical compounds such as ERY, CF and BP-3,

which could be influenced by its proximity to terrestrial urban activities, discharges along the narrow Johor Straits, and contamination from aquaculture setting. ERY, known for its efficacy against gram-positive bacteria like *Streptococcus* species, underscores its extensive application in aquaculture (Lulijwa et al., 2020). The RQ for ERY indicated a

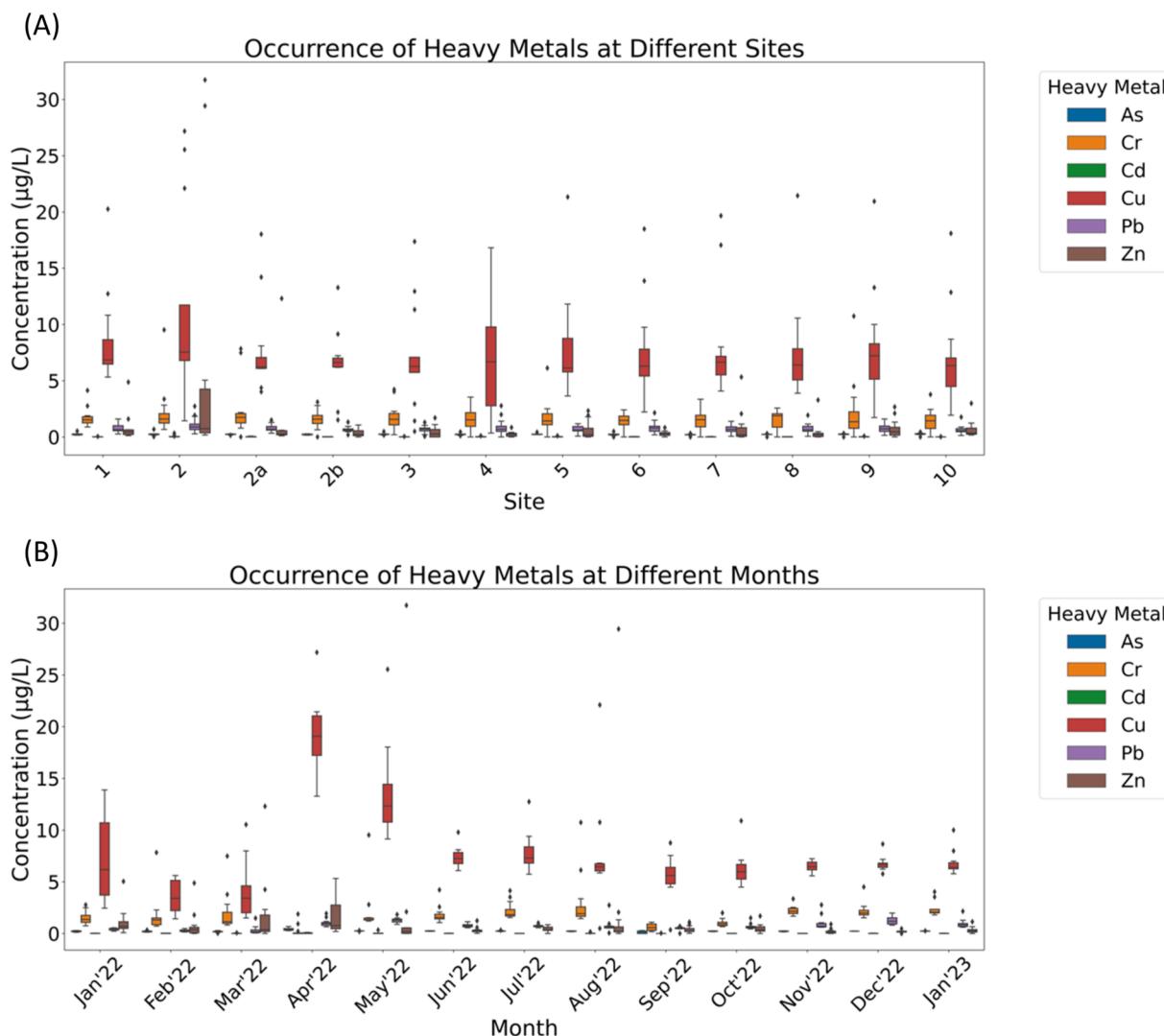


Fig. 8. Concentration of heavy metals ($\mu\text{g}/\text{L}$) measured across (A) different sites and (B) different months. The boxplots represent the distribution of heavy metals including As, Cr, Cd, Cu, Pb and Zn.

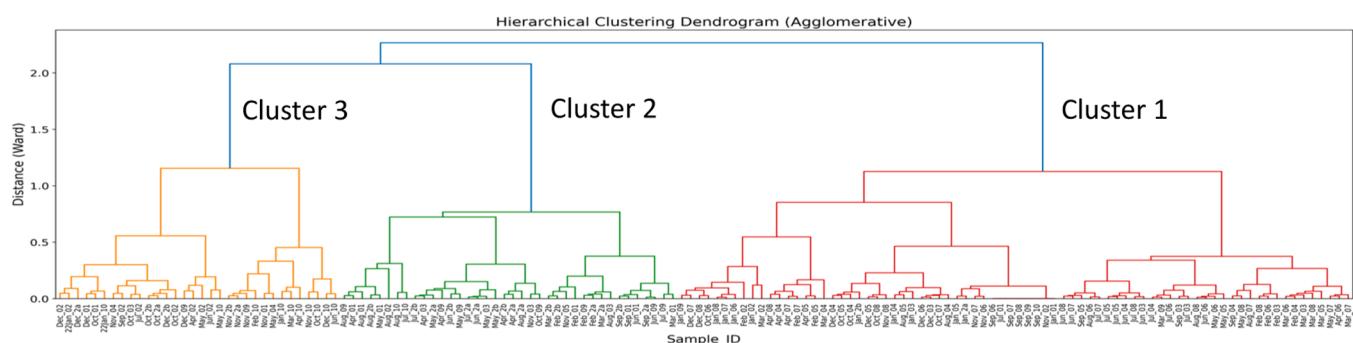


Fig. 9. Agglomerative hierarchical clustering dendrogram based on the combination of microbial, chemical and environmental data. Sample IDs are displayed along the x-axis, and y-axis represents the distance at which clusters are merged. Different colours indicate distinct clusters formed at various level of similarity.

higher risk of eco-toxicity and AMR development at Site 10. These findings suggest that the structural and operational differences between RAS and open cage farm significantly impact the AMR landscape, with implications for the management and design of aquaculture operations to mitigate the risk of AMR propagation.

To understand the influence of chemical compounds on AMR at coastal environments, it is crucial to access their concentrations. This

study indicates that the chemical concentrations are generally low, except for elevated Cu levels in Singapore's coastal areas, and higher levels of ERY-H₂O at Site 10. As specified in the USEPA aquatic life criteria table, criterion maximum concentration (CMC) for acute toxicity and criterion continuous concentration (CCC) for chronic toxicity of Cu are 4.8 and 3.1 $\mu\text{g}/\text{L}$, respectively (Table S6). Alarmingly, Cu concentrations exceeded the chronic CCC in 96 % of the samples and the acute

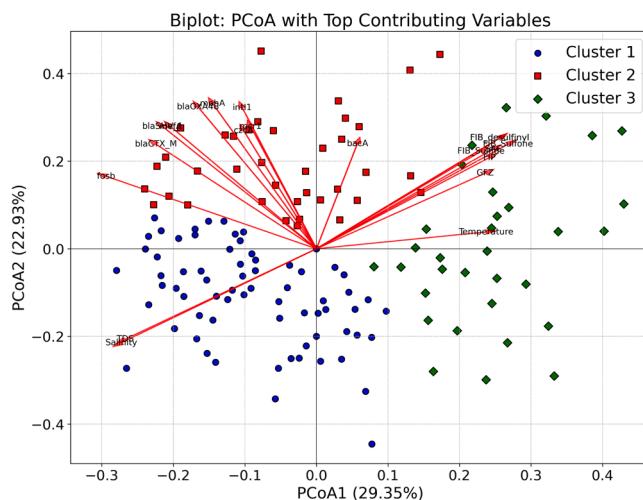


Fig. 10. Biplot of PCoA with top contributing variables. The plot shows the distribution of samples across three clusters (Cluster 1 in circle blue, Cluster 2 in square red, and Cluster 3 in diamond green) based on their microbial, chemical and environmental data. The arrows indicate the top contributing variables driving the separation among the clusters, with the length and direction of arrows representing the influence of each variable. PCoA1 and PCoA2 explain 29.35 % and 22.93 % of the total variance, respectively.

CMC in 87 % of samples, indicating a significant risk of toxicity to aquatic organisms, in particular, fishes in nearby fish farms. In addition, there is evidence that supports co-selection for antibiotic resistance by metals in various environments, as well as laboratory testing (Li et al.,

2021, 2019; Zhang et al., 2018). In contrast, the concentrations of As, Cr, Cd, Pb, Zn, and Ni were all below the USEPA's acute CMC and chronic CCC values, suggesting that these metals are not expected to pose immediate toxicity risks to marine life. While the levels of most chemicals found are relatively low and pose minimal immediate risks, the potential long-term impact of these trace chemicals on the emergence of AMR warrants consideration. Prolonged exposure of microbes to small quantities of these chemicals could potentially act as a source of selective pressure. This sustained low-level exposure may gradually drive the evolution of resistance mechanisms in microbial populations, suggesting a plausible pathway for the development of AMR over time. Therefore, it is essential to explore and monitor the long-term effects of these trace chemicals in the environment to fully understand their role in the emergence and spread of AMR.

The inclusion of the human marker, crAssphage, in this study provides valuable insights into potential faecal contamination from human sources within marine environments. Among the sampling sites, only Site 10 (west Johor Straits) showed the presence of crAssphage, with an average concentration of 1345 GC/L in 5 out of 13 samples. It is observed that most ARGs did not show significant correlations with crAssphage, except the *qacE* gene (Spearman's rho = 0.231, p-value = 0.005) and *sul1* gene (Spearman's rho = 0.246, p-value = 0.003). The *qacE* gene confers resistance to antiseptics (biocides), indicating an anthropogenic contribution, potentially linked to practices such as household cleaning and toilet washing. CrAssphage, on the other hand, shows significant correlations with many chemical compounds, such as lopinavir (LOP), used in treatments for viral infection. The lack of correlations between ARGs and crAssphage and chemical compounds hint at the possibility that ARGs detected in marine samples may be more closely related to environmental sources such as agriculture runoff,

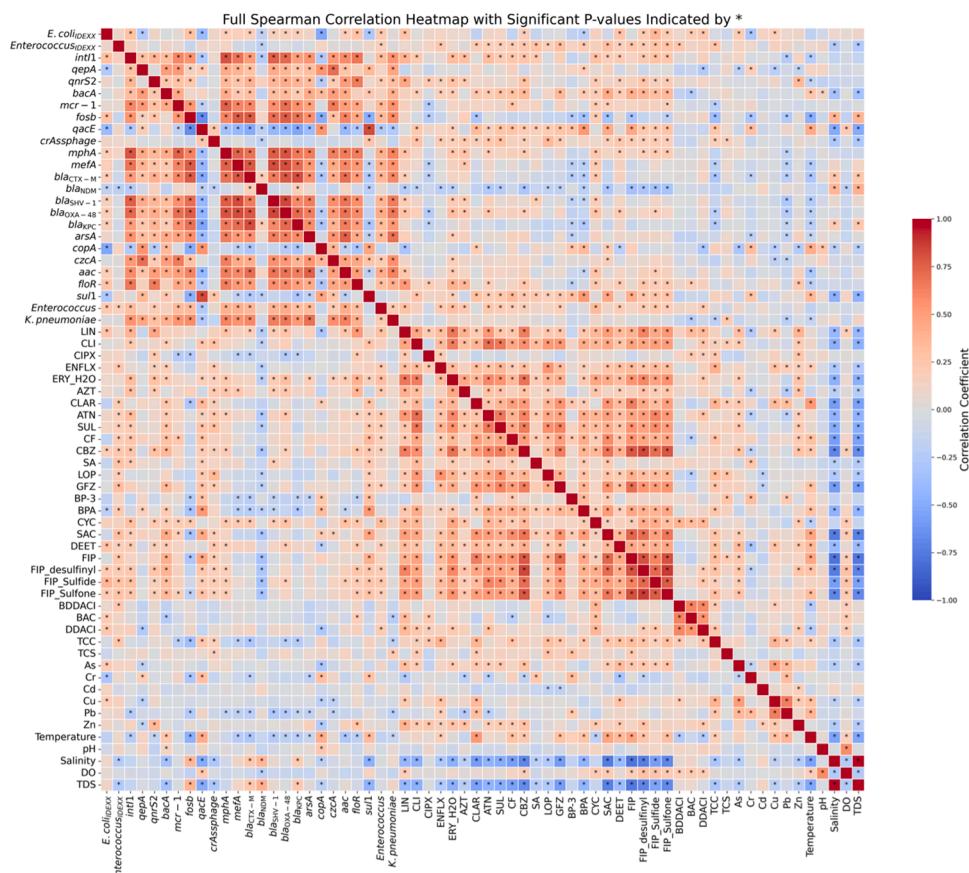


Fig. 11. Spearman correlations heatmap matrix displaying pairwise correlation coefficients among various microbial, chemical, and environmental variables. Pairs with significant p-values are indicated with asterisk (*).

Table 2

Percentage of resistance to antibiotics in fish, sediment and water samples from Sites 2 and 10. The MAR index was calculated according to Eq. (1).

	Fish_2	Fish_10	Sediment_2	Sediment_10	Water_2	Water_10
Ampicillin	57.9	92.9	100.0	27.3	96.6	53.6
Ampicillin/ Sulbactam	7.9	28.6	66.7	0.0	27.6	10.7
Piperacillin/ Tazobactam	0.0	0.0	0.0	0.0	0.0	0.0
Cefazolin	76.3	100.0	100.0	90.9	69.0	89.3
Cefoxitin	81.6	42.9	66.7	9.1	82.8	64.3
Ceftazidime	10.5	14.3	66.7	0.0	24.1	7.1
Ceftriaxone	0.0	0.0	0.0	0.0	0.0	3.6
Cefepime	21.1	14.3	66.7	0.0	24.1	10.7
Meropenem	0.0	7.1	0.0	0.0	3.4	0.0
Amikacin	0.0	0.0	0.0	0.0	0.0	0.0
Gentamicin	0.0	0.0	0.0	0.0	0.0	0.0
Tobramycin	0.0	0.0	0.0	0.0	0.0	0.0
Ciprofloxacin	10.5	0.0	0.0	0.0	24.1	0.0
Nitrofurantoin	0.0	7.1	0.0	0.0	3.4	0.0
Trimethoprim/ Sulfamethoxazole	7.9	0.0	0.0	0.0	13.8	0.0
Number of isolates	38	14	3	11	29	28
MAR Index	0.182	0.205	0.311	0.085	0.246	0.160

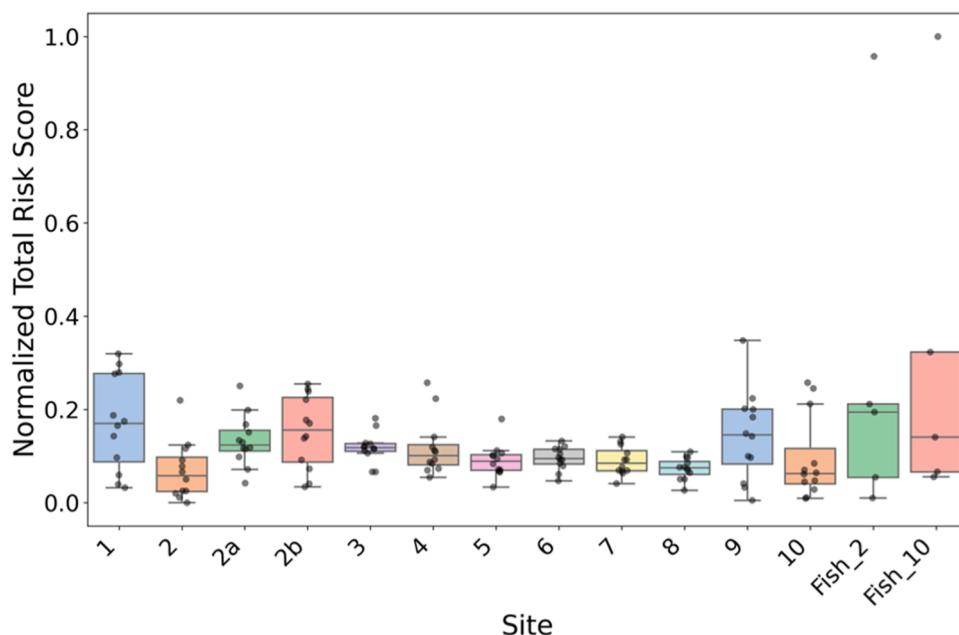


Fig. 12. Boxplot of normalized total risk scores across different sites. The plot displays the distribution of risk scores for each site, with individual data points indicated by dots. Sites include water samples (1–10) and fish samples (Fish_2 and Fish_10). The line in each box shows the median, box edges show the interquartile range (IQR), and whiskers extend to 1.5 times of IQR. Outliers are shown as dots beyond the whiskers.

household or industrial discharges and natural microbial communities or the spread of ARGs to environmental strains rather than being solely attributed to human faecal contamination.

To our knowledge, this study is the first to conduct a comprehensive evaluation of AMR risks by considering three critical determinants: ARB, ARG and antibiotics in aquatic environments influenced by anthropogenic activities. Nevertheless, there are limitations and challenges in the different modes of AMR risk assessment. One of the limitations is the scarcity of comprehensive data, particularly from resource-limited settings. This gap in data hampers the development of global risk models

and the accurate identification of emerging resistance trends. In addition, there is an absence of standardized methods for the detection of AMR determinants, resulting in discrepancies in data across various laboratories and studies. Another challenge is the limited understanding of the impact of selective pressures in natural environments. Unlike controlled laboratory environments, natural settings may feature multiple selective pressures, albeit at lower concentrations. In addition, the dynamics of resistance transmission, such as HGT, still remain unclear in these diverse natural systems.

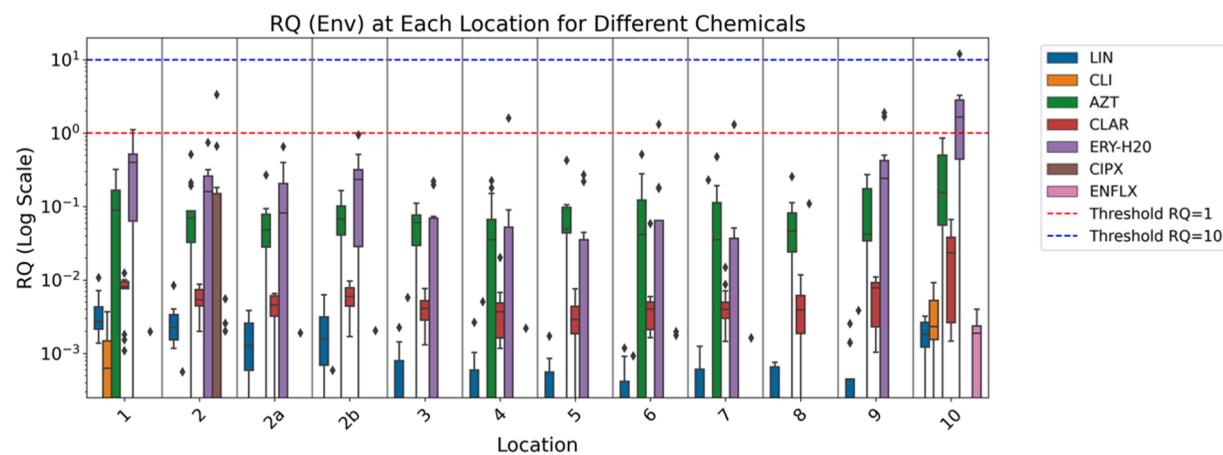


Fig. 13. Boxplot of environmental risk quotients (in log scale) for various antibiotics at each location. The red dashed line represents the threshold RQ = 1, and the blue dashed line represents the threshold RQ = 10. The line in each box shows the median, box edges show the interquartile range (IQR), and whiskers extend to 1.5 times of IQR. Outliers are shown as dots beyond the whiskers.

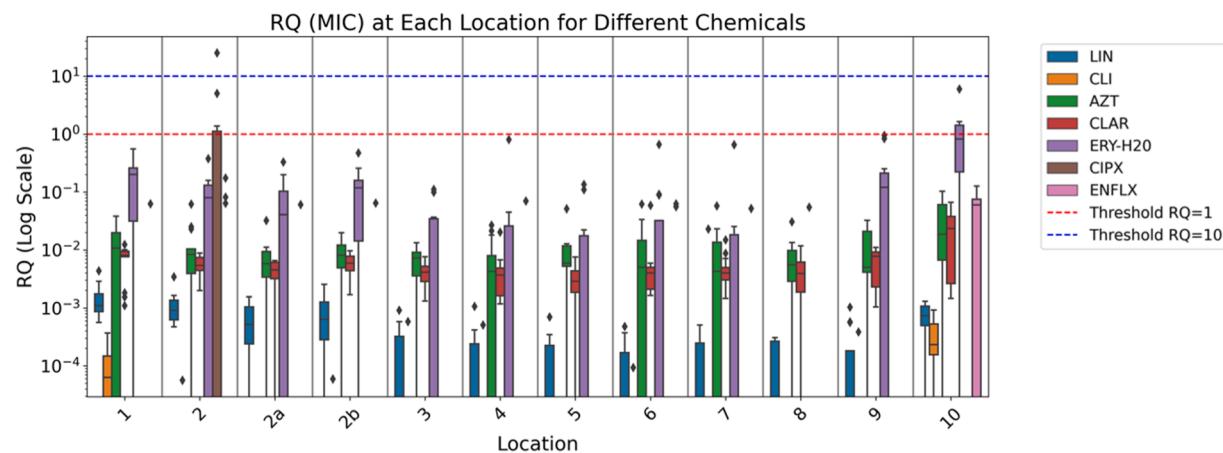


Fig. 14. Boxplot of antimicrobial resistance risk quotients (in log scale) for various antibiotics at each location. The red dashed line represents the threshold RQ = 1, and the blue dashed line represents the threshold RQ = 10. The line in each box shows the median, box edges show the interquartile range (IQR), and whiskers extend to 1.5 times of IQR. Outliers are shown as dots beyond the whiskers.

5. Conclusions

This study identified significant variations in AMR patterns between open cage farm and RAS. Antibiotic-resistant *Vibrio* was more prevalent in RAS water and sediment samples, while specific ARGs (*qepA*, *bla_{CTX-M}*, *bacA*) were more abundant in fish from the RAS. The closed-loop nature of RAS likely promotes nutrients and organic matter accumulation, creating hotspots for ARB, HGT and ARGs. Chemical contaminants varied across different sites, influenced by anthropogenic activities. Higher levels of chemical contaminants were observed in the open cage farm due to greater exposure to external contamination sources. In addition, environmental and seasonal variations also significantly shaped the distribution of ARGs and chemical contaminants in coastal ecosystems.

Hierarchical cluster analysis, based on microbial, chemical and environmental data, revealed three distinct clusters shaped by location, time, and aquaculture activities. The lack of strong correlations between ARGs and chemical compounds suggest that concentrations of chemical compounds in the coastal environment may be too low to exert significant selective pressures for resistance. However, special attention should be given to ERY-H2O and Cu due to their relatively high concentrations, which may contribute to selective pressure on AMR.

The multifaceted risk assessment approach using the MAR index,

CAMRI and Risk Quotient methodologies provide a comprehensive evaluation of AMR risks in coastal waters. These methods emphasize the importance of employing multidimensional approaches in AMR management to address the complexity and variability of resistance patterns, while highlighting the necessity of global standardization methods for detecting AMR determinants.

CRediT authorship contribution statement

Shin Giek Goh: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Luhua You:** Writing – original draft, Methodology, Formal analysis, Data curation. **Charmaine Ng:** Methodology, Data curation. **Xuneng Tong:** Methodology, Formal analysis, Data curation. **Sanjeeb Mohapatra:** Methodology, Formal analysis, Data curation. **Wei Ching Khor:** Writing – review & editing, Validation, Resources, Project administration, Conceptualization. **Hong Ming Glendon Ong:** Writing – review & editing, Validation, Resources, Project administration, Conceptualization. **Kyaw Thu Aung:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation, Conceptualization. **Karina Yew-Hoong Gin:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

Acknowledgements

This research was funded by the Singapore Food Agency. We would like to thank team members, Francis Charles, Jitxin Lim, Benjamin Chun Min Lim, Reuben Gangesh, Jing Jian Koh, Evelyn Quek, Jason Wai Yee Ku, Felix Tandadjaja, Qiyi Yuan, Dawn Zhixin Xiang, Yifan Zhang, Sijie Hong, Callie Cheung Yat Ka, Wu Yue, Shanice Yu En Tam and Kwang Ji Wang for their help with field sampling and lab works. We would also like to express our gratitude to National University of Singapore for providing support to this research.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.watres.2024.122353](https://doi.org/10.1016/j.watres.2024.122353).

References

- Ajayi, A.O., Odeyemi, A.T., Akinjogunla, O.J., Adeyeye, A.B., Ayo-ajayi, I., 2024. Review of antibiotic-resistant bacteria and antibiotic resistance genes within the one health framework. *Infect. Ecol. Epidemiol.* 14 (1), 2312953.
- An, T., Cai, Y., Li, G., Li, S., Wong, P.K., Guo, J., Zhao, H., 2023. Prevalence and transmission risk of colistin and multidrug resistance in long-distance coastal aquaculture. *ISME Commun.* 3 (1).
- Baker-Austin, C., Wright, M.S., Stepanauskas, R., McArthur, J.V., 2006. Co-selection of antibiotic and metal resistance. *Trends Microbiol.* 14 (4), 176–182.
- Bayen, S., Zhang, H., Desai, M.M., Ooi, S.K., Kelly, B.C., 2013. Occurrence and distribution of pharmaceutically active and endocrine disrupting compounds in Singapore's marine environment: influence of hydrodynamics and physical-chemical properties. *Environ. Pollut.* 182, 1–8.
- Carey, D.E., McNamara, P.J., 2015. The impact of triclosan on the spread of antibiotic resistance in the environment. *Front. Microbiol.* 5.
- Chakraborty, S., Owens, G., 2014. Metal distributions in seawater, sediment and marine benthic macroalgae from the South Australian coastline. *Int. J. Environ. Sci. Technol.* 11, 1259–1270.
- Chenia, H.Y., Jacobs, A., 2017. Antimicrobial resistance, heavy metal resistance and integron content in bacteria isolated from a South African tilapia aquaculture system. *Dis. Aquat. Org.* 126 (3), 199–209.
- Cuong, D.T., Karuppiah, S., Obbard, J.P., 2008. Distribution of heavy metals in the dissolved and suspended phase of the sea-surface microlayer, seawater column and in sediments of Singapore's coastal environment. *Environ. Monit. Assess.* 138, 255–272.
- Da Le, N., Hoang, T.T.H., Phung, V.P., Nguyen, T.L., Rochelle-Newall, E., Duong, T.T., Pham, T.M.H., Phung, T.X.B., Nguyen, T.D., Le, P.T., 2022. Evaluation of heavy metal contamination in the coastal aquaculture zone of the Red River Delta (Vietnam). *Chemosphere* 303, 134952.
- Dagostar, P., 2019. Antimicrobial resistance: implications and costs. *Infect. Drug Resist.* 12 (null), 3903–3910.
- Dewi, R.R., Hassan, L., Daud, H.M., Matori, M.F., Nordin, F., Ahmad, N.I., Zakaria, Z., 2022. Prevalence and antimicrobial resistance of escherichia coli, salmonella and vibrio derived from farm-raised red hybrid tilapia (*Oreochromis spp.*) and Asian Sea Bass (*Lates calcarifer*, Bloch 1970) on the west coast of peninsular Malaysia. *Antibiotics* 11 (2), 136.
- Done, H.Y., Venkatesan, A.K., Halden, R.U., 2015. Does the recent growth of aquaculture create antibiotic resistance threats different from those associated with land animal production in agriculture? *AAPS J.* 17 (3), 513–524.
- Ellis, T., North, B., Scott, A.P., Bromage, N.R., Porter, M., Gadd, D., 2002. The relationships between stocking density and welfare in farmed rainbow trout. *J. Fish Biol.* 61 (3), 493–531.
- Faja, O.M., Sharad, A.A., Younis, K.M., Alwan, M.G., Mohammed, B.J., Ahmad, A., 2019. Isolation, detection of virulence genes, antibiotic resistance genes, plasmid profile, and molecular typing among *Vibrio parahaemolyticus* isolated in Malaysian seawater from recreational beaches and fish. *Vet. World* 12 (7), 1140–1149.
- FAO, 2022. The State of World Fisheries and Aquaculture 2022. FAO.
- Fu, J., Yang, D., Jin, M., Liu, W., Zhao, X., Li, C., Zhao, T., Wang, J., Gao, Z., Shen, Z., Qiu, Z., Li, J.-W., 2017. Aquatic animals promote antibiotic resistance gene dissemination in water via conjugation: role of different regions within the zebra fish intestinal tract, and impact on fish intestinal microbiota. *Mol. Ecol.* 26 (19), 5318–5333.
- Goh, S.G., Haller, L., Ng, C., Charles, F.R., Jitxin, L., Chen, H., He, Y., Gin, K.Y.-H., 2023. Assessing the additional health burden of antibiotic resistant Enterobacteriaceae in surface waters through an integrated QMRA and DALY approach. *J. Hazard. Mater.* 458, 132058.
- Goh, S.G., Jiang, P., Ng, C., Le, T.-H., Haller, L., Chen, H., Charles, F.R., Chen, H., Liu, X., He, Y., Gin, K.Y.-H., 2022. A new modelling framework for assessing the relative burden of antimicrobial resistance in aquatic environments. *J. Hazard. Mater.* 424, 127621.
- Higuera-Llantén, S., Vásquez-Ponce, F., Barrientos-Espinoza, B., Mardones, F.O., Marshall, S.H., Olivares-Pacheco, J., 2018. Extended antibiotic treatment in salmon farms select multiresistant gut bacteria with a high prevalence of antibiotic resistance genes. *PLoS ONE* 13 (9), e0203641.
- Hora, P.I., Pati, S.G., McNamara, P.J., Arnold, W.A., 2020. Increased use of quaternary ammonium compounds during the SARS-CoV-2 pandemic and beyond: consideration of Environmental Implications. *Environ. Sci. Technol. Lett.* 7 (9), 622–631.
- Jia, Y., Lu, H., Zhu, L., 2022. Molecular mechanism of antibiotic resistance induced by mono- and twin-chained quaternary ammonium compounds. *Sci. Total. Environ.* 832, 155090.
- Kampouris, I.D., Klümper, U., Kramer, L., Sorum, H., Wedekind, H., Berendonk, T.U., 2022. Dissemination of antibiotic resistance in antibiotic-free recirculating aquaculture systems. *J. Hazardous Mater.* Adv. 8, 100201.
- King, R.K., 2001. The Presence of Pathogenic Bacteria in Recirculating Aquaculture System Biofilms and Their Response to Various Sanitizers. Virginia Polytechnic Institute and State University, Blacksburg, Virginia.
- Lane, D.J., 1991. 16S/23S rRNA Sequencing. John Wiley & Sons, New York.
- Larsson, D.G.J., Flach, C.F., 2022. Antibiotic resistance in the environment. *Nat. Rev. Microbiol.* 20 (5), 257–269.
- Li, F., Chen, L., Chen, W., Bao, Y., Zheng, Y., Huang, B., Mu, Q., Wen, D., Feng, C., 2020. Antibiotics in coastal water and sediments of the East China Sea: distribution, ecological risk assessment and indicators screening. *Mar. Pollut. Bull.* 151, 110810.
- Li, J., Phulpoto, I.A., Zhang, G., Yu, Z., 2021. Acceleration of emergence of *E. coli* antibiotic resistance in a simulated sublethal concentration of copper and tetracycline co-contaminated environment. *AMB Express* 11 (1), 14.
- Li, S., Zhang, S., Ye, C., Lin, W., Zhang, M., Chen, L., Li, J., Yu, X., 2017. Biofilm processes in treating mariculture wastewater may be a reservoir of antibiotic resistance genes. *Mar. Pollut. Bull.* 118 (1), 289–296.
- Li, X., Gu, A.Z., Zhang, Y., Xie, B., Li, D., Chen, J., 2019. Sub-lethal concentrations of heavy metals induce antibiotic resistance via mutagenesis. *J. Hazard. Mater.* 369, 9–16.
- Li, X., Wang, H., Zhao, H., 2020. Propagation of antibiotic resistance genes in an industrial recirculating aquaculture system located at northern China. *Environmental Pollution* 261, 114155.
- Liú, Z., Yao, J., Ma, H., Rukeya, A., Liang, Z., Du, W. and Chen, Y. 2022 Bacterial hosts and genetic characteristics of antibiotic resistance genes in wastewater treatment plants of Xinjiang (China) revealed by Metagenomics.
- Lu, J., Wang, Y., Zhang, S., Bond, P., Yuan, Z., Guo, J., 2020. Triclosan at environmental concentrations can enhance the spread of extracellular antibiotic resistance genes through transformation. *Sci. Total Environ.* 713, 136621.
- Lu, S., Lin, C., Lei, K., Xin, M., Gu, X., Lian, M., Wang, B., Liu, X., Ouyang, W., He, M., 2022. Profiling of the spatiotemporal distribution, risks, and prioritization of antibiotics in the waters of Laizhou Bay, northern China. *J. Hazard. Mater.* 424, 127487.
- Luljija, R., Rupia, E.J., Alfaro, A.C., 2020. Antibiotic use in aquaculture, policies and regulation, health and environmental risks: a review of the top 15 major producers. *Rev. Aquac.* 12 (2), 640–663.
- Makkaew, P., Kongprajug, A., Chyerochana, N., Sresung, M., Precha, N., Mongkolsuk, S., Sirikanchana, K., 2021. Persisting antibiotic resistance gene pollution and its association with human sewage sources in tropical marine beach waters. *Int. J. Hyg. Environ. Health* 238, 113859.
- Martínez, J.L., Coque, T.M., Baquero, F., 2015. What is a resistance gene? Ranking risk in resistomes. *Nature Rev. Microbiol.* 13 (2), 116–123.
- Mills, M., Lee, S., Mollenkopf, D., Wittum, T., Sullivan, S.M.P., Lee, J., 2022. Comparison of environmental microbiomes in an antibiotic resistance-polluted urban river highlights periphyton and fish gut communities as reservoirs of concern. *Sci. Total Environ.* 851, 158042.
- Mohamad, N., Amal, M.N.A., Saad, M.Z., Yasin, I.S.M., Zulkiply, N.A., Mustafa, M., Nasruddin, N.S., 2019. Virulence-associated genes and antibiotic resistance patterns of *Vibrio* spp. isolated from cultured marine fishes in Malaysia. *BMC. Vet. Res.* 15 (1), 176.
- Monjura Afrin, R., Min, O., Benjamin, C.D., Adheesh, J., Connor, L.B., Peter, J.V., Amy, P., Liqiang, Z., 2024. MetaCompare 2.0: differential ranking of ecological and human health resistome risks. *bioRxiv*. 2024.2001.2017.576132.
- Murray, C.J.L., Ikuta, K.S., Sharara, F., Swetschinski, L., Robles Aguilera, G., Gray, A., Han, C., Bisignano, C., Rao, P., Wool, E., Johnson, S.C., Browne, A.J., Chipeta, M.G., Fell, F., Hackett, S., Haines-Woodhouse, G., Kashef Hamadani, B.H., Kumaran, E.A.P., McManigal, B., Achalapong, S., Agarwal, R., Akech, S., Albertson, S., Amusi, J., Andrews, J., Aravkin, A., Ashley, E., Babin, F.-X., Bailey, F., Baker, S., Basnyat, B., Bekker, A., Bender, R., Berkley, J.A., Bethou, A., Bielicki, J., Boonkasidecha, S., Bukosia, J., Carvalheiro, C., Castañeda-Orjuela, C., Chansamouth, V., Chaurasia, S., Chiurchiu, S., Chowdhury, F., Clotaje Donatien, R., Cook, A.J., Cooper, B., Cressey, T.R., Criollo-Mora, E., Cunningham, M., Darboe, S., Day, N.P.J., De Luca, M., Dokova, K., Dramowski, A., Dunachie, S.J., Duong Bich, T., Eckmanns, T., Eibach, D., Emami, A., Feasey, N., Fisher-Pearson, N., Forrest, K., Garcia, C.,

- Garrett, D., Gastmeier, P., Giref, A.Z., Greer, R.C., Gupta, V., Haller, S., Haselbeck, A., Hay, S.I., Holm, M., Hopkins, S., Hsia, Y., Iregbu, K.C., Jacobs, J., Jarovsky, D., Javanmardi, F., Jenney, A.W.J., Khorana, M., Khusuwani, S., Kissoon, N., Kobeissi, E., Kostyaney, T., Krapp, F., Krumkamp, R., Kumar, A., Kyu, H.H., Lim, C., Lim, K., Limmathurotsakul, D., Loftus, M.J., Lunn, M., Ma, J., Manoharan, A., Marks, F., May, J., Mayxay, M., Mturi, N., Munera-Huertas, T., Musicha, P., Musila, L.A., Mussi-Pinhata, M.M., Naidu, R.N., Nakamura, T., Nanavati, R., Nangia, S., Newton, P., Ngoun, C., Novotney, A., Nwakanma, D., Obiero, C.W., Ochoa, T.J., Olivas-Martinez, A., Olliaro, P., Ooko, E., Ortiz-Brizuela, E., Ounchanum, P., Pak, G.D., Paredes, J.L., Peleg, A.Y., Perrone, C., Phe, T., Phommasonne, K., Plakkal, N., Ponce-de-Leon, A., Raad, M., Ramdin, T., Rattanavong, S., Riddell, A., Roberts, T., Robotham, J.V., Roca, A., Rosenthal, V.D., Rudd, K.E., Russell, N., Sader, H.S., Saengchan, W., Schnall, J., Scott, J.A.G., Seekaew, S., Sharland, M., Shivamallappa, M., Sifuentes-Osornio, J., Simpson, A.J., Steenkiste, N., Stewardson, A.J., Stoeva, T., Tasak, N., Thaiprakong, A., Thwaites, G., Tigoi, C., Turner, C., Turner, P., van Doorn, H.R., Velaphi, S., Vongpradith, A., Vongsouvath, M., Vu, H., Walsh, T., Walton, J.L., Waner, S., Wangrangsimakul, T., Wannapinij, P., Wozniak, T., Young Sharma, T.E.M.W., Yu, K.C., Zheng, P., Sartorius, B., Lopez, A.D., Stergachis, A., Moore, C., Dolecek, C., Naghavi, M., 2022. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *The Lancet* 399 (10325), 629–655.
- O'Neill, J. 2014 Review on antimicrobial resistance. antimicrobial resistance: tackling a crisis for the health and wealth of nations.
- Oh, M., Pruden, A., Chen, C., Heath, L.S., Xia, K., Zhang, L., 2018. MetaCompare: a computational pipeline for prioritizing environmental resistome risk. *FEMS Microbiol. Ecol.* 94 (7).
- Ong, H.M.G., Zhong, Y., Hu, C.C., Ong, K.H., Khor, W.C., Schlundt, J., Aung, K.T., 2023. Quantitative risk evaluation of antimicrobial-resistant vibrio parahaemolyticus isolated from farmed grey mullets in Singapore. *Pathogens*. 12 (1).
- Pal, C., Asiani, K., Arya, S., Rensing, C., Stekel, D.J., Larsson, D.G.J., Hobman, J.L., 2017. Advances in Microbial Physiology. Academic Press, pp. 261–313. Poole, R.K. (ed.).
- Pal, C., Bengtsson-Palme, J., Kristiansson, E., Larsson, D.G.J., 2015. Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential. *BMC Genomics* 16 (1), 964.
- Paul, D., Chakraborty, R., Mandal, S.M., 2019. Biocides and health-care agents are more than just antibiotics: inducing cross to co-resistance in microbes. *Ecotoxicol. Environ. Saf.* 174, 601–610.
- Peters, L., Olson, L., Khu, D.T.K., Linnros, S., Le, N.K., Hanberger, H., Hoang, N.T.B., Tran, D.M., Larsson, M., 2019. Multiple antibiotic resistance as a risk factor for mortality and prolonged hospital stay: a cohort study among neonatal intensive care patients with hospital-acquired infections caused by gram-negative bacteria in Vietnam. *PLoS ONE* 14 (5), e0215666.
- Polat, N., Akkan, T., 2016. Assessment of heavy metal and detergent pollution in Giresun coastal zone, Turkey. *Fresenius Environ. Bull.* 25 (8), 2884–2890.
- Poole, K., 2017. At the nexus of antibiotics and metals: the impact of Cu and Zn on antibiotic activity and resistance. *Trends Microbiol.* 25 (10), 820–832.
- Qiu, D., Ke, M., Zhang, Q., Zhang, F., Lu, T., Sun, L., Qian, H., 2022. Response of microbial antibiotic resistance to pesticides: an emerging health threat. *Sci. Total Environ.* 850, 158057.
- Rajeshkumar, S., Li, X., 2018. Bioaccumulation of heavy metals in fish species from the Meiliang Bay, Taihu Lake, China. *Toxicol. Rep.* 5, 288–295.
- Rangasamy, K., Athiappan, M., Devarajan, N., Paray, J.A., 2017. Emergence of multi drug resistance among soil bacteria exposing to insecticides. *Microb. Pathog.* 105, 153–165.
- Schar, D., Zhao, C., Wang, Y., Larsson, D.G.J., Gilbert, M., Van Boeckel, T.P., 2021. Twenty-year trends in antimicrobial resistance from aquaculture and fisheries in Asia. *Nat. Commun.* 12 (1), 5384.
- Shoemaker, C.A., Evans, J.J., Klesius, P.H., 2000. Density and dose: factors affecting mortality of *Streptococcus iniae* infected tilapia (*Oreochromis niloticus*). *Aquaculture* 188 (3), 229–235.
- Siri, Y., Precha, N., Sirikanchana, K., Haramoto, E., Makkaew, P., 2023. Antimicrobial resistance in southeast Asian water environments: a systematic review of current evidence and future research directions. *Sci. Total Environ.* 896, 165229.
- Suyamud, B., Chen, Y., Quyen, D.T.T., Dong, Z., Zhao, C., Hu, J., 2024. Antimicrobial resistance in aquaculture: occurrence and strategies in Southeast Asia. *Sci. Total Environ.* 907, 167942.
- Thiang, E.L., Lee, C.W., Takada, H., Seki, K., Takei, A., Suzuki, S., Wang, A., Bong, C.W., 2021. Antibiotic residues from aquaculture farms and their ecological risks in Southeast Asia: a case study from Malaysia. *Ecosyst. Health Sustain.* 7 (1), 1926337.
- Thompson, J.R., Randa, M.A., Marcelino, L.A., Tomita-Mitchell, A., Lim, E., Polz, M.F., 2004. Diversity and dynamics of a north atlantic coastal Vibrio community. *Appl. Environ. Microbiol.* 70 (7), 4103–4110.
- Thongsamer, T., Neamchan, R., Blackburn, A., Acharya, K., Sutheeworapong, S., Tirachulee, B., Pattanachan, P., Vinithnantharat, S., Zhou, X.-Y., Su, J.-Q., Zhu, Y.-G., Graham, D., Werner, D., 2021. Environmental antimicrobial resistance is associated with faecal pollution in Central Thailand's coastal aquaculture region. *J. Hazard. Mater.* 416, 125718.
- Tran, N.H., Chen, H., Reinhard, M., Mao, F., Gin, K.Y.-H., 2016. Occurrence and removal of multiple classes of antibiotics and antimicrobial agents in biological wastewater treatment processes. *Water Res.* 104, 461–472.
- Tran, N.H., Reinhard, M., Khan, E., Chen, H., Nguyen, V.T., Li, Y., Goh, S.G., Nguyen, Q.B., Saeidi, N., Gin, K.Y.-H., 2019. Emerging contaminants in wastewater, stormwater runoff, and surface water: application as chemical markers for diffuse sources. *Sci. Total Environ.* 676, 252–267.
- Turner, S., Pryer, K.M., Miao, V.P.W., Palmer, J.D., 1999. Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis1. *J. Eukaryotic Microbiol.* 46 (4), 327–338.
- Van Boeckel, T.P., Brower, C., Gilbert, M., Grenfell, B.T., Levin, S.A., Robinson, T.P., Teillant, A., Laxminarayan, R., 2015. Global trends in antimicrobial use in food animals. *Proc. Natl. Acad. Sci.* 112 (18), 5649.
- Vestel, J., Caldwell, D.J., Tell, J., Constantine, L., Häner, A., Hellstern, J., Journel, R., Ryan, J.J., Swenson, T., Xei, W., 2022. Default predicted no-effect target concentrations for antibiotics in the absence of data for the protection against antibiotic resistance and environmental toxicity. *Integr. Environ. Assess. Manag.* 18 (4), 863–867.
- Vezzulli, L., Pezzati, E., Moreno, M., Fabiano, M., Pane, L., Pruzzo, C., The VibrioSea, C., 2009. Benthic ecology of vibrio spp. and pathogenic vibrio species in a coastal mediterranean environment (La Spezia Gulf, Italy). *Microb. Ecol.* 58 (4), 808–818.
- Wang, H., Ren, L., Yu, X., Hu, J., Chen, Y., He, G., Jiang, Q., 2017. Antibiotic residues in meat, milk and aquatic products in Shanghai and human exposure assessment. *Food Control* 80, 217–225.
- Watts, J.E.M., Schreier, H.J., Lanska, L., Hale, M.S., 2017. The rising tide of antimicrobial resistance in aquaculture: sources, sinks and solutions. *Mar. Drugs* 15 (6), 158.
- Wei, C., Wang, Y., Zhang, R., Liu, F., Zhang, Z.-E., Wang, J., Yu, K., 2024. Spatiotemporal distribution and potential risks of antibiotics in coastal water of Beibu Gulf, South China Sea: livestock and poultry emissions play essential effect. *J. Hazard. Mater.*, 133550
- WHO, 2021. Guidelines on Recreational Water Quality. Volume 1: Coastal and Fresh Waters. WHO, Geneva.
- Wozniak, T.M., Dyda, A., Lee, X., 2022. The increased length of hospital stay and mortality associated with community-associated infections in Australia. *Open Forum. Infect. Dis.* 9 (5), ofac133.
- Yern, K.K., Zain, N.A.M., Jaafar, M., Sani, M.A., Suhaimin, M.S.M., 2022. Prevalence of antibiotic resistance bacteria in aquaculture sources in Johor, Malaysia prevalence of antibiotic resistance. *J. Trop. Life Sci.*
- You, K.G., Bong, C.W., Lee, C.W., 2016. Antibiotic resistance and plasmid profiling of *Vibrio* spp. in tropical waters of Peninsular Malaysia. *Environ. Monit. Assess.* 188 (3), 171.
- Zhang, A.-N., Gaston, J.M., Dai, C.L., Zhao, S., Poyet, M., Groussin, M., Yin, X., Li, L.-G., van Loosdrecht, M.C.M., Topp, E., Gillings, M.R., Hanage, W.P., Tiedje, J.M., Moniz, K., Alm, E.J., Zhang, T., 2021a. An omics-based framework for assessing the health risk of antimicrobial resistance genes. *Nat. Commun.* 12 (1), 4765.
- Zhang, M., Hou, L., Zhu, Y., Zhang, C., Li, W., Lai, X., Yang, J., Li, S., Shu, H., 2022a. Composition and distribution of bacterial communities and antibiotic resistance genes in fish of four mariculture systems. *Environ. Pollut.* 311, 119934.
- Zhang, T., Ding, Y., Peng, J., Dai, Y., Luo, S., Liu, W., Ma, Y., 2022b. Effects of broad-spectrum antibiotic (Florfenicol) on resistance genes and bacterial community structure of water and sediments in an aquatic microcosm model. *Antibiotics* 11 (10), 1299.
- Zhang, X., Zhang, J., Han, Q., Wang, X., Wang, S., Yuan, X., Zhang, B., Zhao, S., 2021b. Antibiotics in mariculture organisms of different growth stages: tissue-specific bioaccumulation and influencing factors. *Environ. Pollut.* 288, 117715.
- Zhang, Y., Gu, A.Z., Cen, T., Li, X., He, M., Li, D., Chen, J., 2018. Sub-inhibitory concentrations of heavy metals facilitate the horizontal transfer of plasmid-mediated antibiotic resistance genes in water environment. *Environ. Pollut.* 237, 74–82.