Environmental antimicrobial resistance is associated with faecal pollution in Central Thailand’s coastal aquaculture region

Thunchanok Thongsamer\textsuperscript{c}, Rattikan Neamchan\textsuperscript{c}, Adrian Blackburn\textsuperscript{a}, Kishor Acharya\textsuperscript{a}, Sawanee Sutheeworapong\textsuperscript{b}, Bundit Tirachulee\textsuperscript{b}, Pavinee Pattanachan\textsuperscript{b}, Soydoa Vinitthanharat\textsuperscript{c}, Xin-Yuan Zhou\textsuperscript{d}, Jian-Qiang Su\textsuperscript{d}, Yong-Guan Zhu\textsuperscript{d}, David Graham\textsuperscript{a}, David Werner\textsuperscript{a,}\textsuperscript{*}

\textsuperscript{a} School of Engineering, Newcastle University, Newcastle upon Tyne, United Kingdom
\textsuperscript{b} Pilot Plant Development and Training Institute, King Mongkut’s University of Technology Thonburi, Bangkok 10140, Thailand
\textsuperscript{c} Environmental Technology Program, School of Energy, Environment and Materials, King Mongkut’s University of Technology Thonburi, Bangkok 10140, Thailand
\textsuperscript{d} Institute of Urban Environment, Chinese Academy of Science, Xiamen 361021, China

\textsuperscript{*} Corresponding author.
\textit{E-mail address:} david.werner@ncl.ac.uk (D. Werner).

https://doi.org/10.1016/j.jhazmat.2021.125718

Received 15 October 2020; Received in revised form 18 March 2021; Accepted 19 March 2021

1. Introduction

Considerable debate occurs regarding the importance of aquaculture as a driver for environmental antimicrobial resistance (AMR) (Mo et al., 2017; Hossain et al., 2017; Lai et al., 2018; Mrozik et al., 2019). In response to the rapidly growing global demand for aquaculture produce, aquaculture has become the fastest growing animal food-produce sector in the Asian region (Tacon, 2020). The rapid adaptation of intensive production methods has transformed entire coastal landscapes in Asia with many aquaculture facilities often in closest proximity (Mrozik et al., 2019; Ottinger et al., 2016). This trend has destabilized coastal ecosystems and created vulnerability in the aquaculture industry to water pollution, algae blooms, and the rapid spread of diseases (Brooks and Cooke, 2019; Szuster et al., 2008). Widespread disease outbreaks, such as most recently the acute hepatopancreatic necrosis (AHPN) caused by pathogenic strains of \textit{Vibrio parahaemolyticus}, have had significant economic impacts on the global aquaculture industry, with an estimated US $ 44 billion combined losses from AHPN outbreaks in shrimp aquaculture in China, Malaysia, Mexico, Thailand and Vietnam between 2010 and 2016 alone (Tang and Bondad-Reantaso, 2019). Initially, aquaculture producers responded to such disease outbreaks with the widespread and indiscriminate use of antibiotics, which then led to concerns about impacts on aquaculture practice, human health and the environment (Mo et al., 2017; Holmström et al., 2003). A review of publications on antibiotics use in aquaculture from 2008 to 2018 revealed evidence that directly linked antibiotics use to food safety concerns, occupational...
health hazards and antimicrobial resistance (Lulijwa et al., 2020). Global concerns about antibiotics use in aquaculture relate to the loss of veterinary effectiveness, as more and more pathogenic bacterial strains became antimicrobial resistant (Vincent et al., 2019). Also, antimicrobial residues in aquaculture produce may affect consumers directly (Pham et al., 2015), or antimicrobial use in aquaculture may lead to the dissemination of antimicrobial resistant bacteria via the environment (Shen et al., 2018; Huang et al., 2015; Xiong et al., 2015). In response to such concerns, and because of export bans for aquaculture produce failing European Commission Council and US Food and Drug Administration standards for antibiotic residuals, various Asian countries have started to implement mitigation measures. With such policies in place, compliance monitoring becomes important, and some authors have questioned whether the aquaculture industry adheres to the stricter standards (Lulijwa et al., 2020).

In this broader context, Thailand exemplifies a major Asian aquaculture producer who aims to achieve 20% and 30% reduction of antimicrobial consumption in humans and animals, respectively, in line with a national One Health approach (HPSR-AMR, 2018). The Thailand Surveillance of Antimicrobial Consumption group monitors the National Strategic Plan on Antimicrobial Resistance (2017–2021) (NSP-AMR, Table S1 in Supporting information), and the Thai Department of Fisheries monitors antibiotic residues in aquaculture produce. In Thailand, antimicrobial agents used in aquatic animal farms nowadays must be prescribed by a veterinarian, and only a few antimicrobial agents (oxytetracycline, amoxycillin, sulfadimethoxine/ormetoprim, enrofloxacín) are approved for use in aquaculture (Baoprasertkul et al., 2012).

Our recent investigation of water pollution issues, incl. antibiotics and tetracycline resistance, in the peri-urban environment of Bangkok, Thailand (Mrozik et al., 2019), raised questions about the role of urban versus rural sources as drivers of environmental antimicrobial resistance. To more comprehensively understand the main drivers of antimicrobial resistance in this important aquaculture region, we combined high-throughput qPCR (HT-qPCR) targeting 283 antibiotic resistance genes (ARGs) representing potential resistance to seven major classes of antibiotics, and 12 mobile genetic elements (MGE), with next generation sequencing (NGS), to assess the prevalence resistance to seven major classes of antibiotics, and 12 mobile genetic elements (MGE), with next generation sequencing (NGS), to assess the prevalence resistance to seven major classes of antibiotics, and 12 mobile genetic elements (MGE), with next generation sequencing (NGS), to assess the prevalence and sources of AMR in water and sediment at five aquaculture sites in Central Thailand, including two sites from the previous study. By comparing the occurrence of AMR in canals versus the associated aquaculture ponds we could test the hypothesis that aquaculture is an important driver of local environmental antimicrobial resistance (Rico et al., 2017; Jang et al., 2018; Chen et al., 2018; Gao et al., 2012). By comparing the occurrence of AMR in water versus sediment, we could test the hypothesis that aquaculture sediment acts as reservoir of antimicrobial resistance (Shen et al., 2020). By combining HT-qPCR with NGS of 16S rRNA gene amplicons, we could study relationships between AMR and microbial community characteristics. Our overall aim was to identify and help control the main sources of AMR in an important aquaculture region so that One Health policy
can be focussed accordingly.

2. Materials and methods

2.1. Case study sites, sampling and sample processing

Five aquaculture farms in different locations were selected for this survey (Fig. 1 and Fig. S1 in Supporting information). They were all located in an area of Central Thailand managed under the Marine and Coastal Resources Management Promotion Act B.E.2558(2015). Two farms (locations S1&2) were retained from our previous study to enable cross-comparison with the results from the previous fieldwork in June 2018 (Mrozik et al., 2019). The five aquaculture facilities investigated were earth dyke pond cultures with between 14,400 and 80,000 m² surface area. Aquaculture practices at the five sites followed a similar pattern of pond preparation, seeding, cultivating and harvesting, with sediment dredging typically every 12–14 months, and regular water exchange with the local canals for pond filling, draining, and to maintain pond water levels (Mrozik et al., 2019). The five farms all cultured Vannamei shrimp, in some cases in combination with other species (S2: Tilapia, S3: Mud Crab S4: Mud Crab, Tilapia). Water and sediment samples were collected in January 2019 from each farm (S1–5) in both, the culture pond (P), and the adjacent canal (C). Water (W) and sediment (S) samples were taken from different points to form composite samples. Temperature, pH, dissolved oxygen (DO), turbidity, salinity, and electrical conductivity (EC) was measured on site by a multiparameter water quality instrument (YSI, EXO1). Samples were stored in cooling boxes for transportation to the laboratory at King Mongkut’s University of Technology Thonburi (KMUTT). Sediment samples were frozen upon return to the laboratory, and water samples were stored in the fridge at 4 °C. For conventional microbiology by membrane filtration, triplicates of an appropriate water sample volume were filtered through 0.45 µm membranes for incubation and subsequent plate counting at KMUTT (Mrozik et al., 2019). In brief, the 0.45 µm sterile membrane filters were placed on selective agar for incubation. The media of Faecal Coliform Bacteria, E. coli and Faecal Streptococci were M-FC agar from HIDMEDIA, modified MTEC agar from Difco, and KF streptococcus agar from Difco, respectively. The plates for Faecal Coliform Bacteria and E. coli analysis were placed in the incubator at 44.5 and 35.5 °C, respectively, for 24 h. For Faecal Streptococci, the plates were incubated at 35.5 °C for 48 h.

Basic water quality analysis was also conducted at KMUTT and comprised ammonia (phenate method), nitrite (diazotization method), nitrate (sodium salicylate method), TKN (digestion and distillation), BOD (azide modification method), total phosphate (ascorbic acid method), and alkalinity (titration method) (Mrozik et al., 2019). In addition, triplicate 100 mL samples of canal and pond water were filtered in the evening of the sampling day through 0.22 µm membranes (Sartorius UK Limited, Surrey, UK), and these filters were frozen immediately. The frozen filters were shipped on ice to Newcastle University (NU) for DNA extraction from the retained biomass.

2.2. Molecular microbiology analysis

The total DNA from bacterial biomass retained by the membrane was extracted at Newcastle University (NU) using a PowerWater® DNA Isolation Kit as per the manufacturer’s instructions (QIAGEN, Crawley, UK). DNA purity and concentration was determined using a DS-11 Fluorospectrophotometer/fluorometer (DeNovix, Delaware, USA). To evaluate and quantify the abundance of antimicrobial resistance genes (ARGs) in water and sediment samples, HT-qPCR was performed on freeze-dried DNA shipped to the Institute of Urban Environment, Chinese Academy of Science, Xiamen (IUECAS) using SmartChip Real-time PCR (Wafgergen Inc. USA) following the method described elsewhere (Su et al., 2015). A total of 296 primer sets were used to screen and quantify 283 ARGs, mobile genetic elements (MGEs, 8 transposase genes and 4 integrase genes), and one universal bacterial 16S rRNA gene (Su et al., 2015). Raw HT-qPCR data was cleaned using SmartChip qPCR Software (V 2.7.0.1) that removes data from wells with multiple melting peaks or inefficient amplification (i.e., outside 90–110%). Cleaned data from replicate independent samples were screened according to their threshold cycle value (CT). Any sample with a CT > 31 was removed as, based on experience, a CT threshold ≥ 31 was the method detection limit (Ouyang et al., 2015). Genes that showed amplification for at least two replicates were considered positive and included in the analysis. The relative gene copy number of ARGs, transposase genes, or integrase genes, normalized by 16S rRNA genes, were calculated according to

\[
\frac{N_{\text{ARG/MGE}}}{N_{16S}} = \frac{10^{\left(C_{\text{CT_{target}}}-C_{\text{CT_{16S}}}/2\right)}}{10^{\left(C_{\text{CT_{16S}}}/2\right)}}
\]

Where N is the number of ARG, MGE or 16S rRNA gene copies, with the corresponding CT values. Estimated gene copy numbers were then obtained via absolute quantification of 16S rRNA genes in the samples, as previously described (Ouyang et al., 2015).

Real time qPCR assays were performed to quantify faecal markers genes on a BioRad CFX C1000 system (BioRad, Hercules, CA USA) using the primers shown in Table S2 in Supporting information, which also reports efficiency data. For quantification of target genes, 2 µL template DNA was used in a reaction mixture containing 5 µL 2 × SsoAdvanced Universal SYBR Green Supermix (BioRad), 500 nmol L⁻¹ of each forward and reverse primer, and molecular grade H₂O (Invitrogen, Life Technologies, Paisley, UK) to a final volume of 10 µL. Reaction conditions for quantification of each target gene were 98 °C for 3 min (1 ×), then 98 °C, 15 s, Primer Annealing Temperature (Tₐ), 60 s (40 ×). All samples were run in duplicate and molecular grade H₂O replaced template in control reactions. In order to avoid inhibitor effects, DNA samples were diluted to a working solution of 5 ng/µL. Standards with 10⁸ copies per µL of the target genes were obtained from Thermo Fisher, Cramlington, UK, and used to construct 8 point calibration curves using 10 fold dilutions. The limit of detection for the assays was 1–2 gene copies per µL, using 40 cycles of PCR.

30 ng of DNA was used to build a 16S rRNA sequencing library for nanopore sequencing with a 16S Barcoding kit (SQK-RAB204 from Oxford Nanopore Technologies (ONT), Oxford, UK) as per the manufacturer’s instructions and loaded onto a MinION sequencing apparatus flow cell (R9.4.1, FLO-MIN106). The workflow for the sequencing run to generate HDF5 raw signals, the processing of these raw signals to generate .fastq files (base-calling), and the interpretation of the .fastq files with the FASTQ 16S workflow followed the same procedures as outlined in Acharya et al. (2020), with Guppy software (version v2.1.3) and the EPiZOME platform (version v. 2.59.1896509) of ONT, Oxford, UK. For quality assurance, blanks and a MOCK community (Zymo Research, Irvine, California) consisting of genomic DNA from eight bacterial and two fungal species were included in the NGS analysis. MOCK community results were consistent with those of Acharya et al. (2019), who reported that assignment of partially erroneous reads to species by the MinION 16S bioinformatic workflow can result in mismatched identities for closely related species, while more reliable information is obtained at family and genus level. Highly conserved 16S rRNA genes in some bacterial families and genera hinder taxonomic resolution regardless of the NGS platform used (Maguvu et al., 2020), but 16S rRNA gene amplicon data nonetheless provide reliable information for ecological interpretations (Piwowar et al., 2020). In this study we therefore used genus level identities for the multivariate data analysis, and combined NGS with qPCR and plate count methods for cross-comparison of results (Acharya et al., 2019).
2.3. Data processing and statistical methods

The 283 ARGs quantified with HT-qPCR mostly represented resistance to seven major class of antibiotics, and ARGs were therefore aggregated into groups according to drug type and presumed resistance mechanism, or multidrug resistance. The data were normalized by 16S rRNA gene copies in each sample to account for variation in the bacterial abundance between samples, and these normalized data were square-root transformed and used for the multivariate data analysis. For the NGS data, EPI2ME CSV files were processed for multivariate data analysis using Matlab scripts following procedures outlined in Acharya et al. (2020). In brief, an equal number of reads above the quality threshold (15,000) was drawn without replacement from each barcode (i.e. sample). NGS data were then grouped at genera level, using only reads classified to at least genera level for the multivariate data analysis. The resulting OTU table contained many zeros (as is typical for NGS data (Tromas et al., 2017)), which can be problematic for the calculation of Euclidean distances. Square-root transformed relative abundance data (Hellinger transformation) was therefore used for the principal component analysis (PCA) to minimize this problem (Legendre and Gallagher, 2001). Cluster analysis was performed using average Euclidean distance for the linkage tree, and PCA was performed using Euclidean distance and other default Matlab © PCA settings. Three-way crossed ANOSIM was performed using Primer7 (primer-e, Auckland, New Zealand), to investigate effects of the factors 1) geographic location, 2) sediment versus water, and 3) canal versus pond, on the sample microbiome characteristics. An initial cluster analysis with MOCK community and blank samples is included in Fig. S2 in Supporting information. This identified one out of 60 samples as an outlier in the NGS data set with evidence for cross-contamination of this sample from the MOCK community samples during processing (Fig. S2 in supporting information). This sample was not only dissimilar from all the other environmental samples, but also more similar to the Mock community samples, indicating cross-contamination, and was therefore removed from the data set. Furthermore, Ralstonia contamination was indicated in some of the blank samples and reads classified to this genus were also removed from all data sets. Ralstonia is a common contaminant of DNA extraction kit or PCR reagents (Salter et al., 2014).

3. Results

3.1. Multivariate data analysis

A principal component analysis (PCA) of the prevalence of ARGs and

![Principal component analysis for the prevalence of ARGs and MGEs](image-url)

![Scores, 16S rRNA genes, NGS](image-url)

![Loadings, 16S rRNA genes, NGS](image-url)
MGES (Fig. 2 a and b), and microbial genera (Fig. 2 c and d) in the water and sediment samples provided an initial overview of the multivariate data. Additional PCA plots for subsets of samples are provided in Figs. S3 and 4 in Supporting information. Prevalence was measured as the ratio of ARGs or MGES to 16S rRNA genes, whereas the prevalence of microbial genera derived from NGS data was also measured as the ratio of 16S rRNA genes from each genus to total 16S rRNA genes classified for each sample. The average estimated absolute abundances of ARG copies in each sample aggregated into different antibiotic resistance classes together with standard deviations is shown in Table S3 in Supporting information.

In the PCA of the ARG and MGE data, principal component 1 (PC1) accounted for 88.16% of the observed variance, and water samples S1CW and S2CW clearly separated away from all the other environmental samples (Fig. 2a). These water samples were from the Hua Krabue canal at the inland edge of the coastal aquaculture region and in proximity to Bangkok (Fig. 1). The loadings plot (Fig. 2b) shows that all ARG and MGE classes, except for vancomycin resistance, contributed positively to PC1, meaning that the relative abundance of ARGs and MGEs was high in the Hua Krabue canal water. A three-way crossed ANOSIM confirmed sampling location as a significant factor in shaping the prevalence of antibiotic resistance genes (Table 1).

In ANOSIM, the R-value compares the mean of ranked dissimilarities between groups (i.e. between sampling locations) to within groups (i.e. from the same sampling location), and a R-value of 1 indicates dissimilarity between the groups that is greater than within groups, whereas an R-value of −1 indicates the opposite. Pairwise tests provided R values close to 1 for all locations with high statistical significance (Table 1). But relatively speaking, the lowest R value was obtained for locations S1 versus S2, on Hua Krabue canal (Fig. 1). Grouping canals versus ponds, and water samples versus sediment samples, also had significant ANOSIM testing outcomes with Global R values close to 1 (Table 1), indicating dissimilarity between the groupings. For the NGS data, PC1 accounted for 34% of the observed variance between microbiomes and separated water samples in a positive sign direction from the sediment samples (Fig. 2c). Several genera linked to the sulfur cycle had negative PC1 loadings and were prevalent in the sediment microbiomes (Fig. 2d). PC2 accounted for 18.04% of the observed variance and separated microbiomes from locations S1 and S2 in a positive sign direction from the other samples (Fig. 2c). This was particularly notable for the water samples. Hua Krabue canal water samples (S1CW and S2CW), and the corresponding aquaculture pond water samples (S1PW and S2PW), formed two distinct clusters away from the other water samples along PC2 (Fig. 2c), likely because these locations were the furthest from the coastline (Fig. 1). The planktonic freshwater genus *Pseudomonas* had positive, and the marine/estuary genera *Marinobacterium* and *Flumabilis* had negative PC2 loadings (Fig. 2d). ANOSIM confirmed significant dissimilarity between the microbiomes of sample groupings sediment versus water, canal versus pond and according to location (Table 1). In the pairwise comparison of locations, the microbiomes from S1 and S2 on the Hua Krabue canal had the lowest dissimilarity (i.e. lowest R value), which also was observed in the ARG and MGE data. In a nutshell, the multivariate data analysis found significant effects of the factors sampling locations, sample type (water versus sediment) and system type (canal versus pond) on the antimicrobial resistance gene profiles and microbiomes compositions in Thailand’s coastal aquaculture region. The PCA highlighted Hua Krabue canal water samples S1CW and S2CW as having high prevalence of antimicrobial resistance genes, compared to the other samples (Fig. 2a), and a microbiome distinct from the other samples investigated in this study (Fig. 2c).

### 3.2. ARG, MGE and faecal indicator genera profiles in canal and aquaculture pond water and sediment samples

Following on from the findings of the multivariate data analysis, Fig. 3 illustrates the prevalence of resistance to major classes of antibiotics (ARG, Fig. 3a and b) and mobile genetic elements (MGE, Fig. 3c and d) together with patterns observed for the prevalence of faecal indicator bacteria (FIB, Fig. 3e and f). Multidrug resistance from non-specific ARGs (Fig. 3a and b, yellow shading), which is often linked to generic defense mechanisms such as efflux pumps, was found across all water and sediment samples, with no obvious pattern distinguishing samples from different locations, canals vs ponds, or water vs sediment. On the other hand, the prevalence of resistance to major classes of antibiotics, most notably aminoglycoside (light blue shading in Fig. 3a), MLSB (green shading in Fig. 3a), and sulphonamides (dark blue shading in Fig. 3a), was elevated in the water samples from the Hua Krabue canal (samples S1CW and S2CW), which was similar to MGE patterns (Fig. 3c and d). These patterns caused the separation of Hua Krabue canal water from all the other water and sediment samples in the PCA (Fig. 2a and b). There was a strong positive Pearson correlation between the ARGs and MGEs data (r = 0.9356, p < 0.001), mainly because of the high prevalence of ARGs and MGES in Hua Krabue canal water samples (Fig. S5a in supporting information). Without the Hua Krabue canal water samples, the Pearson correlation was weaker, but still statistically significant (r = 0.5562, p = 0.017).

Because faecal indicator bacteria (FIB) were not among the most predominant genera in the sample microbiomes, they were not highlighted in the PCA loadings plot (Fig. 2d), and their relative abundance in the various microbiomes is therefore illustrated in Fig. 3e and f. The average relative abundances of the FIB genera in each sample together with standard deviations is provided in Table S4 in Supporting information. Water samples from the Hua Krabue canal (S1CW and S2CW) had higher relative abundance of faecal pollution indicator genera as compared to the other water and sediment samples. There was a strong positive Pearson correlation between the ARG&MGE and FIB data (r = 0.9357, p < 0.001), mainly because of the high prevalence of ARG&MGE and FIB in Hua Krabue canal water samples (Fig. SSb in Supporting information). Without the Hua Krabue canal water samples, the Pearson correlation was weaker, and marginally not statistically significant (r = 0.4526, p = 0.059).

#### 3.3. Microbiological and chemical metadata

Conventional plate count methods (Fig. 4a) and quantitative PCR of

| Table 1 Three-way crossed ANOSIM results. |
|-------------------------------------------|
| **ARG and MGE, HT-qPCR**                  |
| **Genera, NGS**                           |
| Factor | R-value | p-value | Factor | R-value | p-value |
| Location | 0.997 | 0.001 | Location | 0.919 | 0.001 |
| (Global) | (Global) |
| S1, S2 | 0.944 | 0.002 | S1, S2 | 0.694 | 0.002 |
| (Pairwise) | (Pairwise) |
| S1, S3 | 1 (Pairwise) | 0.001 | S1, S3 | 1 (Pairwise) | 0.002 |
| S1, S4 | 1 (Pairwise) | 0.002 | S1, S4 | 1 (Pairwise) | 0.001 |
| S1, S5 | 1 (Pairwise) | 0.002 | S1, S5 | 1 (Pairwise) | 0.001 |
| S2, S3 | 1 (Pairwise) | 0.002 | S2, S3 | 0.981 | 0.001 |
| S2, S4 | 1 (Pairwise) | 0.001 | S2, S4 | 0.907 | 0.001 |
| S2, S5 | 1 (Pairwise) | 0.001 | S2, S5 | 0.909 | 0.001 |
| S3, S4 | 0.981 | 0.001 | S3, S4 | 0.889 | 0.001 |
| (Pairwise) | (Pairwise) |
| S3, S5 | 1 (Pairwise) | 0.001 | S3, S5 | 1.000 | 0.002 |
| S4, S5 | 1 (Pairwise) | 0.002 | S4, S5 | 0.949 | 0.001 |
| Water/Sediment | 1 (Global) | 0.001 | Water/Sediment | 1 (Global) | 0.001 |
| Canal/Pond | 0.989 | 0.001 | Canal/Pond | 0.946 | 0.001 |
| (Global) | (Global) |
Fig. 3. Ratio of (a, b) antibiotic resistance genes (ARGs), (c, d) mobile genetic elements (MGE), and (e, f) 16S rRNA genes from faecal indicator bacteria (FIB), relative to total 16S rRNA genes as marker of the overall abundance of bacteria/archaea, for DNA from (a, c, e) water (W), and (b, d, f) sediment (S) samples from canals (C) and the associated aquaculture ponds (P). See Fig. 1 for the site locations S1-S5. The figures show the average for triplicates, and the error bar indicates the standard deviation for the sum of genes. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)
Aquaculture facilities do not appear to be the primary driver of environmental AMR spread in Central Thailand’s coastal aquaculture region. Instead, relative ARG prevalence was the highest in Hua Krabue canal water (Fig. 3) at the inland edge of the coastal aquaculture region (Fig. 1), where faecal pollution markers were also elevated (Figs. 3 and 4). Notably, ARG & MGE prevalence at the two Hua Krabue canal locations (S1 and S2) was consistently and substantially higher in canal water compared with local aquaculture ponds. In a separate PCA of the water samples (Fig. S3a in Supporting information), PC1 explained 92.01% of the ARG&MGE variance, and clearly separated Hua Krabue water samples in a positive sign direction away from all the other water samples. All ARG&MGE classes had positive PC1 loadings except for vancomycin, which had near neutral PCI loadings (Fig. S3b in Supporting information). Vancomycin is not permitted for use in aquaculture (Table S1 in Supporting information), and some environmental bacteria in the order Lactobacillales have intrinsic vancomycin resistance (Orberg and Sandine, 1984; Ekwanzala et al., 2020), which may explain the neutral observation. Less clear-cut, but similar trends were observed in a PCA of the sediment samples. All ARG&MGE classes had positive loadings for PC1 which separated Hua Krabue canal location S2 in a positive sign direction away from the other sediment samples (Fig. S3c and d in Supporting information). The findings mirror our earlier observations from June 2018 that showed elevated tetracycline resistance tetC gene copy numbers in canal as compared to pond water samples in this periurban area of Bangkok (Mrozik et al., 2019). Antibiotics can provide selective advantage for bacteria with resistance traits, although the scale of impact varies from place to place. For example, Knapp et al. (2008) demonstrated an increase in the ratio of tetB to 16S rRNA genes in mesocosm units containing pristine surface water treated with oxytetracycline at 250 µg L⁻¹ for 56 days. However, the antibiotic concentrations which we had previously measured at locations S1 and S2 and an additional nearby location were at least three orders of magnitude below this concentration level (i.e. were < 250 ng/L) for both aquaculture pond and canal water samples (Mrozik et al., 2019). In that study the measured antibiotic residues were also low in local sediment and shrimp tissue samples. These observations make it unlikely that environmental antibiotic concentrations would have provided selective advantages for resistant bacteria in the canal and pond environments. Instead, the highest relative ARG prevalence coincided with a high relative prevalence of FIB in the Hua Krabue canal. The FIB paradigm does not allow one to determine the nature of faecal sources, as most animals shed these bacteria, and naturalized or environmentally adapted strains of FIB can persist in many habitats (Harwood et al., 2014). However, Hua Krabue canal drains water from central Bangkok, and Human E. coli qPCR assay data indicates human faecal contamination in the canal (Fig. 4b and Mrozik et al. (2019)), which together suggests urban wastewater as a likely FIB pollution source. While implicit, evidence here suggests the main driver of elevated AMR in the study area is faecal releases from Bangkok and inadequate wastewater treatment rather than antibiotic use in coastal aquaculture. In Bangkok, only 45–53% of total domestic wastewater is treated which also impacts eutrophication in canals and the Gulf of Thailand (Buathong et al., 2013; Thitanuwat et al., 2016). Here we show that urban pollution also appears to drive environmental antibiotic resistance in Central Thailand’s coastal aquaculture region between the Gulf of Thailand and Bangkok.

4. Discussion

4.1. Drivers of environmental antibiotic resistance in Central Thailand’s coastal aquaculture region

Aquaculture facilities do not appear to be the primary driver of environmental AMR spread in Central Thailand’s coastal aquaculture region. Instead, relative ARG prevalence was the highest in Hua Krabue canal water (Fig. 3) at the inland edge of the coastal aquaculture region (Fig. 1), where faecal pollution markers were also elevated (Figs. 3 and 4). Notably, ARG&MGE prevalence at the two Hua Krabue canal locations (S1 and S2) was consistently and substantially higher in canal water compared with local aquaculture ponds. In a separate PCA of the water samples (Fig. S3a in Supporting information), PC1 explained 92.01% of the ARG&MGE variance, and clearly separated Hua Krabue water samples in a positive sign direction away from all the other water samples. All ARG&MGE classes had positive PC1 loadings except for vancomycin, which had near neutral PCI loadings (Fig. S3b in Supporting information). Vancomycin is not permitted for use in aquaculture (Table S1 in Supporting information), and some environmental bacteria in the order Lactobacillales have intrinsic vancomycin resistance (Orberg and Sandine, 1984; Ekwanzala et al., 2020), which may explain the neutral observation. Less clear-cut, but similar trends were observed in a PCA of the sediment samples. All ARG&MGE classes had positive loadings for PC1 which separated Hua Krabue canal location S2

Fig. 4. Absolute abundance of faecal indicator bacteria (FIB) by (a) plate count methods, and (b) qPCR methods, in water samples from canals (C) and the associated aquaculture ponds (P). See Fig. 1 for the site locations S1–S5. The figures show the average for triplicates, and the error bar indicates the standard deviation.
assays were elevated in a reach of the River Melayu receiving discharge of wastewater oxidation ponds (Ho et al., 2021). Similar to our observations in Figs. 2d and 4a and b, the bacterial genera *Polynucleobacter* and *Novosphingobium*, *E. coli* marker gene copies by qPCR, and *E. coli* plate counts were all elevated near the wastewater discharge, and then decreased towards the coast due to increased dilution with marine water by tidal mixing. Similarly, more saline canal water from sampling locations S3–S5 nearer the Gulf of Thailand, had lower prevalence of ARG, MGE and faecal pollution indicators, compared with canal water from locations S1–2 nearer Bangkok. Such a pattern suggests that the high prevalence of ARGs and MGEs in urban polluted drainage water from the city of Bangkok becomes diluted by tidal mixing with marine water from the Gulf of Thailand as it passes through the coastal aquaculture region. It is increasingly recognized that inadequate sanitation is a major driver of antibiotic resistance spread in the emerging country environments (Graham et al., 2019), with implications for public health (WHO, 2020). Other studies have additionally linked antibiotic resistance in the environment to geochemical metal conditions (Knapp et al., 2011) and spread from manure to soil and surface water (Macedo et al., 2020). Such factors may explain a more ubiquitous background level of environmental antibiotic resistance, which in this study was mostly linked to multidrug resistance from non-specific ARGs, often linked to generic defense mechanisms such as efflux pumps.

Antibiotic use in aquaculture has been suspected to be an additional important driver of environmental antibiotic resistance (Rico et al., 2017; Jang et al., 2018; Chen et al., 2018; Gao et al., 2012). For example, Shen et al. studied a large-scale aquaculture farm cultivating fish in the Baima Lake, Jiangsu Province, China, and identified aquaculture sediment as reservoir of a more limited number of ARGs identified with conventional qPCR (Shen et al., 2020). Such findings differ from our findings here of relatively low relative abundance of antibiotic resistance genes in sediments with no evidence for higher ARG prevalence for sediment from aquaculture ponds versus canals (Fig. 3). In a separate PCA of the AMR&MGEs in aquaculture pond samples, there were no obvious sediment versus water groupings, whereas in a PCA of all the canal samples, it was the water samples from locations S1&2, and not the sediment samples, which were distinguished by high prevalence of AMR&MGEs (Fig. S4 in Supporting information). Indiscriminate use of antibiotics can undoubtedly promote environmental antibiotic resistance. However, based on owners’ testimonials, the relatively small-scale aquaculture systems investigated in this study were not using antibiotics, despite concerns about decreasing yields and water pollution impacts on the enterprises (Mrozik et al., 2019). Our environmental monitoring data corroborates these testimonials and suggests good compliance with Thai policies to reduce antibiotics use in aquaculture. At the same time, we show the impact of urban pollution on periurban aquacultures, including elevated antibiotic resistance in canals draining the city of Bangkok.

4.3. Policy implications

Aquaculture systems are highly complex, dynamic and interconnected systems, and surveillance of antibiotic usage and resistance is critical to address current knowledge gaps for risk assessment (Brunton et al., 2019). Our study findings show that Thai policies to mandate prescription for antibiotic use in aquaculture is effective. With a sound policy in place compliance monitoring becomes critical. With its report of antimicrobial consumption in humans and animals in 2017 in Thailand, NSP-AMR has established an important reference point for monitoring national progress towards reducing antibiotics use in line with national targets (HPSR-AMR, 2018). Introducing reporting requirements to disaggregated consumption by animal species would enhance understanding of antibiotics use in aquaculture at a national level. This top-down approach can be strengthened by the bottom-up approach of monitoring environmental antibiotic resistomes. To this end, we have shown the value of high-throughput metagenomic methods for comprehensive screening of environmental antibiotic resistomes in surface waters and sediments, and the added value of well-characterized environmental microbiomes for pollution source tracking. In this study, HT-qPCR was performed in China, and through the collaboration Thai partners became competent in NGS of 16S rRNA gene amplicons which they are now using independently. Our transnational collaboration thus demonstrates the feasibility of using these powerful and readily automated metagenomic tools in two middle-income countries which are globally amongst the most significant aquaculture producers. Wide and regular monitoring of environmental antibiotic resistomes can identify pollution hot-spots and sources to pinpoint the most effective countermeasures. In Central Thailand’s coastal aquaculture region our findings identified an urgent need to improve urban sanitation for the protection of periurban food-production systems. In this context, efforts by the Bangkok Metropolitan Administration to collect more revenue for wastewater infrastructure expansion and operation via a wastewater treatment fee (Wancharoen, 2018) are to be warmly welcomed. The city dwellers of Bangkok are typically more affluent than the national average, and can contribute to more resilient and eco-friendly food-production in Central Thailand’s coastal aquaculture region, in line Thai National Strategy 2018–2037 to preserve and restore natural resources and the environment for sustainable development (ONESDB, 2018). Our study provides a further line of evidence for the importance of safely managed sanitation for combating antibiotic resistance (WHO, 2020) and the inter-linkage of the United Nation’s Sustainable Development Goals, in particular between the targets 6.3 to halve the proportion of untreated wastewater, 6.6 to protect and restore water-related ecosystems, and 2.4 to ensure sustainable food production systems.

5. Conclusions

- There was no evidence to support the hypothesis that aquaculture is a major driver of environmental AMR spread in Central Thailand’s coastal region.
- There was also no evidence to support the hypothesis that aquaculture pond sediment serves as reservoir for AMR in the environment.
- Instead, relative ARG and MGE prevalence was found to be highest in peri-urban Hua Krabue canal water at the inland edge of the coastal aquaculture region.
- High relative ARG and MGE prevalence in Hua Krabue canal water was associated with high relative prevalence of faecal pollution indicator bacteria.
- The monitoring data suggested compliance of small-scale aquaculture farmers with Thai government One Health policies to reduce antimicrobial use in aquaculture.
- The findings raised concerns about the impacts of only partially treated urban water pollution on environmental AMR and water quality in Central Thailand’s coastal aquaculture region.

CRediT authorship contribution statement

Thunchanok Thongsamer: Methodology, Investigation, Writing - review & editing. Rattikan Neamchan: Methodology, Investigation, Writing - review & editing. Adrian Blackburn: Methodology, Investigation, Writing - review & editing. Kishor Acharya: Methodology, Formal analysis, Data curation, Writing - review & editing. Sawannee Sutheeworapong: Conceptualization, Supervision, Writing - review & editing. Bundit Tirachulee: Methodology, Investigation, Writing - review & editing. Pavinee Pattananach: Conceptualization, Supervision, Writing - review & editing. Soydoa Vinitnantharat: Conceptualization, Resources, Supervision, Writing – review & editing. Xin-Yuan Zhou: Methodology, Investigation, Writing - review & editing. Jian-Qiang Su: Methodology, Investigation, Writing - review & editing. Yong-Guan Zhu: Resources, Writing - review & editing. David Graham: Writing -
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
Raw sequencing data from the 16S rRNA gene sequencing are registered on the NCBI biosample database with sequence read archive (SRA) accession number PRJNA699700 (https://www.ncbi.nlm.nih.gov/sra/PRJNA699700). Additional data created during this research are openly available (https://doi.org/10.25405/data.ncl.13637684). Please contact Newcastle Research Data Service at rdm@ncl.ac.uk for access instructions.

Acknowledgements
This work was mainly funded by an Institutional Links grant between Newcastle University and KMITL (414469402), awarded by the Newton Fund via the British Council, and by the Office of Higher Education Commission (OHEC), Thailand. Additional support was provided by the UK Research and Innovation’s Engineering and Physical Sciences Research Council (EPSRC), project EP/P028527/1, and Biotechnology and Biological Sciences Research Council, project BB/T012471/1, and the Key Collaborative Research Program of the Alliance of International Science Organizations, Grant no. ANSO-CR-KP-2020-03.

Appendix A. Supporting information
Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.125718.

References
ONESDB. 2018. National Strategy 2018–2037, in, Office of the National Economic and Social Development Board, Bangkok, pp. 68.

Acharya, K., Khanal, S., Pantha, K., Amatya, N., Davenport, R.J., Werner, D., 2019. Antibiotic resistance gene abundances correlate with metal and geochemical conditions in archived scottish soils. PLoS One 6, e23735.

Laliyia, 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, 640–663.

Macedo, G., Hernandez-Leal, L., van der Maas, P., Heederik, D., Mevius, D., Schmitt, H., 2011. Antibiotic resistance in urban streams of Jiulongjiang River, China. Appl. Microbiol. 48, 1129–1136.

Orberg, P.K., Sandine, W.E., 1984. Common occurrence of plasmid DNA and vancomycin resistance in Leuconostoc spp. Appl. Environ. Microbiol. 48, 1129–1133.

Ottinger, M., Claus, K., Kuenzer, C., 2016. Aquaculture: relevance, distribution, impacts and spatial assessments – a review. Ocean Coast. Manag. 119, 244–266.

Ouyang, W.-Y., Huang, F.-Y., Zhao, Y., Li, H., Su, J.-Q., 2015. Increased levels of antibiotic resistance in urban stream of Jiulongjiang River. China. Appl. Microbiol. Biotechnol. 99, 5697–5707.

Pham, D.K., Chu, J., Do, N.T., Bree, F., Degand, G., Delahaut, P., De Pauw, E., DeMaeyer, E., Kautsky, N., 2003. Antibiotic use in shrimp farming and implications for antibiotic resistance in leuconostoc bacteria. J. Infect. 46, 197–203.

Ricco, A., Jacobs, R., Van den Broek, P.J., Teille, A., 2017. A probabilistic approach to assess antibiotic resistance development risks in environmental compartments and its application to an intensive aquaculture production scenario. Environ. Pollut. 231, 100–109.

Salter, S.J., Cox, M.J., Turek, E.M., Calus, S.T., Cookson, W.O., Moffatt, M.F., Turner, P., Parkhill, J., Loman, N.J., Walker, A.W., 2014. Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. BMC Biol. 12, 87.

Shen, Y., Jin, G., Zhao, Y., Shao, X., 2020. Prevalence and distribution analysis of antibiotic resistance genes in a large-scale aquaculture environment. Sci. Total Environ. 695, 139523.

Garlock, T., Asche, F., Anderson, J., Bjørdal, T., Kumar, G., Lorenzen, K., Ropicki, A., Smith, M.D., Tvetens, R., 2020. A Global blue revolution: aquaculture growth across regions, species, and countries. Rev. Fish. Sci. Aquac. 28, 107–116.

Graham, D.W., Giesen, M.J., Bunce, J.T., 2019. Strategic approach for prioritising local and regional sanitation interventions for reducing global antibiotic resistance. Water Air Soil Pollut. 238, 1–46.
associated with high incidence of mcr-1 carriage in humans across China. Nat. Microbiol. 3, 1054–1062.
Su, J.-Q., Wei, B., Ou-Yang, W.-Y., Huang, F.-Y., Zhao, Y., Xu, H.-J., Zhu, Y.-G., 2015. Antibiotic resistome and its association with bacterial communities during sewage sludge composting. Environ. Sci. Technol. 49, 7356–7363.
Szuster, B.W., Chalermwat, K., Flaherty, M., Intacharoen, P., 2008. Peri-urban oyster farming in the upper gulf of Thailand. Aquac. Econ. Manag. 12, 268–288.
Tacon, A.G.J., 2020. Trends in global aquaculture and aquafeed production: 2000–2017. Rev. Fisheries Sci. Aquac. 28, 43–56.
Tang, K.F.J., Bondad-Reantaso, M.G., 2019. Impacts of acute hepatopancreatic necrosis disease on commercial shrimp aquaculture. Rev. Sci. Tech. 38, 477–496.
Thitanuwat, B., Polprasert, C., Englande, J., 2016. Quantification of phosphorus flows throughout the consumption system of Bangkok Metropolis, Thailand. Sci. Total Environ. 542, 1106–1116.
Tromas, N., Fortin, N., Bedrani, L., Terrat, Y., Cardoso, P., Bird, D., Greer, C.W., Shapiro, B.J., 2017. Characterising and predicting cyanobacterial blooms in an 8-year amplicon sequencing time course. ISME J. 11, 1746–1763.
Vincent, A.T., Gauthier, J., Derome, N., Charette, S.J., 2019. The rise and fall of antibiotics in aquaculture. In: D. N. (Ed.), Microbial Communities in Aquaculture Ecosystems. Springer, Cham.
Wancharoen, S., 2018. Wastewater treatment fee plan resurfaces, 15 years on. In: Bangkok Post. Kowit Sanandang, Bangkok.
Technical brief on water, sanitation, hygiene and wastewater management to prevent infections and reduce the spread of antimicrobial resistance, 2020. Food and Agriculture Organization of the United Nations (FAO) and World Organisation for Animal Health (OIE), Geneva, Switzerland, p. 32.
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. Microb. Ecol. 70, 425–432.