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A case study on the distribution of the environmental resistome in Korean shrimp farms

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ABSTRACT

Hundreds of tons of antibiotics are widely used in aquaculture to prevent microbial infections and promote fish growth. However, the overuse of antibiotics and chemical products can lead to the selection and spreading of antibiotic-resistant bacteria (ARB) and antimicrobial resistance genes (ARGs), which are of great concern considering the threat to public health worldwide. Here, in-depth metagenome sequencing was performed to explore the environmental resistome and ARB distribution across farming stages in shrimp farms and examine anthropogenic effects in nearby coastal waters. A genome-centric analysis using a metagenome binning approach allowed us to accurately investigate the distribution of pathogens and ARG hosts in shrimp farms. The diversity of resistomes was higher in shrimp farms than in coastal waters, and the distribution of resistomes was dependent on the farming stage. In particular, the tetracycline resistance gene was found mainly at the early post-larval stage regardless of the farm. The metagenome-assembled genomes of *Vibrio* spp. were dominant at this stage and harbored *tet34*, which is known to confer resistance to oxytetracycline. In addition, opportunistic pathogens such as *Francisella*, *Mycoplasma*, *Photobacterium*, and *Vibrio* were found in abundance in shrimp farms, which had multiple virulence factors. This study highlights the increased resistance diversity and environmental selection of pathogens in shrimp farms. The use of environmental pollutants on farms may cause an increase in resistome diversity/abundance and the transmission of pathogens to the surrounding environment, which may pose future risks to public health and aquatic organisms.

1. Introduction

As the spread of antimicrobial resistance (AMR) increases, threats to public health around the world are of concern. AMR is a natural process of bacteria and is one of their common defense mechanisms (D'Costa et al., 2011). A number of antibiotic-resistant bacteria (ARB) have been identified in patients and hospital settings where antimicrobials have been used (Finland, 1955; Haller et al., 2018). In addition, the emergence of bacterial pathogens with extensive drug resistance and multidrug resistance can make treatment difficult and increase the cost of health care (Roca et al., 2015). The number of deaths per year from AMR so far is around 700,000, and the continued emergence of multidrug-resistant bacteria could result in 10 million deaths per year in the near future (Tagliabue and Rappuoli, 2018).

There has been rapid growth in aquaculture over the past few decades. Approximately 50% of the consumed seafood was produced from aquaculture in 2009 (Diana et al., 2013), and in 2014, fisheries and aquaculture generated around 56.6 million jobs worldwide (FAO, 2016). However, studies on the distribution of antimicrobial resistance genes (ARGs) in aquaculture are limited. The growth of aquaculture suggests that freshwater and marine environments may serve as reservoirs for ARGs and ARB (Cabello et al., 2016, 2013). Moreover, the use of antimicrobials in livestock farms is far more common than in human medicine. This may also be the same for aquaculture in some countries; for example, fluoroquinolone has been used more than 10 times the amount used in human medicine over several years in Chile (Millanao et al., 2011).

One of the major concerns associated with intensive aquaculture

Abbreviations: AMR, Antimicrobial resistance; ARB, Antibiotic-resistant bacteria; ARG, Antimicrobial resistance gene; EPL, Early post-larval; MAG, Metagenome-assembled genome; MQ MAG, Medium-quality metagenome-assembled genome; PERMANOVA, Permutational multivariate analysis of variance; VFDB, Virulence Factor Database.

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production is the use of oxytetracycline, which is one of the most widely used antibiotics in aquaculture (Leal et al., 2019). Oxytetracycline is a broad-spectrum antibiotic belonging to the tetracycline class (Chopra and Roberts, 2001). It is used in the treatment of bacterial diseases of fish caused by *Aeromonas*, *Pseudomonas*, *Lactococcus*, and *Vibrio*. Furthermore, it is frequently used at the sub-treatment level in fish feed to promote growth, improve feed efficiency, and prevent diseases (Cruz-Lacierda et al., 2000; Miller and Harbottle, 2018; Sangrungruang et al., 2004). The indiscriminate use of oxytetracycline in aquaculture has led to an increase in ARB, and antibiotic resistance to oxytetracycline is the most frequently reported antibiotic resistance (Leal et al., 2019). It has been reported that the abundance of the tetracycline resistance (*tetR*) gene is higher in aquaculture facilities using oxytetracycline than in those not using oxytetracycline (Seyfried et al., 2010). In addition, antibiotic residues can accumulate in aquaculture products; thus, frequent consumption can cause health problems. Therefore, it is crucial to minimize bacterial resistance problems by removing antibiotics from the water and eliminating biological and by-product activity (Mog et al., 2020).

Vibrio and *Photobacterium* spp. have been reported to account for more than 70% of the bacteria isolated from the intestine of *Penaeus monodon*, a species of shrimp. In particular, many *Vibrio* spp. produce chitinolytic enzymes (Sugita and Ito, 2006) in a chitin-rich environment, providing niche substrates for utilization. However, these enzymes can have negative effects on the carapace of the animal and other health complications (Jayasree et al., 2006; Lee et al., 2002). Several *Vibrio* spp. often cause mass mortality (Lavilla-Pitogo et al., 1998), and seemingly non-pathogenic *Vibrio* spp. also express virulence in compromised hosts (Manilal et al., 2010) and have historically resulted in significant losses to aquaculture farming. Nevertheless, *Vibrio* is described as the dominant genus within the shrimp gut microbiota, where most species live in harmony with the host.

Organic and inorganic toxicants from anthropogenic sources have become an environmental concern (Tauqeer et al., 2021a). The source of these environmental toxicants is diverse, ranging from agriculture to ammunition wastes and mining activities (Iftikhar et al., 2021; Tauqeer et al., 2021a). Recently, heavy metal contamination in the sediments and water of shrimp farms due to the use of chemical products (fertilizers, feed additives, and fungicides containing copper sulfate, manganese sulfate, zinc sulfate, etc.) has been reported (Lacerda et al., 2006, 2011; León-Cañedo et al., 2017). Wastewater from coastal aquaculture is commonly used for irrigation to address issues related to food scarcity and large populations (Tauqeer et al., 2021b). This can cause heavy metals to accumulate in the immediate environment and crops, resulting in indirect bioaccumulation (water-soil-plant-animal) and threatening human health (Tauqeer et al., 2021b). Among the different heavy metals, nickel, lead, and arsenic are hazardous pollutants in the soil, which can affect crop quality and productivity. In addition, contamination can cause serious human and animal health problems, such as metal poisoning. Therefore, methods of remediation with various biochar combinations or phytoremediation in heavy metal-contaminated environments with the enhancement of soil enzymatic activity are being actively investigated (Turan, 2021a, 2021b, 2020, 2019).

Aquaculture farming that intensively uses several environmental toxicants simultaneously can lead to harmful outcomes in the future. Antibiotics and chemical products can change the microbial community of fish and shellfish and increase their susceptibility to infection by antimicrobial-resistant fish pathogens. Therefore, this study investigated the types and distribution patterns of resistomes (antimicrobial, metal, and biocide resistance genes) in shrimp farms in Korea. This study provided baseline data on the resistome profile of shrimp aquaculture environments in Korea. We showed that anthropogenic contamination increased the diversity of resistomes and altered the community structure from a genome-centric perspective.

2. Methods

2.1. Sample collection

Shrimp farm samples were collected from four sites in Asan (Farm1) and Taean (Farm2) in South Korea: Asan aquaculture (Farm1), coastal water near Asan aquaculture (Coastal1), Taean aquaculture (Farm2), and coastal water near Taean aquaculture (Farm2) (Table S1). Each collection date represents a different growth stage (April for early post-larval (EPL) stage and September for harvest stage). All water samples were collected in a 20 L bottle and transported to the laboratory immediately.

2.2. Sample filtration

For each experiment, water samples were filtered through a pre-filter (Mixed Cellulose Esters, 3.0 μm pore size, 142 mm; Merck Millipore) and a collection filter (Mixed Cellulose Esters, 0.22 μm, 142 mm; Merck Millipore) using a peristaltic pump (Masterflex L/S Peristaltic Pump) at 4 mL/min. After filtration, the filters were finely shredded, and DNA was extracted using the DNeasy PowerSoil Kit (Qiagen, Germany) according to the protocol of the manufacturer.

2.3. Shotgun metagenomics and bioinformatic analysis

To identify the microbiome and resistome of the shrimp farm samples, a shotgun metagenomic library was prepared using TruSeq DNA Nano and sequenced with a paired-end run (2 × 150 bp) using the Illumina HiSeq X platform (Macrogen Inc., Seoul, Korea). Raw reads were quality-filtered using FaQCs (v.2.09) (Lo and Chain, 2014) with the default setting. FastUniq (Xu et al., 2012) was used to eliminate duplicate paired-end reads among the filtered reads. After quality filtering, 11–14 Gb of clean data were obtained for each sample. Each sample was de novo assembled separately using MEGAHIT (v.1.1.3) (Li et al., 2015) with the "–presets meta sensitive and –min-contig-len 500" options.

2.4. Resistome analysis

Quality-filtered reads were aligned to a curated ARG database named MEGARes (v.2.0) (Doster et al., 2020) using the AmrPlusPlus (v 2.0) pipeline (Doster et al., 2020) with the default option. To reduce false positives, resistome profiles were obtained by selecting only genes mapped to 30% or more of each gene length. In addition, in order to correct the sequencing depth between samples, the read count of ARGs was normalized to the 16S rRNA abundance. METAXA2 (v2.2.3) (Bengtsson-Palme et al., 2015) was used to quantify the 16S rRNA gene of each sample, and the normalized ARG abundance was specified as "copy of ARG per copy of 16S rRNA gene" (Liu et al., 2019b) (Eq. (1)).

$$Abundance = \sum_{1}^{n} \frac{Read\,count\,of\,ARG}{Length\,of\,ARG} \Big/ \frac{Read\,count\,of\,16S\,rRNA}{1432\,bp\,(Average\,length\,of\,16S\,rRNA)}$$

2.5. Genome-resolved metagenomic analysis

For genome binning, the clean reads of each sample were mapped to the contigs using BWA-MEM (v.0.7.17) (Li, 2013) and Samtools (v.1.3.1) (Li et al., 2009). Then, the abundance of each sample was calculated with "jgi_summarize_bam_contig_depths" from MetaBAT2 (v.2.12.1) (Kang et al., 2019a). The contigs of each sample were then binned with MetaBAT2 to reconstruct metagenome-assembled genomes (MAGs) using default parameters. We used CheckM (Parks et al., 2015) to estimate the completeness and contamination of each MAG (Table S2). Only MAGs with a completeness above 50% and a contamination below 10% were used for further analysis. As multiple samples may have the same MAG, dRep (v.2.3.2) (Olm et al., 2017) was used to

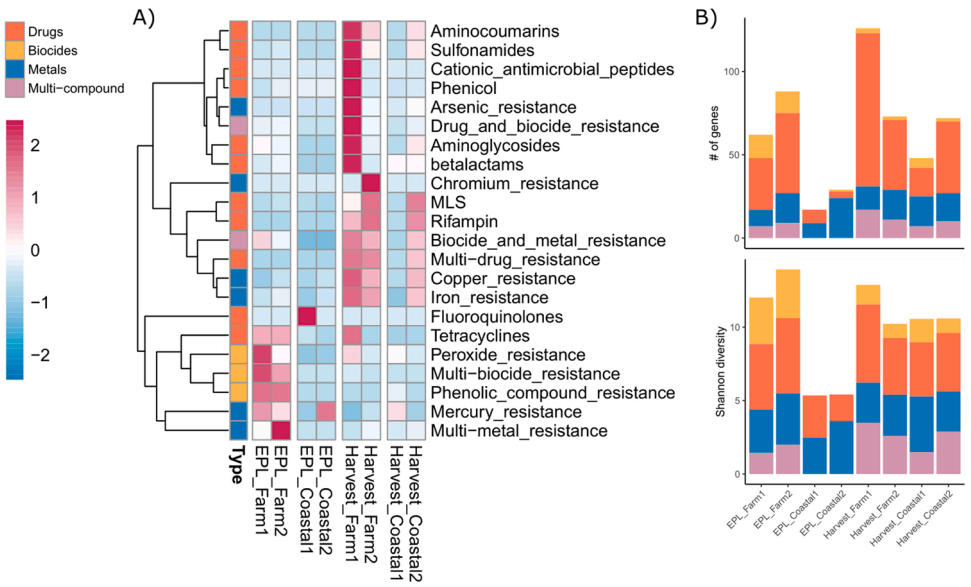

Fig. 1. Characterization of the resistome of shrimp farm and coastal marine samples. (A) Comparison of the resistome abundance according to the 22 resistome classes with four types. (B) Cumulative number and diversity of genes for each resistome class.

cluster the genomes on a defined average nucleotide identity (ANI, 0.99) to obtain a set of non-redundant MAGs for the combined dataset. The taxonomy of MAGs was assigned using the Genome Taxonomy Database Toolkit (GTDB-Tk; v.0.1.3) (Chaumeil et al., 2020), and the taxonomic tree was visualized by iTOL v6.

The quality-filtered reads from each sample were mapped against non-redundant MAGs using Bowtie2 (Langmead and Salzberg, 2012) with the '–very-sensitive' parameter. The relative abundance of each MAG was calculated as the number of reads mapped to each MAG dividing by the total reads of the sample. To determine the number of distinct MAGs in each sample, the presence of MAGs was determined when the relative abundance value was greater than 0.1.

The open reading frames of each MAG were predicted using Prodigal (v.2.6.2) (Hyatt et al., 2010) with default parameters. Then, ARGs in MAGs were identified using NCBI's AMRFinder (v.3.1.1b) (Feldgarden et al., 2019), and virulence factors were annotated using VFanalyzer (Liu et al., 2019a) by uploading the pathogen genome into the Virulence Factor Database (VFDB) (http://www. mgc.ac.cn/VFs/). We then used PlasFlow (v.1.1) (Krawczyk et al., 2018) to determine whether the ARG-carrying contigs of MAGs are plasmids.

2.6. Statistical analysis

Similarities in the resistome and MAG composition among samples were examined by principal coordinates analysis (PCoA). Data preprocessing reduced the influence of abundant species through square root transformations, and Bray-Curtis dissimilarity values were calculated using the 'vegdist' module. Subsequently, PCoA was performed using 'cmdscale'. To assess similarities between the resistome and MAG ordinations, Procrustes and PROTEST permutation (nperm = 999) analyses were performed. We performed multivariate analysis (PERMANOVA) to determine the effect of the farming stage and habitat (farm and coastal water) on Bray-Curtis dissimilarity using the 'adonis' module. These modules were loaded from the vegan library, and the data were visualized by the ggplot2 library in R.

3. Results

3.1. Diversity and distribution of the environmental resistome in shrimp farms

Four surface water samples from two shrimp farms were collected at the EPL stage and harvest stage (Table S1). In addition, four nearby coastal marine surface water samples were collected to compare the anthropogenic effects of aquaculture farming (Fig. S1). Shotgun metagenomic reads were sequenced at an average of 77.6 million pairs of reads for each sample, and read-based resistome analysis was performed using AmrPlusPlus. A total of 22 classes of resistance genes against various compounds (including antimicrobials, metals, and biocides) were characterized from the farm and coastal marine samples (Table S3). The abundance of antimicrobial, metal, and biocide resistance genes (referred to as resistomes) was estimated and normalized with the copy number of 16S rRNA genes. In all samples, resistance genes against metals were the most abundant, followed by genes against multiple compounds, biocides, and drugs. They were present in the samples at 15.29–4.36 times higher than the average 16S rRNA gene levels (Table S3). The abundance of these four resistome types was increased according to the farming stage; specifically, the abundance of drug resistance genes was increased by 5.21 and 4.54 times in the coastal and farm samples, respectively (Table S4). The prevalence of resistome classes differed between shrimp farm samples and nearby coastal marine samples (Fig. 1A) and was notably different according to the shrimp farming stage (Fig. 3A, Fig. S2A, PERMANOVA, $p = 0.036$). Tetracycline and three classes (peroxide, phenolic compound, and multi-biocide) of biocide resistance genes were abundant in quantity and diversity at the EPL stage in shrimp farm samples regardless of the farm (Fig. 1A). However, samples from shrimp farms at the harvest stage showed different distributions of ARGs between the two farms. Specifically, the number and abundance of genes with aminoglycoside, beta-lactam, sulfonamide, aminocoumarin, cationic antimicrobial peptide, and phenicol resistance were increased at the harvest stage for Farm1 (Fig. 1A). Generally, the diversity and number of ARGs were increased in shrimp farm samples compared with coastal marine samples (Fig. 1B), particularly ARGs targeting aminoglycosides and tetracyclines (Fig. S3, Table S3).

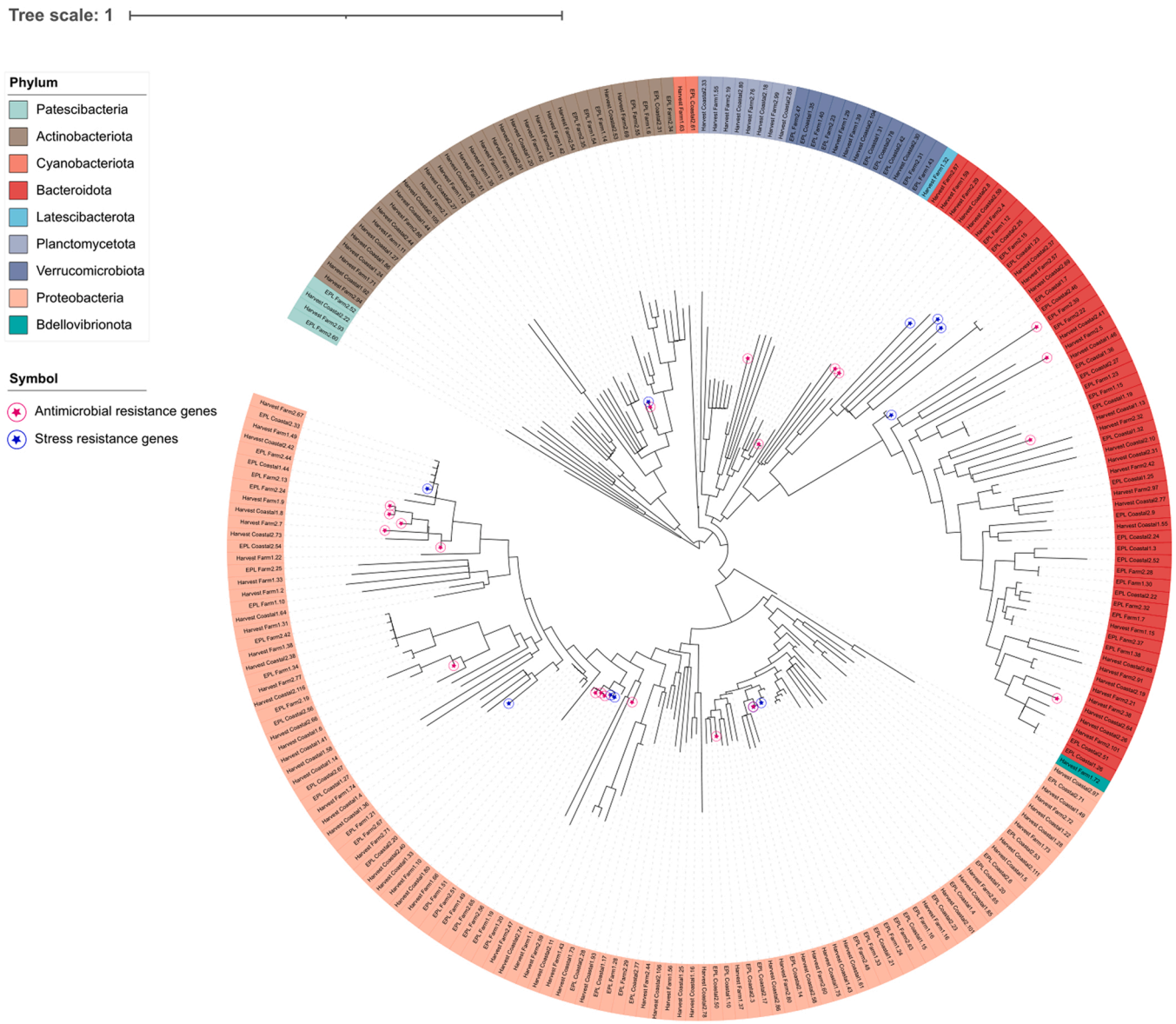

Fig. 2. Phylogenetic tree of MAGs inferred from the GTDB based on conserved single-copy marker genes. Star rings in the branch of tree show the resistome. The red color indicates antimicrobial resistance genes, and the blue color indicates stress resistance genes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Microbial MAGs according to the farming stage

A total of 645 bins were constructed using MetaBAT2, and 234 filtered medium-quality (MQ) MAGs were recovered according to the MIMAG criteria (Table S2) (Bowers et al., 2017). In addition, the dereplication step was performed to select representative MAGs at the strain level (< 99 ANI value), and 234 MAGs were finally recovered including 3 archaeal and 231 bacterial MAGs (Fig. 1). Among the MAGs spanning 11 phyla, Proteobacteria (111 MAGs) is the most common, followed by Bacteroidota (57 MAGs), Actinobacteriota (34 MAGs), Verrucomicrobiota (13 MAGs), Planctomycetota (8 MAGs), Patescibacteria (4 MAGs), Cyanobacteriota (2 MAGs), Thermoplasmatota (2 MAGs), Asgardarchaeota (1 MAG), Bdellovibrionota (1 MAG), and Latescibacterota (1 MAG). Of the 234 MAGs recovered, 79 and 200 MAGs could not be classified at the genus and species level, respectively, when compared with the GTDB database (Fig. 2) (Chaumeil et al., 2020).

The relative abundance of MAGs was calculated based on the number of mapped reads and indicated distribution differences between samples. PCoA clustering based on the Bray-Curtis distance of the samples showed distinct grouping between shrimp farm samples and coastal marine samples according to the farming stage. Moreover, significant differences were observed between the shrimp farming stages (Fig. S2B, PERMANOVA, $p = 0.03$). At the phylum level, Proteobacteria and Bacteroidota were dominant in all samples; however, even at the phylum level, there were differences between the farming stages, for example, the predominance of Verrucomicrobiota at the EPL stage and Bdellovibrionota at the harvest stage (Fig. 3). Moreover, among the EPL samples, there were notable differences in certain MAGs according to the habitat (Table 1). At the EPL stage, the abundance of Verrucomicrobiota was increased especially in coastal marine samples, of which the BACL24 MAG was dominant (Fig. 3). In contrast, in shrimp farm samples, the *Marinobacter*, *Polaribacter*, *Nonlabens*, and *Vibrio* MAGs were dominant (Fig. 3, Table S5). These results showed that the microbial community was influenced by the habitat, such as a farm or coastal marine environment; moreover, it was significantly affected by the farming stage.

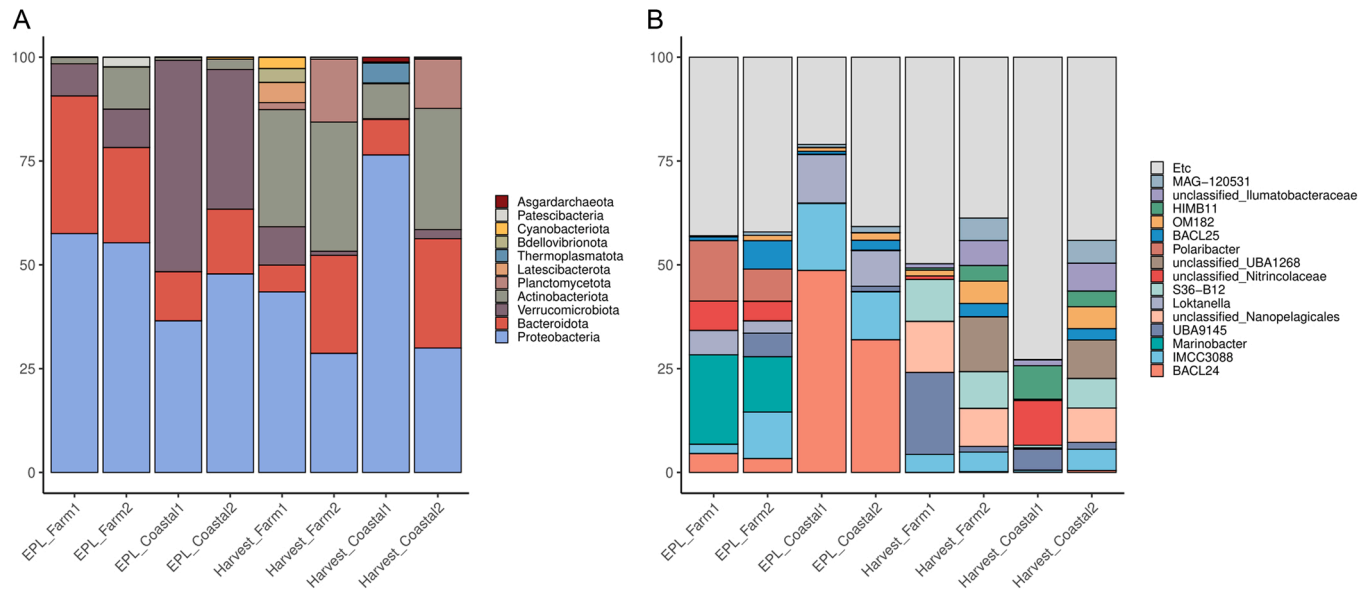

Fig. 3. Relative abundance of MAGs in each sample according to the taxonomic order. (A) Phylum level of MAG abundance. (B) Genus level of MAG abundance. Low-abundance MAGs at the genus level are colored in gray and grouped into "Etc".

Table 1
Number of unique MAGs in each group at the early post-larval stage.

Species	EPL	
	Farm	Coastal
Hyunsoonleella jejuensis	2	0
Polaribacter tangerinus	2	0
unclassified_Nonlabens	2	0
unclassified_Verruco-01	2	0
unclassified_Zobellella	2	0
Vibrio cyclitrophicus	2	0
Marinobacter adhaerens	1	0
Nautella italica	1	0
unclassified_Nitrincolaceae	1	0
unclassified_Photobacterium	1	0
unclassified_BACL24	0	2
unclassified_Fabibacter	0	2
unclassified_Roseovarius	0	2
Lentibacter algarum	0	1
Loktanella vestfoldensis_B	0	1
unclassified_Opitutaceae	0	1
unclassified_SCGC-AAA027-K21	0	1
unclassified_SCGC-AAA160-P02	0	1

3.3. Resistome host identification through genome-resolved metagenomics

Of the 234 MAGs recovered, only 30 MAGs had two types of resistomes (metal and drug). These MAGs were assigned to 4 phyla: Proteobacteria (17 MAGs), Bacteroidota (10 MAGs), Verrucomicrobiota (2 MAGs), and Actinobacteriota (1 MAG). Most of the MAGs had ARGs that could confer resistance to beta-lactam, except for the 5 MAGs with ARGs that could confer resistance to oxytetracycline, fosfomycin, and macrolide. In addition, most metal resistance genes were found in 10 MAGs for mercury, except for 1 MAG for arsenite; only 2 of the MAGs had two types of resistomes (drug and metal). For example, a MAG of the *Mycobacterium* genus carried resistance genes conferring resistance to beta-lactam (*bla*) and arsenite (*arsD*). Another MAG of *Rhodobacteraceae* bacterium HIMB11 harbored resistance genes against fosfomycin (*fosX*) and mercury (*merA* and *merF*). Moreover, 6 of the MAGs with resistomes belonged to opportunistic pathogenic bacteria. Two *Vibrio* MAGs and a *Photobacterium* MAG harbored ARGs that could confer resistance to oxytetracycline (e.g., *tet34*) and beta-lactam (*ampC*), respectively (Table 2). The *Mycobacterium* MAG also belonged to opportunistic pathogenic bacteria with resistance genes (Table 2).

Of the 15 MAGs with beta-lactam resistance genes, 7 MAGs belonged to Proteobacteria, specifically Gammaproteobacteria, and 6 MAGs belonged to *Pseudomonadales*. MAGs belonging to *Pseudomonadales* were found irrespective of the habitat and farming stage. Moreover, a comparison of the topology of the phylogenetic tree revealed that *bla* was intrinsic in the UBA9145 genus. In addition, Procrustes analysis found that the structure of taxonomic composition was associated with the distribution of resistomes between samples (Fig. S2C, Procrustes, $p = 0.001$). The findings indicated that some bacteria exhibited a pronounced tendency to carry specific ARGs (Fig. 2).

3.4. Prevalence of pathogenic bacteria in shrimp farm samples

A total of 7 MAGs spanning 4 genera were found, which are known opportunistic pathogenic genera (*Francisella*, *Mycoplasma*, *Photobacterium*, and *Vibrio*). Two *Vibrio* and *Photobacterium* MAGs were only found at the EPL stage in shrimp farm samples (Fig. 4). At the harvest stage, *Mycobacterium* and *Francisella* were detected. A *Francisella* MAG was only found in a shrimp farm, whereas 2 *Mycobacterium* MAGs were present in different proportions between the two shrimp farms (Fig. 4).

A total of 15, 11, and 9 putative virulence classes with 19, 47, and 24 virulence factors in *Vibrio*, *Mycobacterium*, and *Francisella* MAGs, respectively, were identified by VFanalyzer (Liu et al., 2019a). Ten virulence factors (LPS O-antigen, O-antigen, capsule, phytotoxin phaseolotoxin, urease, capsule biosynthesis and transport, AcrAB, LOS, allantoin utilization, and two-component system) were found in *Vibrio* MAGs, which were not found among other *Vibrio* spp. of the VFDB. In particular, phytotoxin phaseolotoxin (cysC1), which is mainly present in *Pseudomonas*, was found in 2 *Vibrio* MAGs and was not found in the *V. cyclitrophicus* FF75 strain. *Mycobacterium* and *Francisella* MAGs did not have reference genomes at the species level; thus, we compared them with the genomes in the VFDB (Liu et al., 2019a). *Mycobacterium* MAGs had the largest number of virulence-related genes; however, when compared with other *Mycobacterium* spp., only one more gene was identified as associated with immune evasion. In contrast, in the *Francisella* MAG, 6 virulence factors related to iron uptake, secretion system, antiphagocytosis, colonization, glycosylation system, and immune evasion were found, which were not found in other *Francisella* spp.

Table 2
Bacterial hosts of resistance genes.

Phylum	Class	Order	Family	Genus	Species	MAG ID	Resistance genes	CLASS
Bacteroidota	Rhodothermia					EPL_Farm1.12	*merA*	MERCURY
Proteobacteria	Gammaproteobacteria	Enterobacterales	Aeromonadaceae	Zobellella		EPL_Farm1.19	merA, merP, merR	MERCURY
Proteobacteria	Gammaproteobacteria	Enterobacterales	*Vibrionaceae*	*Vibrio*	*Vibrio cyclitrophicus*	EPL_Farm1.49	*tet(34)*	OXYTETRACYCLINE
Proteobacteria	Gammaproteobacteria	Peudomonadales	*Nitrincolaceae*			EPL_Farm1.21	*merC*	MERCURY
Actinobacteriota	Actinobacteria	Corynebacteriales	*Corynebacteriaceae*	*Mycobacterium*		Harvest_Farm1.42	*bla*	BETA-LACTAM
							arsD	ARSENITE
Proteobacteria	Alphaproteobacteria	Rhodobacterales	*Rhodobacteraceae*	*Roseovarius*		Harvest_Farm1.37	*fosX*	FOSFOMYCIN
Proteobacteria	Gammaproteobacteria	Francisellales	*Francisellaceae*	*Francisella*		Harvest_Farm1.1	*bla*	BETA-LACTAM
Proteobacteria	Gammaproteobacteria	Pseudomonadales	*Pseudohongiellaceae*	*UBA9145*		Harvest_Farm1.9	*bla*	BETA-LACTAM
Bacteroidota	Rhodothermia	Balneolales				EPL_Coastal1.23	*merA*	MERCURY
Verrucomicrobiota	Verrucomicrobiae	Opitutales	*Opitutaceae*			EPL_Coastal1.35	*bla*	BETA-LACTAM
Bacteroidota	Bacteroidia	Flavobacteriales	*1G12*			Harvest_Coastal1.13	*bla*	BETA-LACTAM
Proteobacteria	Alphaproteobacteria	Rhodobacterales	*Rhodobacteraceae*	*HIMB11*	*GCA_001510135.1*	Harvest_Coastal1.43	*fosX*	FOSFOMYCIN
							merA, merF	MERCURY
Proteobacteria	Gammaproteobacteria	Pseudomonadales	*Halieaceae*	*Luminiphilus*	*Luminiphilus sp2*	Harvest_Coastal1.6	*bla*	BETA-LACTAM
Proteobacteria	Gammaproteobacteria	Pseudomonadales	*Pseudohongiellaceae*	*UBA9145*		Harvest_Coastal1.8	*bla*	BETA-LACTAM
Bacteroidota	Bacteroidia					EPL_Farm2.39	*bla*	BETA-LACTAM
Bacteroidota	Rhodothermia	Balneolales				EPL_Farm2.15	*merA*	MERCURY
Proteobacteria	Alphaproteobacteria	Rhodobacterales	*Rhodobacteraceae*	*Nautella*	*Nautella italica*	EPL_Farm2.48	*merF*	MERCURY
Proteobacteria	Gammaproteobacteria	Pseudomonadales	*Pseudohongiellaceae*	*UBA9145*		EPL_Farm2.24	*bla*	BETA-LACTAM
Proteobacteria	Gammaproteobacteria	Pseudomonadales	*Pseudohongiellaceae*	*OM182*		EPL_Farm2.44	*merA*	MERCURY
Proteobacteria	Gammaproteobacteria	Enterobacterales	*Aeromonadaceae*	*Zobellella*		EPL_Farm2.56	*merA, merP, merR*	MERCURY
Proteobacteria	Gammaproteobacteria	Enterobacterales	*Vibrionaceae*	*Photobacterium*		EPL_Farm2.51	*ampC*	BETA-LACTAM
Proteobacteria	Gammaproteobacteria	Enterobacterales	*Vibrionaceae*	*Vibrio*	*Vibrio cyclitrophicus*	EPL_Farm2.65	*tet(34)*	OXYTETRACYCLINE
Verrucomicrobiota	Verrucomicrobiae	Opitutales	*Puniceicoccaceae*	*BACL24*	*GCA_002480015.1*	EPL_Farm2.31	*bla*	BETA-LACTAM
Bacteroidota	Kapabacteria	Kapabacteriales	*GCA-002839825*			Harvest_Farm2.29	*bla*	BETA-LACTAM
Bacteroidota	Bacteroidia	UBA7662				Harvest_Farm2.5	*bla*	BETA-LACTAM
Proteobacteria	Gammaproteobacteria	Pseudomonadales	*Pseudohongiellaceae*	*UBA9145*		Harvest_Farm2.7	*bla*	BETA-LACTAM
Bacteroidota	Bacteroidia	Cytophagales	*Cyclobacteriaceae*	*Fabibacter*		EPL_Coastal2.46	*merA*	MERCURY
Proteobacteria	Gammaproteobacteria	Pseudomonadales	*Pseudohongiellaceae*	*UBA9145*	*UBA8143*	EPL_Coastal2.54	*bla*	BETA-LACTAM
Bacteroidota	Bacteroidia	Flavobacteriales	*Flavobacteriaceae*	*MAG-120531*		Harvest_Coastal2.64	*erm*	MACROLIDE
Bacteroidota	Kapabacteria	Kapabacteriales	*GCA-002839825*			Harvest_Coastal2.8	*bla*	BETA-LACTAM

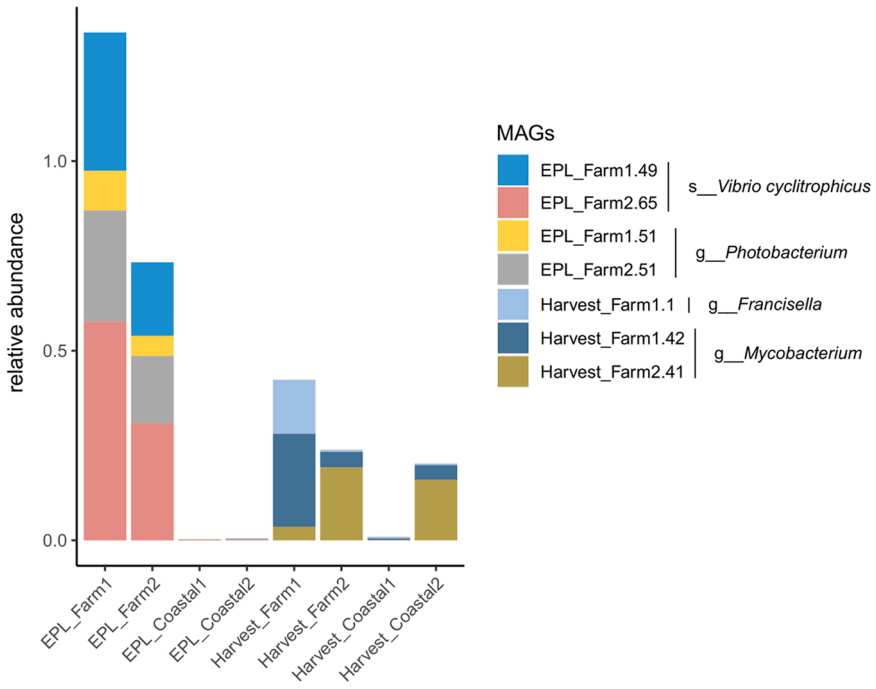

Fig. 4. Relative abundance of pathogenic MAGs including *Francisella*, *Mycoplasma*, *Photobacterium*, and *Vibrio* MAGs in each sample.

4. Discussion

Some studies have shown that antimicrobial agents in aquatic environments are diluted (Coyne et al., 2001). However, most antimicrobials are enriched in aquatic systems, and their active antimicrobial metabolites exert selective pressures (Björklund et al., 1991; Hektoen et al., 1995). In addition, it has been reported that tetracycline and oxolinic acid accumulate in sediments at concentrations sufficient to inhibit bacterial growth (Hektoen et al., 1995). The indiscriminate use of antibiotics can lead to accumulation in marine and aquafarm environments, affecting microbial communities. This could threaten global public health and consequently increase health-related costs (He et al., 2020; O'Neill, 2016). This study aimed to determine the distribution of resistance genes and changes in the microbial community due to antibiotic use in shrimp farms. We used a metagenomic approach to determine the distribution of resistomes in shrimp farms and their surrounding environments according to the farming stage. Additionally, to provide insights into the resistomes in the context of the genome and to identify hosts, we used a metagenomic binning approach, which allowed us to obtain a genome-centric perspective on the aquaculture microbial community.

Although the diversity of resistomes varied significantly according to the farming stage, antibiotic selection pressures resulted in a substantial increase in the amount and diversity of biocide, metal, and antimicrobial drug resistomes in the shrimp farms compared with the nearby coastal seawater. Overall, at the EPL stage, some resistomes were found in different farms, which included tetracycline (*tet34*, *tet35*, *tet59*, and *tetX*), aminoglycoside (*aph*(3') and *aph*(6)), and multi-biocide resistance genes. Biocide resistance genes were found primarily in farms at the EPL stage, and these genes have been reported to be primarily associated with effluent pumps (Mandal and Paul, 2019). The findings suggest the presence of metals, biocides, and antibiotics in artificial feeds used in the fields of agriculture and aquaculture (Pal et al., 2014). The distribution of resistomes at the harvest stage showed a different distribution from that at the EPL stage. In particular, genes conferring resistance to MLS (macrolides, lincosamides, and streptogramin A and B), rifampin, multiple drugs, copper, and iron were identified. Resistance genes against MLS and rifampin were mainly found in water samples from shrimp farms, as reported in a previous study (Zhao et al., 2018). The predominance of copper resistance at the harvest stage may be attributed to dietary supplementation with copper sulfate, which is commonly used as an algicide, fungicide, and antimicrobial agent (Borkow and Gabbay, 2004). Copper sulfate may have been used to control *Vibrio* spp. in the shrimp gut during the farming process (Zhou et al., 2017). In addition, it is possible that the abundance of metal resistance genes was increased due to continuous metal accumulation (Lemonnier et al., 2021) in adult shrimps. The similar distribution of resistomes at the harvest stage between farm samples (Farm2) and nearby coastal marine samples suggests a possible anthropogenic effect on the surrounding environment, which may be attributed to coastal aquaculture systems, where seawater near the farm may be contaminated with farm wastewater (Jang et al., 2018). As biocides and heavy metals can promote the spread of ARGs (Emerging and Risks, 2010), the environment around the farm requires remediation of heavy metal contamination from wastewater. Monitoring may be necessary to prevent the selection of metal resistance genes from the environment and ARG transmission, which can pose a risk to public health.

Through draft genome recovery using a culture-independent method, we attempted to characterize the microbial community of shrimp farms. This could effectively address the low proportion of culturable microorganisms in the culture-dependent method (Manaia et al., 2018) and identify bacterial hosts. As demonstrated by Procrustes analysis (Fig. S2C), the bacterial community structure and resistome distribution showed a significant correlation, which could promote ARG dissemination with ARB propagation. We also investigated whether the ARG-carrying contigs of MAGs are plasmids. Except for two contigs carrying a mercury resistance gene (*merF*), all contigs were not classified as plasmids. Therefore, in this study, most of the resistome, especially ARGs, may be spread by ARB to coastal areas through the aquaculture flow system. However, elucidating the overall relationship between mobilomes, such as plasmids, may be difficult with genome binning approaches (Maguire et al., 2020). Modern long-read sequencing techniques such as Nanopore or SMRT sequencing should be used (van Dijk et al., 2018), which are advantageous for understanding the genomic context and identifying bacterial hosts.

Oxytetracycline, which has a broad antibacterial spectrum, is one of

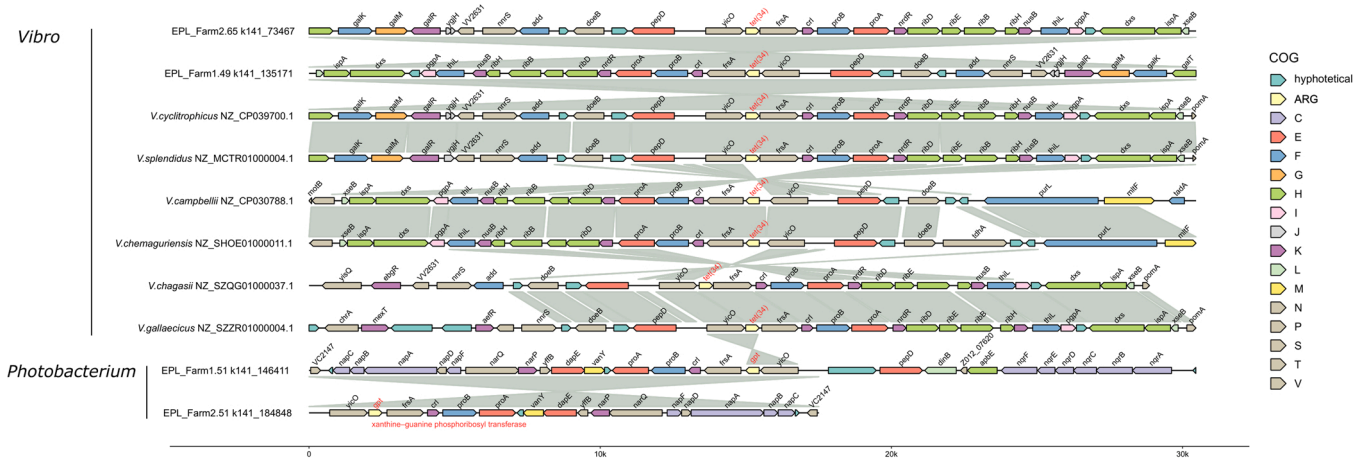

Fig. 5. Conserved oxytetracycline resistance gene (*tet(34)*) in the *Vibrio* and *Photobacterium* genome. *tet(34)* in the 15 kbp flanking region was compared.

the most commonly used antibiotics in fisheries for disease treatment and growth purposes (Cruz-Lacierda et al., 2000; Miller and Harbottle, 2018; Sangrungruang et al., 2004). As oxytetracycline is regularly used in Korean fisheries (Jee et al., 2014; Kang et al., 2019b), oxytetracycline resistance genes are likely to be abundant in shrimp farms at the EPL stage. Environmental changes according to the farming stage were accompanied by alterations in the microbial community and resistome profile, which were similar between the two farms at the EPL stage; specifically, 2 *Vibrio* MAGs harboring genes (e.g., *tet34*) conferring resistance to oxytetracycline were selected in this environment. We found that *tet34* was conserved across various *Vibrio* spp. In addition, in the *Photobacterium* MAG observed in the farm at the EPL stage, the gene encoding xanthine-guanine phosphoribosyl transferase (homologous to *tet34*) and surrounding genes were conserved with similar gene synteny as in *Vibrio* MAGs (Fig. 5). These results demonstrated that several *Vibrio* spp. and *Photobacterium*, high-risk opportunistic pathogens that are oxytetracycline-resistant, are offered the opportunity to be selected at the EPL stage (Fig. 5).

Vibrio, ubiquitous in marine environments with varying temperatures and salinities around the world, and *Photobacterium* are opportunistic pathogens for aquatic organisms and humans (Rivas et al., 2013; Robert-Pillot et al., 2014; Thompson et al., 2004). In the present study, *Vibrio* MAGs associated with *V. cyclitrophicus* and *Photobacterium* MAGs were the most abundant in shrimp farm samples; however, they were rarely found in nearby coastal samples at the EPL stage. *Mycobacterium* sp., which has been found to be associated with necrotizing granuloma and lung disease, can induce infection in fresh and marine water fish (Alexander et al., 2017; Hashish et al., 2018). The abundance of 2 *Mycobacterium* MAGs was increased in shrimp farm samples at the harvest stage in the present study. *Francisella* sp. causes pyogranulomatous and granulomatous infections in fish (Duodu et al., 2012; Gonçalves et al., 2016). A *Francisella* MAG was only found in a shrimp farm in this study. The high abundance of pathogens in farm samples may be attributed to the reduced diversity of normal flora in shrimps under the influence of antimicrobials.

5. Conclusions

This study is a brief case study of the resistome and microbial communities at two shrimp farms according to the farming stage in Korea. The analysis of samples at different farming stages would allow a broader understanding of the dynamics of the microbial communities, resistomes, and pathogens in aquaculture, which have not been well studied. Metagenome analysis revealed the greater abundance and diversity of resistomes and opportunistic pathogens in shrimp farms compared with the surrounding coastal marine environment. In particular, their distributions were different according to the farming stage. This suggests that microbial communities and resistomes may be affected by anthropogenic activity, and genome-centric metagenomics may be used to accurately identify specific pathogens without culture. The results also demonstrated how pathogens could be selected and alter microbial communities in the shrimp farm ecosystem, posing a potential risk to humans and aquatic organisms.

CRediT authorship contribution statement

Hoon Je Seong: Conceptualization, Methodology, Writing – reviewing & editing. **Jin Ju Kim**: Software and Reviewing. **Taeyune Kim**: Investigation. **Sung Jae Ahn**: Investigation. **Mina Rho**: Conceptualization, Funding acquisition. **Woo Jun Sul**: Supervision and Reviewing.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112858.

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