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RESEARCH ARTICLE

Impact of biochar on persistence and diffusion of antibiotic resistance genes in sediment from an aquaculture pond

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Abstract

Aquaculture sediments are a purported sizable pool of antibiotic resistance genes (ARGs). However, the pathways for transmission of ARGs from sediments to animals and humans remain unclear. We conducted an ARG survey in sediments from a bullfrog production facility located in Guangdong, China, and simulated zebrafish breeding systems were constructed, with or without biochar addition in sediments, to explore the effects of biochar on ARGs and their precursors of the sediment and zebrafish gut. After 60 days, 6 subtypes of ARGs and intI1 were detected, with sediments harboring more ARGs than zebrafish gut. The addition of biochar reduced the abundance of ARGs in the sediment and zebrafish gut, as well as suppressed the horizontal transmission of ARGs from sediment to zebrafish gut. Network analysis and partial least squares path modeling revealed that ARG enrichment was mainly affected by bacterial groups dominated by Nitrospirae, Gemmatimonades, Chloroflexi, and Cyanobacteria and intI1. Our findings provide insights into the transmission of ARGs from sediment to animals and highlight the efficacy of biochar amendments to aquaculture sediments to reduce the transmission of ARGs.

Keywords Bullfrog breeding facility · Resistome · Zebrafish · Gut microbiome

Introduction

Antibiotic resistance genes (ARGs) increasingly pose a potential risk to public health and are an emerging pollutant/hazard in the environment due to the indiscriminate global use of antibiotics in human and animal health (Anjum and Krakat 2016; Cohen et al. 2017; D'Costa et al. 2011; Knapp et al. 2010). ARGs can be exchanged and disseminated throughout antibiotic-resistant bacteria to the wider environment (Chen et al. 2019). For example, antibiotic-resistant bacteria including *Klebsiella pneumonia*,

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Highlights
- Amendment of biochar decreases ARGs abundance in bullfrog breeding sediment and guts of zebrafishes.
- Biochar's impacts on inhibiting ability for all ARGs weaken after excessive addition.
- IntI1 significantly affected the horizontal gene transfer of ARGs in sediment.
- Possible transfer mechanism and pathway of ARGs between sediment and animals were proposed.

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Shigella sonnei, and *Staphylococcus haemolyticus* isolates were found to harbor at least two different classes of ARGs (Zhang et al. 2016). Further, ARGs can cluster in the integron as an ecological advantageous unit, thus traversing through different ecosystems with those gene-recruiting elements (Martinez 2009). Moreover, ARGs may be transferred to plants, domestic animals, drinking water, and exotic foods (Allen et al. 2010; Duan et al. 2019; McKinney et al. 2018). Importantly, the pervasiveness of ARGs significantly reduces the efficacy of several antibiotics causing the failure of antibiotic therapies against prominent pathogens (Iwu and Patrick 2021).

Notably, the effect of antibiotic resistance genes distribution, mobilization, and dissemination in the environment has been extensively investigated (Pal et al. 2016; Yu et al. 2022; Zhang et al. 2018, 2019). However, the transmission of ARGs to intermediate hosts such as animals through water environment is rarely mentioned, thereby further investigation in this area is required. Horizontal acquisition of antibiotic resistance genes can elicit antibiotic resistance in animals (Ochman et al. 2000). Previous studies reported that several mobile genetic elements (MGEs), such as transposases, integrases (Ellabaan et al. 2021), and other recombinases, play a crucial role in organisms acquiring and transferring ARGs. For example, a report concerning large-scale farms in Shaanxi Province (bovine, chicken, and pig) revealed the widespread co-occurrence of ARGs and 10 MGEs in animal wastes (eight transposase genes and two class 1 integrase genes) using network analysis (Qian et al. 2018). Guts are an important niche for dissemination of ARGs as they contain both cultivable and uncultivable microbiota having a high diversity and abundance (Kim et al. 2007; Shterzer and Mizrahi 2015). Previous studies have further demonstrated that ARGs are enriched in aquatic animal guts through conjugation, transformation, and transduction, particularly via conjugation, due to the dynamic surface contact prevalent in the gut (Guglielmetti et al. 2009). This infers that the biological environment in an animal gut plays a key role in the transmission of ARGs through the food chain (Zhu et al. 2019). Notably, detailed knowledge concerning diffusion of ARGs between sediment and animal guts is largely unknown.

Antibiotics are extensively used in aquaculture to control aquatic diseases and ensure profits. Consequently, aquaculture wastes often contain ARGs creating a vast reservoir of ARGs in aquaculture sediments (Martinez 2009). Attempts to control antibiotic resistance in aquaculture sediment include fertilization, amending soil with sorptive materials and anaerobic digestion (Sun et al. 2018). Biochar is widely used for environmental remediation of ARGs in sediment to attenuate transfer to animals (Chen et al. 2018; Duan et al. 2017; Jiao et al. 2018; Liang et al. 2017). Biochar is a pyrolysis product of biomass and is characterized as having relatively large specific surface area and high porosity for interaction with pollutants, including ARGs. A study demonstrated that biochar can effectively mitigated ARGs and intI1 genes in soils; however, biochars have widely vary physicochemical properties providing inconsistent results for ARG remediation (Sun et al. 2019). For example, a HT-qPCR investigation of biochar effects on *Brassica chinensis* L. revealed that a majority of ARGs were significantly decreased in non-planted soil, but no significant decrease of ARGs occurred in the rhizosphere or phyllosphere following application of rice straw biochar (Chen et al. 2018). Further, the application of fresh biochar increased the relative abundance of ARGs in soil, with the enrichment capacity related to field aging of biochar and microbial composition (He et al. 2019). Hence, ARGs in sediment might be attenuated by the use of biochar, but the process and mechanisms require further elucidation.

In this study, we investigated aquaculture sediments from a bullfrog breeding facility having a high bullfrog density and experiencing large applications of antibiotics. We simulated a bullfrog production environment with and without biochar addition to the sediment to investigate the persistence and diffusion of ARGs using zebrafish as a test organism. We employed HT-qPCR to quantitative detect ARGs and Illumina Miseq high-throughput sequencing to characterize the microbiome in sediments and zebrafish gut to (1) characterize the ARG profiles in sediments with different contents of biochar, (2) explore the relationships of ARG abundance with microbiome and physicochemical factors of the sediment, and (3) determine the persistence and diffusion of ARGs during the growth of zebrafish. Results of this study provide a mechanistic proof-of-concept for designing remediation strategies for reducing the transmission risk of ARGs in aquaculture activities.

Materials and methods

Sampling sites

Sampling sites were located in a bullfrog aquaculture area in Chenghai District, Shantou city, Guangdong province (23° 35′ 49.63″ N, 116° 52′ 22.61″ E), China (Fig. 1). Shantou is currently the main area for bullfrog production in China, annually exporting ~ 2535 t of bullfrog products, which account for 40% of bullfrog production in China. The bullfrog ponds have an area of approximately 8 m × 25 m with bullfrog densities of 170–220 individuals m^{-2}. The production cycle of bullfrogs is about 6–10 months and requires a suitable temperature of 25–30 ℃, high humidity and ~ 40% of the pond water volume to be exchanged daily with fresh water. Antibiotics including florfenicol, amoxicillin, and neomycin sulfate are regularly used for prophylactic

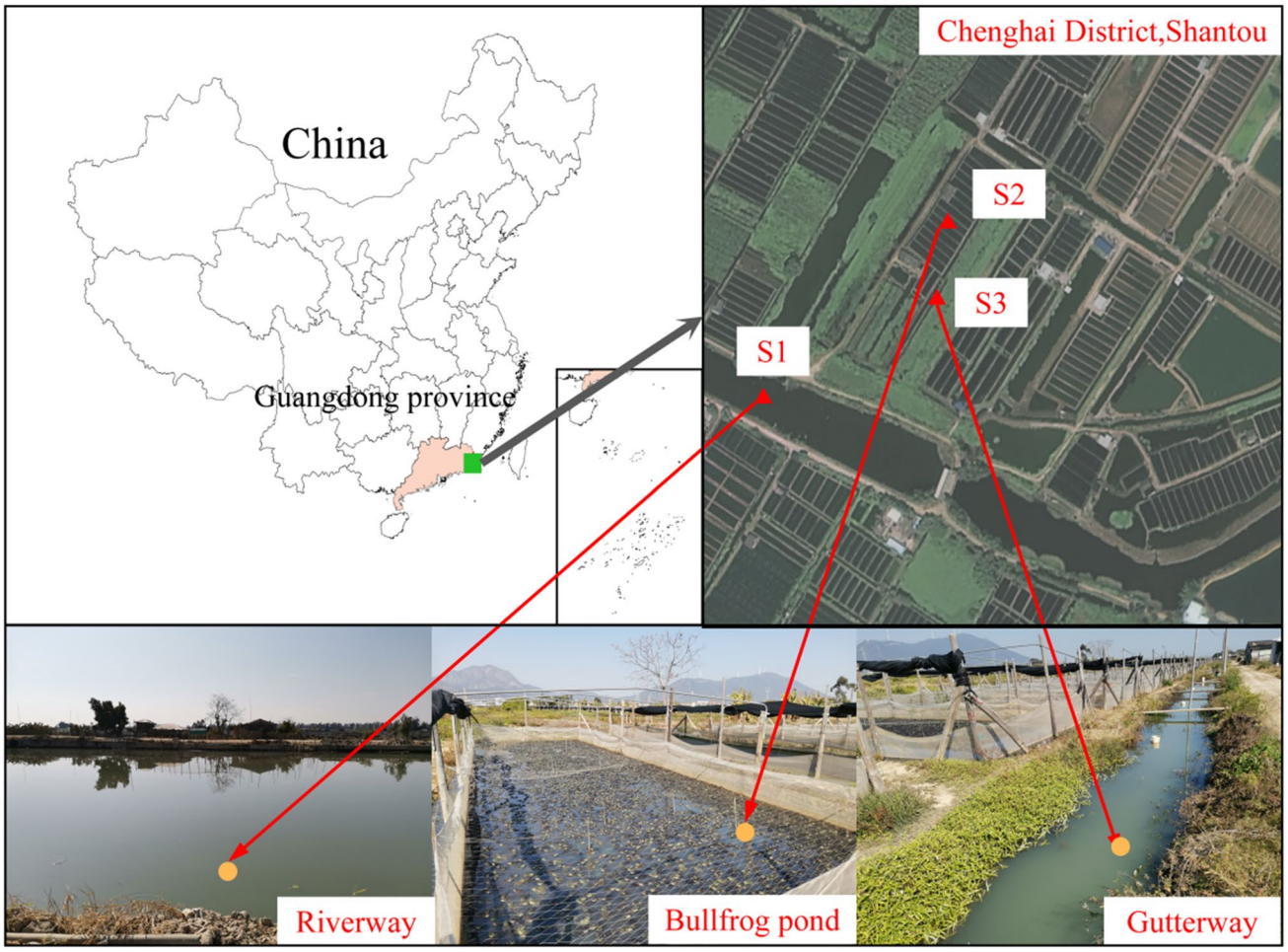

Fig. 1 Study area showing the three sediment sampling sites in the bullfrog production facility: S1 = riverway (source water), S2 = bullfrog pond, and S3 = gutterway (wastewater discharge)

purposes due to the high stocking density. The feed is supplemented with antibiotics and provided twice daily. Additionally, enrofloxacin is used before the bullfrog breeding period to ensure bullfrog health (Yuan et al. 2019). A large amount of fecal material, sick/dead frog bodies, feed residues, and polluted water transport antibiotics and ARGs to downstream surface waters.

Sample collection and sediment characterization

Sediment samples were collected in September 2020 from a representative bullfrog breeding facility (Fig. 1). Sediment samples were collected from three distinct operational areas within the facility to follow the fate and transport of ARGs: S1 (riverway alongside the bullfrog pond, water source for the production ponds), S2 (bullfrog production pond), and S3 (gutterway, water outlet from production facility).

Five replicate sediment samples (0–10 cm) were collected from each of the three site categories (S1, S2, S3) using a grab sampler. About 2 kg of sample was thoroughly mixed, placed in a sterile polyethylene bag, stored on ice and transferred to the laboratory. A sediment subsample for physicochemical analyses and zebrafish incubations was stored at 4 ℃ before use, and the remaining sample for ARG analysis and DNA extraction was stored at − 20 ℃ before use. Physicochemical characterization of sediments, including water content (WC), pH, electrical conductivity (EC), organic matter content (organic matter), ammonia (NH_4-N), and available phosphorus (P), were determined using the methods reported in SI methods. The physicochemical properties of the sediment samples are summarized in Table 1.

Experimental animals and ARG exposure experimental design

A water tank with a glass partition was used for the zebrafish incubation experiments (Fig. 2). Sediment from the bullfrog pond, with or without addition of biochar, was

Table 1 Physicochemical properties of sediments

Sample sites	WC (%)	pH	EC (μS cm^{-1})	Organic matter (%)	NH_4-N (mg kg^{-1})	P (mg kg^{-1})
Riverway (S1)	59.9	7.49	401	4.93	265	11.6
Bullfrog pond (S2)	48.1	6.87	187	4.50	281	19.2
Gutterway (S3)	44.9	6.93	555	3.04	830	19.2

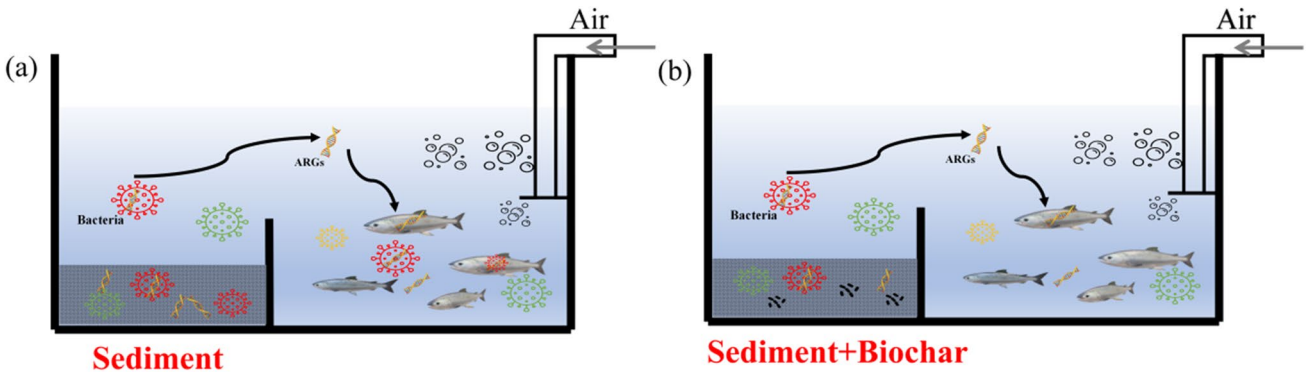

Fig. 2 Experimental incubation chambers with (**a**) and without (**b**) biochar addition for zebrafish incubation

placed on one side of the glass partition, whereas the other side was aerated and filled with dechlorinated tap water for zebrafish incubation, thereby ensuring that the sediment layer and zebrafish experienced the same experimental conditions. Four-month-old zebrafish were purchased from Shanghai FishBio Company. Before the experimental treatment, fish were allowed to acclimatize for 2 weeks in 50 cm × 30 cm × 25 cm tanks with aeration of dechlorinated tap water and a natural photoperiod of 14 h light/10 h dark. The water temperature and pH were maintained at 26 ℃ and 6.8–7.5, respectively. During the 15-day acclimatization period, fish were fed paramecium (Shengsuo Co., Shandong, China) twice daily. After 2 weeks of acclimatization, 400 zebrafish were randomly divided into four groups for biochar treatments.

Four biochar-sediment dosages (0, 2, 5, and 8% by weight) were prepared, and 200 g of sediment (net dry weight) was added to each experimental chamber. Two days after biochar-sediment addition, dechlorinated tap water and zebrafish were added to form the four biochar treatment groups (20 fish in each group). The incubation experiment lasted for 60 days, and sediment and water samples were collected on days 0, 15, 30, 45, and 60 for physicochemical analyses. Biochar produced by slow pyrolysis (500 ℃) of corn stover was purchased from Lize Ecotechnology (Zhengzhou, China) and the following characteristics were detected: pH = 9, carbon content = 41.09%, total nitrogen content = 8.51%, total phosphorus content = 2.34%, Brunauer–Emmett–Teller(BET) surface area = 75.29 m^2 g^{-1}, cation exchange capacity(CEC) = 63.05 cmol kg^{-1}, C:N molar ratio = 5.6. The properties were determined using the methods reported in SI methods.

DNA extraction and high-throughput qPCR

DNA samples were extracted from sediment samples and zebrafish gut tissues using the DNeasy PowerSoil Kit (QIAGEN, Netherlands) following manufacturer's instructions, and stored at − 20 °C before further analysis. A NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine the amount of extracted DNA, and agar-gel electrophoresis was used to determine the quality of extracted DNA.

Based on previous studies, tetracycline, sulfonamide, and β-lactams resistance genes are the most abundant ARGs in bullfrog pond sediments; hence, we specifically focused on these genes in this study. ARGs of sul1, sul3, tetA, tetX, blaTEM, and blanps, as well as mobile genetic elements (MGEs) of intI1, and 16S rRNA of total bacteria1 genes were quantified using high throughput qPCR(HT-qPCR) via MA6000 real-time qPCR (Yarui, China). The primers and annealing temperature are reported in Table S1. Plasmids containing these specific primer sequences were manufactured by Pyrobest DNA Polymerase (TaKaRa, DR500A) following manufacturer's instructions. A threshold cycle of 18.45 was set as the detection limit, three replicates were regarded as positive (Su et al. 2015), and all genes achieved amplification efficiencies between 86.0% and 95.3%. Details of HT-qPCR protocols for ARG analysis are reported in SI methods.

16S rRNA gene amplicon sequencing

At the end of experimental trials (60 days), sediment and zebrafish gut tissues were extracted for subsequent 16S rRNA Gene Amplicon Sequencing and HT-qPCR. For each group, the guts of ten individual zebrafish with approximately the same body size were randomly selected for analysis. Zebrafish gut tissues were extracted with a scalpel under aseptic conditions, washed with phosphate buffer (pH = 7.2), quick-frozen in liquid nitrogen for 15 min, and stored at − 20 ℃ for subsequent DNA extraction.

The V4-V5 region of the bacterial 16S rRNA gene of sediment samples and zebrafish gut tissues were amplified with primers 515F and 907R. A unique barcode for each sample was present on the reverse primer. DNA was amplified three times before subsequent amplification on an Illumina Miseq platform using a Miseq Reagent Kit V3 (Personal Biotechnology, China).

Quantitative Insights Into Microbial Ecology (QIIME2, 2019.4) pipeline and R package were used to process sequencing data. High-quality sequences were grouped into operational taxa (OTUs) based on Vsearch (V2.13.4) (Caporaso et al. 2010). OTUs were identified at 100% sequence similarity using UCLUST clustering. Alpha diversities for each sample were determined using Shannon, Simpson, observed species, and Chao1 indices. Beta diversities for each treatment were generated by principal components analysis (PCA) based on the Bray–Curtis distance matrix. OTU taxonomic classification was conducted using PyNAST and the Greengenes Database(McDonald et al. 2012). The functional abundance of samples was predicted by PICRUSt2 based on the abundance of marker gene sequences (Langille et al. 2013).

Statistical analysis

Excel (Microsoft Office 2019) was used to determine descriptive statistics for ARG abundance data. SPSS-Ver. 25 (IBM, Armonk, USA) was used for one-way analysis of variance (ANOVA) to test for differences in abundance of ARGs between different biochar contents. Histograms were generated through Origin 2021. Differences among resistance gene abundance and bacterial taxa in each sediment and zebrafish sample were assessed by principal components analysis (PCA). PCA was performed based on the Bray–Curtis method using function PCA from the Vegan package in RStudio version 1.4.1717 (RStudio, Boston, MA). The gradient length in the detrended correspondence analysis (DCA) was less than 3; hence, redundancy analysis (RDA) as a multivariate regression analysis was applied to investigate relationships between ARGs, intI1 and bacterial community (phylum level) using Canoco 5.0 (Microcomputer Power, USA). Mantel, adonis, and Spearman procedures were performed in RStudio with package "psych" to assess differences in ARG abundance and bacterial community composition among all samples.

Bacterial genera and intI1 with a correlation index higher than 0.7 ($p < 0.05$) were used to calculate the co-occurrence network. Visualization of network analysis was facilitated using Gephi (V0.9.2) and the Fruchterman Reingold layout. The absolute abundance of ARGs, intI1, and bacterial community was used for co-occurrence network analysis. Partial least-squares path modeling (PLS-PM) was employed to explore the direct, indirect, and interactive effects between biochar content, sediment properties (e.g., pH, NH_4-N, EC, moisture content, available P, organic matter), diversity of sediment and zebrafish gut microbiota (at the phylum level), and diversity of ARGs and MGE in sediment and zebrafish gut through "plmsm" package in RStudio. Goodness-of-fit (GoF) statistics for the developed model was tested by multiple criteria to assess the model's overall predictive power.

Result

Bacterial community and ARG analysis of sediment samples

Significant differences in ARG abundance were observed among sediment types: gutterway > pond > riverway (Fig. 3a). The most abundant ARG in riverway sediments was blaTEM, whereas ARGs in pond and gutterway sediments were dominated by ARGs sul1.

Differences in bacterial community composition at the phylum level were documented among sediment types within the bullfrog production facility (Fig. 3b). High-throughput sequencing results showed that the dominant bacterial communities among the three sediment types were Proteobacteria, Firmicutes, Chloroflexi, and Actinobacteria. Proteobacteria showed higher abundance in the pond and gutterway, whereas Chloroflexi and Actinobacteria showed lower abundances, consistent with the distribution of ARGs in the different sample types.

Characteristics of ARGs and MGE in pond sediments and zebrafish with and without biochar treatment

In the four biochar-sediment treatment levels, we identified 6 subtypes of ARGs and intI1 in the sediments and zebrafish guts (Table 2). Consistent with other studies, we found that bullfrog pond sediment is an ARB repository (Abe et al. 2020; Ding et al. 2021). Consequently, the pond sediments serve as a source of ARGs during bullfrog production, as well as the zebrafish we grew in contact with pond sediments. In the sediment, the sulfonamide-resistant gene sul1 dominated, representing over 80% of the 6 ARG

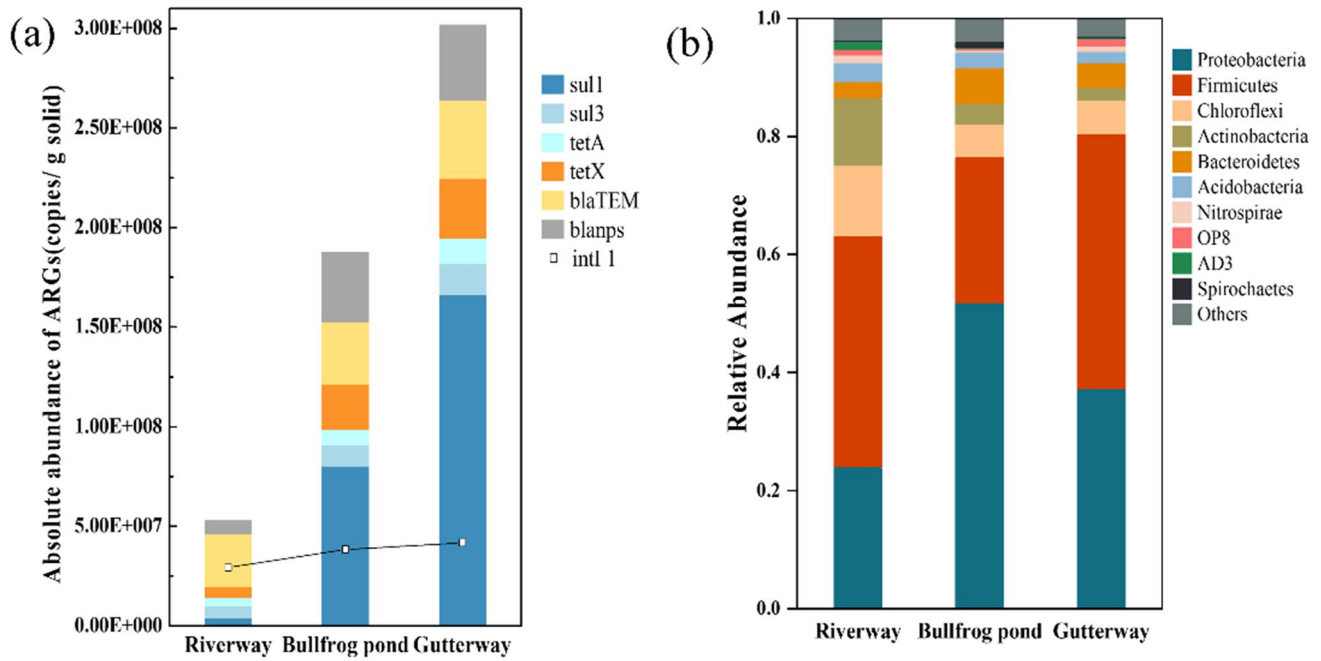

Fig. 3 The absolute abundance of ARGs (bars) and intI1(line) (**a**) and phylum distribution of OTUs (**b**) in sediments from the riverway, bullfrog pond, and gutterway

Table 2 Absolute abundance of ARGs and intI1 in bullfrog pond sediment and zebrafish gut

	Pond sediment ($\times 10^6$ copies g^{-1})				Zebrafish gut ($\times 10^6$ copies g^{-1})			
	Biochar concentration				Biochar concentration			
	0%	2%	5%	8%	0%	2%	5%	8%
sul1	465a	356b	230c	367b	16.8a	9.4b	2.2d	4.3c
sul3	18.2a	9.2b	4.97c	6.6b	1.2a	0.5b	0.1c	0.1c
tetA	2.1a	1.5b	1.0c	2.1a	0.3a	0.2c	0.1d	0.2b
tetX	14.5a	8.6b	2.37c	12.4a	2.5a	1.1c	0.2d	1.5b
blaTEM	40.2a	20.69b	23.6bc	21.1c	7.4a	3.0b	3.7d	2.3c
blanps	39.0a	19.4b	15.4c	9.4b	12.6a	4.2b	1.6c	4.0b
IntI1	33.6a	14.6bc	13.5c	16.2b	14.2a	6.1c	5.2c	10.2b

Values with the same superscript letter are not significantly different across columns ($p < 0.05$).

subtypes with abundances of 9.58×10^5 to 4.65×10^8 copies g^{-1}. In contrast, the content of other individual ARG subtypes was below 8%.

Abundances of the 6 ARG subtypes in zebrafish gut samples ranged from 8.32×10^4 to 1.68×10^7 copies g^{-1}. The sul1 and β-lactam resistance gene blanps accounted for the largest proportion (48.4–74.1%), followed by blaTEM, tetA, tetX, and sul3. Finally, the abundance of intI1 was 1.35×10^7 to 3.36×10^7 copies g^{-1} in sediment samples and 5.15×10^6 to 1.42×10^7 copies g^{-1} in zebrafish gut samples. These intI1 results present the same degree of variation as that of ARGs implying the diffusion of ARGs from sediment to zebrafish (Fig. 4).

Effects of biochar dose on ARGs in pond sediment and zebrafish gut

The overall distribution of ARGs in sediment and zebrafish gut was significantly affected by biochar dose in the pond sediments (Adonis test, $R^2 = 0.20$, $p < 0.05$). The distribution of ARGs from sediment samples and zebrafish gut at different biochar doses were differentiated into distinct clusters by PCA analysis (Fig. 5a).

Changes in the abundance of ARGs and intI1 during the 60-day zebrafish incubation and the fates of various ARGs were appreciably different (Fig. 4). The addition of biochar to sediment led to a similar decreasing trend for all detected

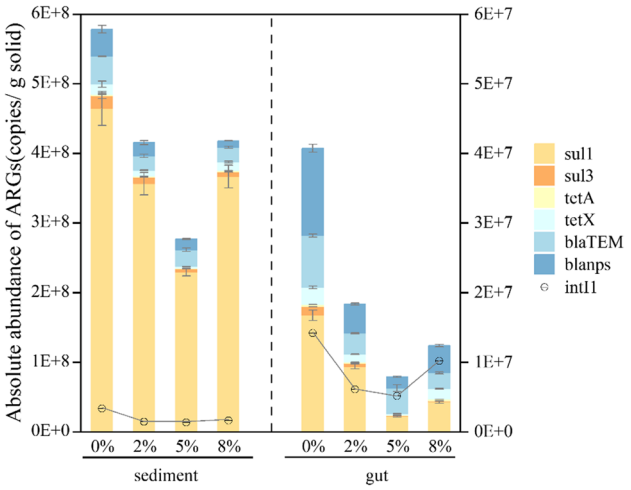

Fig. 4 Absolute abundance of ARGs (bars) and intI 1 (line) detected in bullfrog pond sediment and zebrafish gut. Error bars represent the standard errors for each type of ARG; 0%, 2%, 5%, and 8% represent biochar content of sediment samples, w/w

ARGs in the sediment for biochar concentrations up to 5%, followed by a notably loss of effectiveness for the 8% treatment relative to the 5% treatment. Overall, removal efficiencies ranged from 20 to 70% ($p < 0.05$) for ARGs, whereas the abundances of sul3 and blanps were reduced by more than 50%.

The absolute abundance of intI1 in the sediment was 3.36×10^7 copies g^{-1}, indicating that the untreated pond sediment possessed great ecological risk for the spread/transfer of ARGs (Fig. 4). The abundance of intI1 in the zebrafish gut was reduced by 63.7% (from 1.42×10^7 to 5.15×10^6 copies g^{-1}) in the 5% biochar treatment compared with the control (0%) group.

Microbial community composition and structure after biochar treatment

Sequencing results identified 310,311 and 397,304 high-quality sequences with total average lengths of 413 bp and 419 bp from the pond sediment and zebrafish gut samples

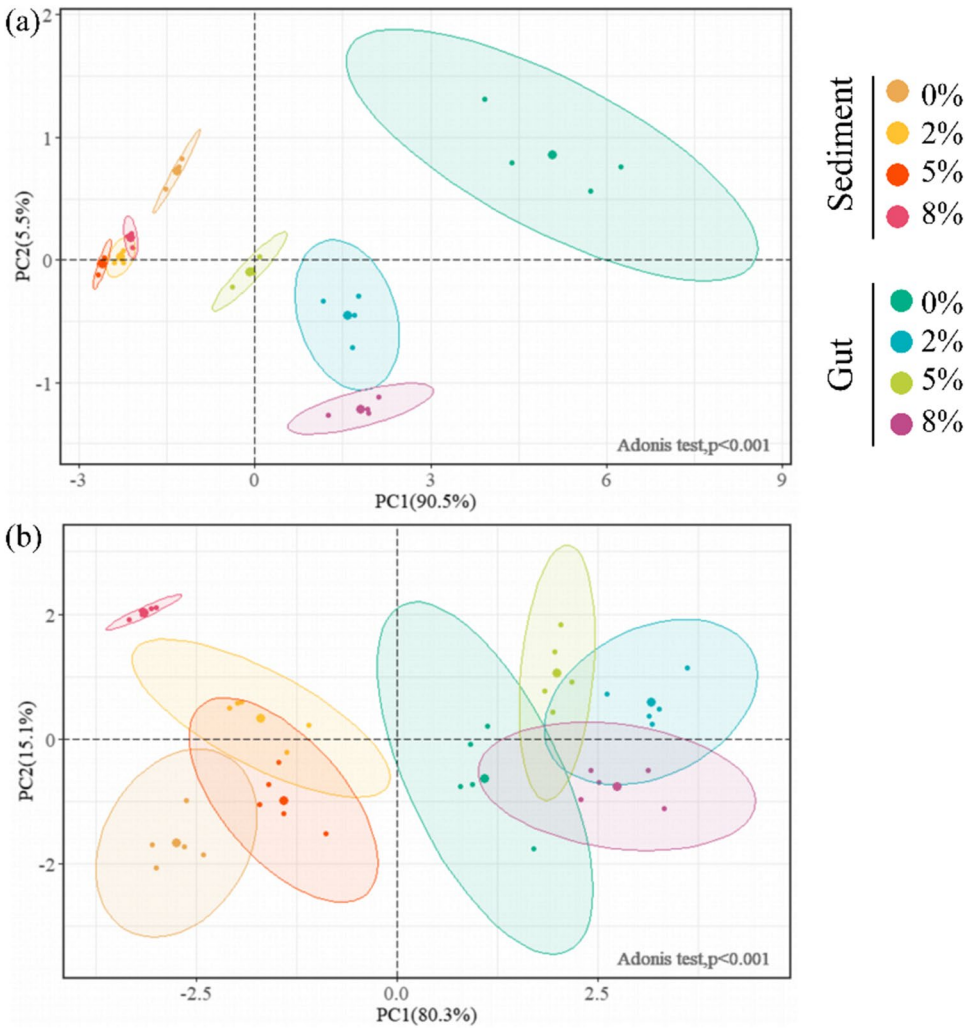

Fig. 5 Bray–Curtis distance based principal components analysis (PCA) showing the distribution patterns of sediment ARGs (**a**) and bacterial composition (**b**) across different biochar treatments (0%, 2%, 5%, and 8% represent biochar content of sediment samples, w/w)

of the four biochar treatments, respectively (Fig. 5b). The similarity of sequencing results indicates that the overall distribution of the bacterial community was correspondingly clustered in both sediment and gut samples, which was further confirmed by the Adonis test ($p < 0.001$).

Shannon and Simpson indices (Table S2) indicated that the alpha diversity of the bacterial community was higher in the sediments than in the zebrafish gut. OTUs were assembled into 35 phyla, 89 classes, and 201 orders in sediment samples, and into 29 phyla, 78 classes, and 157 orders in gut samples. At the phylum level, Proteobacteria, Chloroflexi, and Firmicutes were the dominant bacteria in both sediments and gut samples, which accounted for 68.3–89.6% of the overall communities (Fig. 6a). Addition of biochar promoted the growth of Firmicutes ($p < 0.01$) with a maximal proportion of 17.5% in the 5% biochar treatment. The enhanced Firmicutes fraction might contribute to the transfer and spread of ARGs. The dominant OTUs based on class level were γ-Proteobacteria, Anaerolineae, and Clostridia in the sediment (comprising 12.5–31.4%), and γ-Proteobacteria and Anaerolineae in the zebrafish gut (comprising 10.9–42.6%) (Fig. 6b).

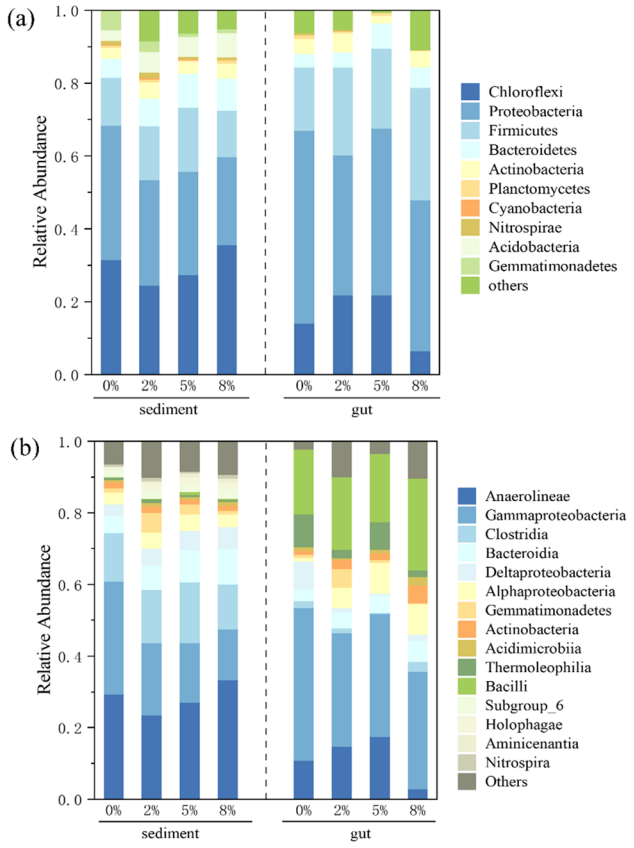

Fig. 6 Phylum (**a**) and class (**b**) distributions of OTUs in pond sediment and zebrafish gut among different treatments

Correlation analysis and co-occurrence patterns between bacterial taxa and ARGs in pond sediment and zebrafish gut

RDA was performed to assess relationships between bacteria at the phylum level and the abundance of MGEs on ARGs during the zebrafish incubation (Fig. 7a). The RDA revealed two components (RDA1 and RDA2) that explained 74.9% and 10.2% of the total variation in ARGs, respectively. In terms of bacteria, ARGs were positively correlated with Nitrospirae, Gemmatimonades, Chloroflexi, and Cyanobacteria, suggesting that these four bacteria types may exert a disproportionate effects on the variation of ARGs in zebrafish gut.

Generally, network analysis is adopted for the analysis of the symbiosis of a single ARG with intI1 and the

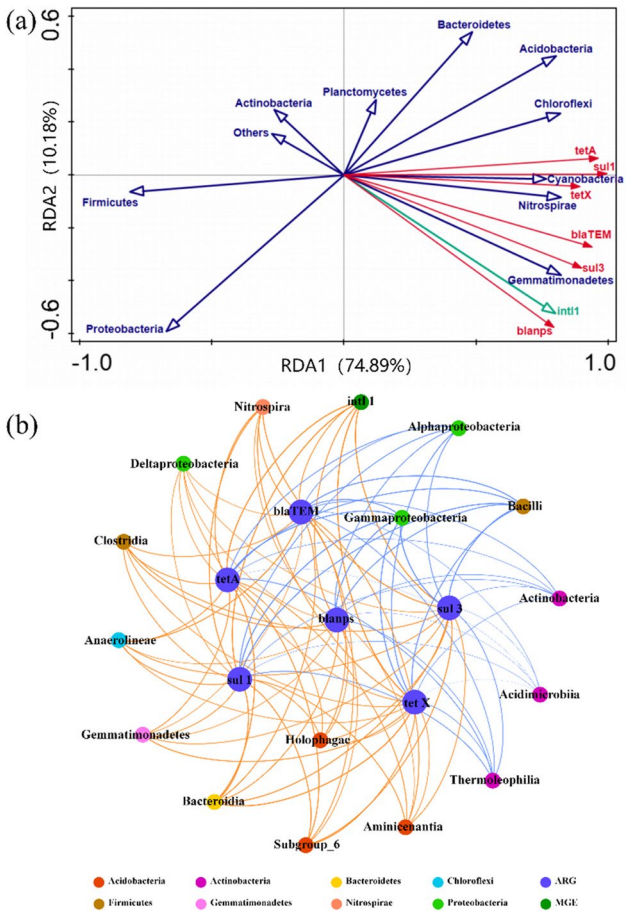

Fig. 7 Redundancy analysis (RDA) examining relationships among ARGs, intI1 and microbial community (phylum level) (**a**). Network analysis shows co-occurrence patterns between bacteria classes and ARGs, as well as intI1 introgen (**b**). Nodes are colored according to ARG type and bacterial taxa; node size is proportional to the number of connections. Thickness of lines is based on Pearson's correlation coefficient; red lines indicate positive correlations and blue line negative correlations

microbial community (at the class level) based on Spearman's correlation analysis. In our network analysis, several bacterial groups presented correlations with the 6 ARG subtypes detected ($R^2 > 0.7$, $p < 0.05$) (Figs. 7b and 8). Specifically, Gammaproteobacteria, Bacteroidia, Nitrospira, Bacilli, Aminicenantia, Alphaproteobacteria, Clostridia, and Anaerolineae showed the strongest correlations with ARGs. Among them, Bacilli, belonging to Firmicutes, displayed prominent correlations with sul1, sul3, and blaTEM ($R^2 > 0.9$, $p < 0.001$), which is consistent with the findings of (Song et al. 2017). Additionally, Gammaproteobacteria, Bacteroidia, and Nitrospira were significantly correlated with some ARGs ($R^2 > 0.8$, $p < 0.01$).

Relationships of sediment properties, microbiome, intI1, and ARGs between sediment and zebrafish gut

Sediment physicochemical properties were altered by biochar application and the degree of alteration became more pronounced over time (0 to 60 days) (Fig. S3). Among measured sediment properties, pH, organic matter, electrical conductivity, and ammonia nitrogen showed significant ($p < 0.05$) positive correlations with biochar application time. Further, RDA results (Table S3) revealed that ARGs in zebrafish gut microbiomes exhibited correlations with sediment pH ($p < 0.01$) and ammonia nitrogen ($p < 0.05$).

PLS-PM was further adopted to evaluate direct and indirect correlations among factors detected with potential structure assumptions (Fig. 8). The results illustrated that biochar content exerted remarkably positive or negative effects on the bacterial community composition ($p < 0.01$), sediment physicochemical properties ($p < 0.01$), and intI1 content ($p < 0.001$), but did not directly affect ARG abundance. Moreover, sediment physicochemical properties had significant direct and positive impacts on the composition of the bacterial community and intI1 content in sediment and zebrafish gut microbiomes ($p < 0.001$), but no obvious effect on ARGs. Composition of the bacterial community and intI1 content showed several correlations with the abundance of ARGs in zebrafish gut ($p < 0.001$), although the extent of specific bacteria or intI1 influence on ARGs varied.

Discussion

Continuous exposure to various antibiotics contributes to proliferation of ARGs

In this study, we found that the abundance of ARGs in bullfrog pond and gutterway was higher than that in riverway in bullfrog breeding farms and surrounding environments. This proliferation of ARGs along the hydrologic flowpath can be attributed to selection pressure of sediment bacteria to continuous exposure time to various antibiotics or different antibiotic levels. Increasing antibiotic exposure time and concentrations along the water flowpath facilitates the development of multi-resistance and core resistance, especially

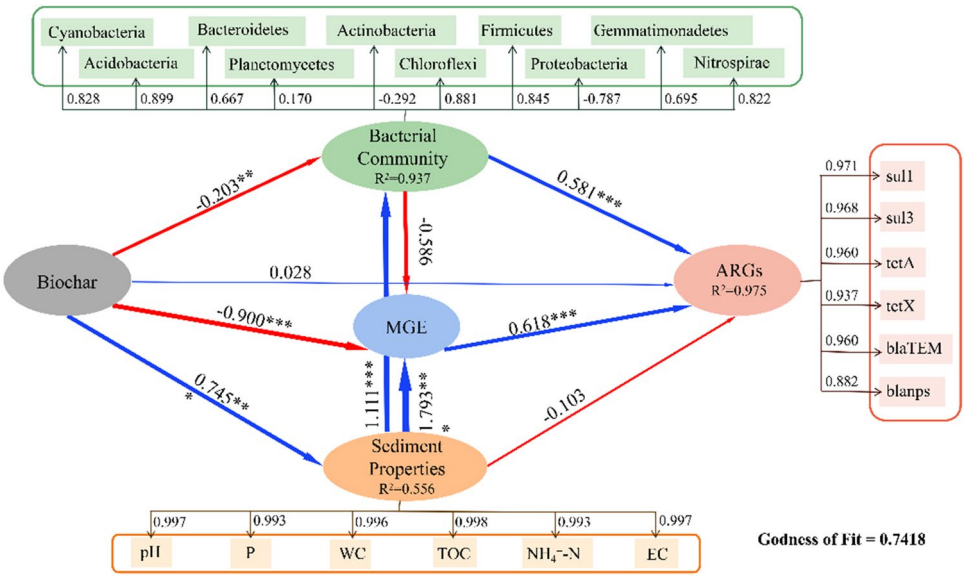

Fig. 8 Partial least-squares path model (PLS-PM) showing direct and indirect effects of different factors on ARG abundances in zebrafish. The PLS-PM describes relationships among biochar, sediment properties, bacterial community composition, and intI1 abundances with respect to ARG abundances. Larger path coefficients are shown as wider arrows; blue and red colors indicate positive and negative effects, respectively. Path coefficients and coefficients of determination (R^2) were calculated after 999 bootstraps with significance levels reported as $p < 0.05$*, $p < 0.01$**, and $p < 0.001$***. Goodness of fit (GoF) was 0.7418

with the continuous addition of antibiotics to the bullfrog production ponds (Wang et al. 2021). The detection of ARGs in all three sediment types demonstrates the migration and accumulation of ARGs in the sediments, which is closely related to horizontal gene transfer of resistance genes in the environment. The large increase in ARGs from input to output sites clearly identifies the bullfrog production ponds as an ARG source to downstream waterways. Similar to our findings, resistance genes were observed to enter the soil through the manure of animals treated with antibiotics. In the case of pig manure, sulfa-resistant bacteria were found to disperse through the soil bacteria (Gillings 2013).

Notably, there is a large change in bacterial abundance and community composition between the sediments of pond source waters and the sites impacted by high antibiotic loads (ponds/gutterways). This implicates the aquaculture activities as a driver of bacterial communities. Further, these results indicate that there is a correlation between the abundance of ARGs and the bacterial community, in which Proteobacteria contribute to the proliferation and enrichment of resistance genes as a potential host of ARGs. It manifests the strong correlation between ARGs and bacteria may enhance the occurrence and transmission of antibiotic resistant bacteria in soil ecosystems.

Biochar addition in the sediment inhibits the spread of antibiotic resistance genes

The pond sediments serve as a source of ARGs during bullfrog production, as well as the zebrafish we grew in contact with pond sediments. We found that sul1 was the dominant resistance gene in both biochar-doped sediment and gut of zebrafish. We posit that the dominance of the sulfonamide-resistant gene sul1 results from the use of sulfonamide antibiotics that are widely used in the treatment of bacterial infections in bullfrogs, fish and livestock (Gao et al. 2012; Razavi et al. 2017; Wang et al. 2016). However, due to proprietary restrictions, we were not able to obtain the antibiotic use information from the bullfrog facility to confirm the use of sulfonamide antibiotics.

In addition, we found that biochar doping in sediment significantly reduced the abundance of resistance genes. Our findings are consistent with those of (Duan et al. 2017) who found that bamboo biochar significantly reduced the abundances of tetC and tetG in soil, with a reduction of 32.5 to 84.3%. Similarly, (Zheng et al. 2021) found that wood biochar effectively reduced the abundance of ARGs in the soil with an overall reduction rate of 47.5%. They posited that the ARG reduction was related to changes in the bacterial community, biochar amendments altered the bacterial community composition by changing the nutrient composition of the sediment, thereby resulting in a reduction in the hosts for some ARGs (Zhi et al. 2020).

Importantly, the addition of biochar changed the horizontal transfer of ARGs. MGE intI1 as an integron-integrase gene common in gut microbiomes, which is a critical carrier responsible for the transfer and spread of resistance genes. Regression analysis indicated that the abundance of intI1 in pond sediment and zebrafish gut was linearly correlated with the abundance of total ARGs ($R^2 = 0.766$, $p < 0.001$) (Fig. S1). Furthermore, the Mantel test ($R^2 = 0.7508$, $p = 0.024$) revealed that the abundance of intI1 was positively related to the measured ARGs, inferring that intI1 was crucial for the horizontal transmission of ARGs. MGEs provide a broad platform for the acquisition, rearrangement and expression of gene cassettes(Zhang et al. 2020b).

In general, the abundances of detected ARGs were reduced by 42.9%, 60.6%, and 39.6%, relative to the no biochar treatment, with the addition of 2%, 5%, and 8% (w/w) of biochar, respectively. This study only considered three biochar levels, and future research should consider testing more biochar concentrations to optimize the concentration for attenuation of antibiotic-resistant genes in the sediment. We posit that the highly porous nature of biochar increases the spacing between microbes and thereby reduces the contact rate of microorganisms and subsequent transfer of ARGs. Our study also provides insights into the role of MGEs in the horizontal transmission of ARGs. Overall, these results indicate that the addition of biochar is an effective remediation measure to attenuate the persistence of some ARGs in the soil/sediment environment.

Biochar alters the bacterial community composition and ARG–bacterial taxa associations

Aquaculture systems affect the composition of the bacterial community in both the sediment and water column. The bacterial community in the water column shares OTUs with animals, indicating that microorganisms are successfully transferred from water to gut by ingestion (Giatsis et al. 2015). Microbes are the primary host of ARGs; hence, variations in the microbial community may strongly influence ARG abundance and transmission through the environment (Guo et al. 2020). The symbiosis model for ARGs and microbes indicated that 8 types of bacteria were the potential hosts of ARGs. As the composition of the bacterial community in sediment was similar to that in zebrafish gut (Fig. 6a), it is probable that a certain number of bacteria in sediment were subsequently transmitted to the zebrafish gut microbiome where they became the dominant strains. Furthermore, the absolute abundance of total ARGs in zebrafish gut varied with different dose rates of biochar application to sediment. Since biochar led to differences in the microbial community composition, the biochar is inferred to affect the persistence and diffusion of ARGs in sediments. The co-application of biochar and compost to soil was previously shown to alter

bacterial communities and reduce the variations in ARGs by 44.2% (Cheng et al. 2021). Similarly, Sun et al. (2018) found that the microbial community and MGEs reduced ARG variation by 81.3% in an anaerobic digestion process of cattle wastewater. As microbes serve as carriers for the horizontal transmission of ARGs, the role of specific microbe taxa responsible for the transmission of ARGs warrants further investigation.

Besides, the network analysis is usually performed to display the relationship between a single ARG subtype and the microbial community (Zhang et al. 2020a). Notably, intI1 has the able to influence the fate of ARGs, which suggests that the integration and transmission of gene cassettes play a leading role in the transmission of ARGs (Ma et al. 2019; Sandberg and LaPara 2016). Together, these results suggest that both microbial community and intI1 improved the removal of ARGs in gut of zebrafishes by reducing the degree of horizontal gene transfer.

Potential mechanisms for ARG removal in aquaculture

The physical and chemical properties of sediment are expected to have a strong influence on the microbial community and intI1, and consequently on the migration of ARGs. These correlations between the physical and chemical properties of sediment and abundance of ARGs suggest that sediment physicochemical properties may affect the distribution of ARGs in sediment and zebrafish microbiomes. The addition of biochar to soils accelerates of the removal of ARGs, which could be a consequence of pH difference in these soils, which affected the soil microbial communities as demonstrated by the dissemination of ARGs in a paddy soil environment (Zhi et al. 2020).

The PLS-PM model showed the correlation between ARGs and biochar content, physicochemical properties of sediment, intI1 abundance, and bacterial community distribution. The correlations were consistent regardless of their size, indicating that MGEs and the bacterial community composition directly determine the migration of ARGs. As the direct carrier of ARG migration, MGEs are considered more crucial for the horizontal transmission of ARGs than bacterial community composition. Notably, intI1 shows a close relationship with many ARGs and responds quickly to environmental stress, making them a sensitive indicator of environmental pollution (Gillings et al. 2015). In this study, intI1 had a relatively high abundance and was positively correlated to the 6 types of ARGs ($p < 0.05$). As a biomarker, intI1 contributed more to the transmission of ARGs in zebrafish gut microbiomes than the bacterial community, consistent with previous studies (Tiimub et al. 2021). Ding et al. (2019) posited that the strong correlation between the abundance of ARG subtypes and MGEs in the host microbial community positively influenced the spread of ARGs, which was further related to the physicochemical properties of the soil environment. Collectively, we found sediment properties, microbial community and intI1 were identified as the main factors contributing to the removal of ARGs in sediment and guts of zebrafishes. Our data infer that the addition of biochar affects the physicochemical properties of the sediment, the bacterial community and intI1 content, thereby attenuating the diffusion of ARGs and eco-environmental risks. There were still some shortcomings in our study; for example, many antibiotic resistance genes in this study are not covered, and we could not detect all ARGs that might occur in bullfrog pond.

Conclusions

This study confirms that ARGs can accumulate and migrate in the aquaculture pond sediments because of the indiscriminate use of antibiotics in aquaculture, and revealed the feasibility of using biochar for mitigating the migration of ARGs in sediments. Moreover, we posit an integrated mechanism whereby biochar has a direct negative effect on potential ARG host bacteria and intI1, which consequently alters the potential ARG host bacteria and MGE, thereby having a direct positive impact on ARGs. Our results indicate that intI1 is the main factor affecting the transmission of ARGs, with MGE directly inducing the horizontal transmission of ARGs from sediment to zebrafish. Therefore, biochar application to aquaculture sediment provides a compelling method for attenuating the secondary transmission of ARGs in aquaculture sediment to the intermediate host and decreasing the risk to human health. For a better application to aquaculture sediment, different biochar types and contents, sediment conditions, and ARG class determination are needed, especially in the field environment.

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Author contribution Fengjie Hu: conceptualization, methodology, software, data curation, writing—original draft preparation. Randy Dahlgren: visualization, investigation. Taiping Zhang: writing—reviewing and editing. Jinni Liang: validation. Jiahui Xiao: validation. Zidan Liu: investigation, visualization.

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Data availability All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate All experiments were performed according to the guidelines laid down by the Institutional Animal Care and Use Committee of South China University of Technology.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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