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Enrofloxacin Induces Intestinal Microbiota-Mediated Immunosuppression in Zebrafish

Wenhui Qiu, Tang Liu, Xinjie Liu, Honghong Chen, Shusheng Luo, Qiqing Chen, Jason T. Magnuson, Chunmiao Zheng,* Elvis Genbo Xu,* and Daniel Schlenk

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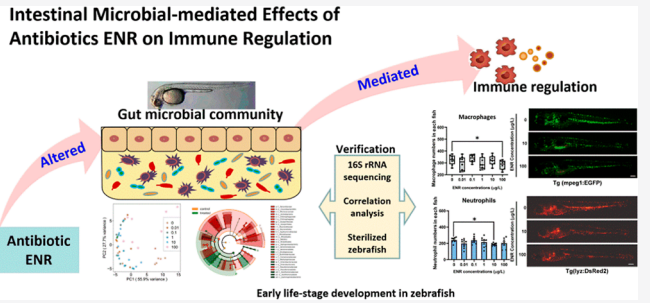
ABSTRACT: The immunosuppressive effects of antibiotics and the potential associations with the intestinal microbiota of the host have been increasingly recognized in recent years. However, the detailed underlying mechanisms of immune interference of antibiotics in environmental organisms remain unclear, particularly at the early life stage of high sensitivity. To better understand the gut microbiome and immune function interactions, the vertebrate model, zebrafish, was treated with environmentally relevant concentrations of a frequently detected antibiotic, enrofloxacin (ENR), ranging from 0.01 to 100 μg/L. 16S ribosomal RNA sequencing indicated diminished diversity, richness, and evenness of intestinal flora following ENR treatment. Twenty-two taxa of gut bacteria including *Rickettsiales*, *Pseudomonadales*, and *Flavobacteriales* were significantly correlated with immunosuppressive biomarkers, including a significant decrease in the abundance of macrophages and neutrophils. To validate the immunomodulatory effects due to altered intestinal microbial populations, zebrafish reared under sterile and non-sterile husbandry conditions were compared after ENR treatment. A significant inhibitory effect was induced by ENR treatment under non-sterile conditions, while the number of macrophages and neutrophils, as well as biomarkers of immunosuppressive effects, were significantly salved in zebrafish under sterile conditions, confirming for the first time that immunosuppression by ENR was closely mediated through alterations of the intestinal microbiome in fish.



KEYWORDS: *enrofloxacin, fish, Danio rerio, gut microbiota, immune function, rRNA seq*

INTRODUCTION

The increasing consumption, misuse, and release of antibiotics into the environment have raised global concern about the potential health risks of antibiotic residues on wildlife and human beings.[1,2] The use of antibiotics increased by 65% from 2000 to 2015 globally and is expected to increase by up to 200%, with 128 billion daily doses, by 2030.[3] The release of antibiotics from sewage treatment plants and wastewater runoff into the receiving natural environments has been shown to result in aquatic ecotoxicity at environmental concentrations and the development of resistant microbial species.[4,5] Enrofloxacin (ENR), a fluoroquinolone antibiotic, is among the most frequently detected, broad-spectrum bacteriostatic antibiotics in the aquatic environment.[6] ENR can hardly be degraded by microbes in the environment but, in turn, influences the microbial diversity and community structure.[7] ENR was frequently detected in river water samples.[8] Elevated concentrations of ENR have been detected in ranges of over 700 μg/L in wastewater treatment plant effluent, 34.4 μg/L in animal wastewater runoff, and 978.8 ng/L in river water.[8−10] These high concentrations posed ecological risks to aquatic organisms as the detected concentrations exceed acute and chronic toxicity threshold levels.[8,11]

ENR has long been reported to be among the most acutely toxic antibiotics to invertebrate and vertebrate model organisms.[12] Recent studies suggest the general immunotoxicity of ENR in different fish species. For example, dysfunction of the immune system was shown in American shad (*Alosa sapidissima*) after a dietary exposure of 100 mg/kg ENR for 30 days.[13] Our previous study found that ENR targeted primary fish macrophages and reduced the phagocytic function of macrophages that were mediated but not limited to the NF-kB pathway.[6] Other potential pathways and detailed mechanisms of ENR immunotoxicity are still largely unknown, particularly at the early life stage of high sensitivity. Several studies link immune functions to altered microbial populations.[14] More recently, it was suggested that persistent antibiotic residues in the environment can interact with the microbiome of humans and dysregulate the homeostatic balance of the microflora in

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the intestine, which may increase the susceptibility of disease.[15] However, the impacts of antibiotics at environmental concentrations on the gut microbiome of environmental organisms and the health implications post-gut microbiome interference have not been characterized. A recent paper published in the PNAS showed that the presence of pharmaceuticals in global surface water poses a threat to aquatic environments.[16] Thus, it is urgent to investigate the potential adverse effects of antibiotics at environmental concentrations. In our previous study, we showed a reduced phagocytic function in fish macrophages following a waterborne exposure to ENR.[6] Here, we hypothesize that ENR exposure induces immunosuppression, which is mediated via alterations of intestinal microbiota in fish. The study aims to illustrate whether ENR can induce changes to gut microbiota and how immune functions are impaired by using rRNA sequencing, bioinformatic analysis, chemical measurements, biochemical assays, mRNA protein expression analysis, correlation network analysis, the transgenic zebrafish assay, and immunoblotting. Sterile zebrafish experiments were further designed to explore the intestinal microbial-mediated effects of ENR treatment on immune-regulated pathways.

2. MATERIALS AND METHODS

2.1. Chemicals. ENR was obtained from Sigma-Aldrich (St. Louis, MO). The internal standard, ENR-d5, was purchased from ANPEL Laboratory Technologies (Shanghai, China). All solvents used were high-performance liquid chromatography (HPLC)-grade and purchased from Sigma-Aldrich (St. Louis, MO). Ultrapure water was produced using a Milli-Q unit (Millipore, USA).

2.2. Experimental Design. Adult wildtype zebrafish (AB) were fed the live brine shrimp (*Artemia nauplii*) twice daily in a flow-through aquarium system under a 14 h light/10 h dark photoperiod. Embryonic zebrafish were collected and assessed for egg quality following fertilization. Embryos (<4 hpf) were exposed to nominal antibiotic ENR concentrations of 0, 0.01, 0.1, 1, 10, or 100 μg/L. These ENR concentrations were selected based on environmentally relevant concentrations detected in the aquatic environment, ranging from a few nanograms per liter to micrograms per liter.[8,9] Each control or treatment group consisted of eight replicate glass Petri dishes, and each Petri dish contained a total of 200 embryos. After a 10 day exposure, 60 zebrafish larvae (240 hpf) from each replicate Petri dish were pooled as one sample for different assays and measurements, including 16S rDNA sequencing, biochemical and gene expression assays, and immunoblotting analysis (n = 8). A complete renewal of exposure solutions was conducted daily. Larval zebrafish until 240 hpf were chosen for parameter testing as the embryo-to-larval stage is when the colonization of microorganisms in the body is established and represents a developmental timepoint that is generally more sensitive to chemicals than adult stages. Each replicate was flash-frozen in liquid nitrogen and stored at −80 °C for 16S rRNA sequencing and immune indicator assays. Tg (mpeg1:EGFP) transgenic zebrafish and Tg (lyz:DsRed2) transgenic zebrafish, utilized for in vivo visualization of macrophages (labeled with green fluorescence) and neutrophils (labeled with red fluorescence), respectively, were subsequently exposed to ENR under the same exposure conditions as those for the above wildtype AB embryos. The macrophages and neutrophils were imaged using a LEICA M205 FCA microscope and counted by using ImageJ software (version 1.8.0) at 120 hpf. The parameters tested, macrophage and neutrophil numbers, had eight replicates, and each replicate contained 10 zebrafish (n = 8).

Sterile zebrafish experiments were conducted to assess intestinal microbial-mediated effects of ENR treatment on immune-regulated pathways. Zebrafish (<4 hpf) embryos were exposed until 240 hpf to 0, 10, or 100 μg/L ENR in both normal fish water (non-sterile maintain) or sterile fish water (sterilized maintain), with eight replicates per treatment (each treatment group had eight replicate Petri dishes, and each Petri dish contained 200 zebrafish). Samples were stored at −80 °C for immune indicator assessment.

2.3. Sterile Zebrafish Embryos. Sterile zebrafish embryos were generated by following a previously published method.[17] Briefly, embryos were collected in sterile fish water, assessed for egg quality, washed using sterile techniques, and then transferred into sterile tissue culture flasks where they were maintained in sterile fish water in a sterile incubator set at 28.5 °C. To validate that the zebrafish were sterile, agarose gel electrophoresis and subsequent quantitative polymerase chain reaction (qPCR) were conducted using the hypervariable region V3−V4 of the bacterial 16S rRNA gene with primer pairs 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′).

2.4. Gut Microbial Community and 16S rDNA Sequencing. Microbial community genomic DNA was extracted from 240 hpf larvae for gut bacterial community assessment using an E.Z.N.A. Soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.). DNA concentration and purity were determined using a NanoDrop 2000 UV−vis spectrophotometer and 1% agarose gel. A TransStart Fastpfu DNA polymerase kit (TransGen, China) was used to assess PCR amplification of V3−V4 of the bacterial 16S rRNA gene using the following thermal cycling conditions: 95 °C for 3 min; 29 cycles of 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 45 s; and 72 °C for 10 min. The PCR product was extracted from a 2% agarose gel, purified using the AxyPrep DNA gel extraction kit (Axygen Biosciences, Union City, CA, USA), and quantified using a Quantus fluorometer (Promega, USA). Purified amplicons were pooled in equimolar concentrations and then paired-end sequenced on an Illumina MISeq PE300 platform (Illumina, San Diego, USA) according to the standard protocols published by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: SUB10396477).

2.5. Diversity Analyses of Microbial Communities. The raw 16S rRNA gene sequencing reads were demultiplexed, quality-filtered using fastp (version 0.20.0), and merged using FLASH (version 1.2.7). Operational taxonomic units (OTUs) with a 97% similarity cutoff were clustered using Vsearch (version 2.7.0), and chimeric sequences were identified and removed.[18] The taxonomy of each OTU representative sequence was analyzed using classify-sklearn (version 2021.4.0) against the 16S rRNA database (Silva v138). α-Diversity (OTU richness and Shannon index) was calculated using R-4.0.0 with the vegan 2.5-6 package.[19] Principal coordinates analysis (PCoA) was employed for the visualization of the Bray−Curtis distance matrices. Biomarkers were identified for ENR-treated and control groups via LEfSe (linear discriminant analysis effect size).[20] The Sloan et al. neutral model and the null model were based on the β-nearest taxon index (βNTI), and the Bray−Curtis-based Raup−Crick

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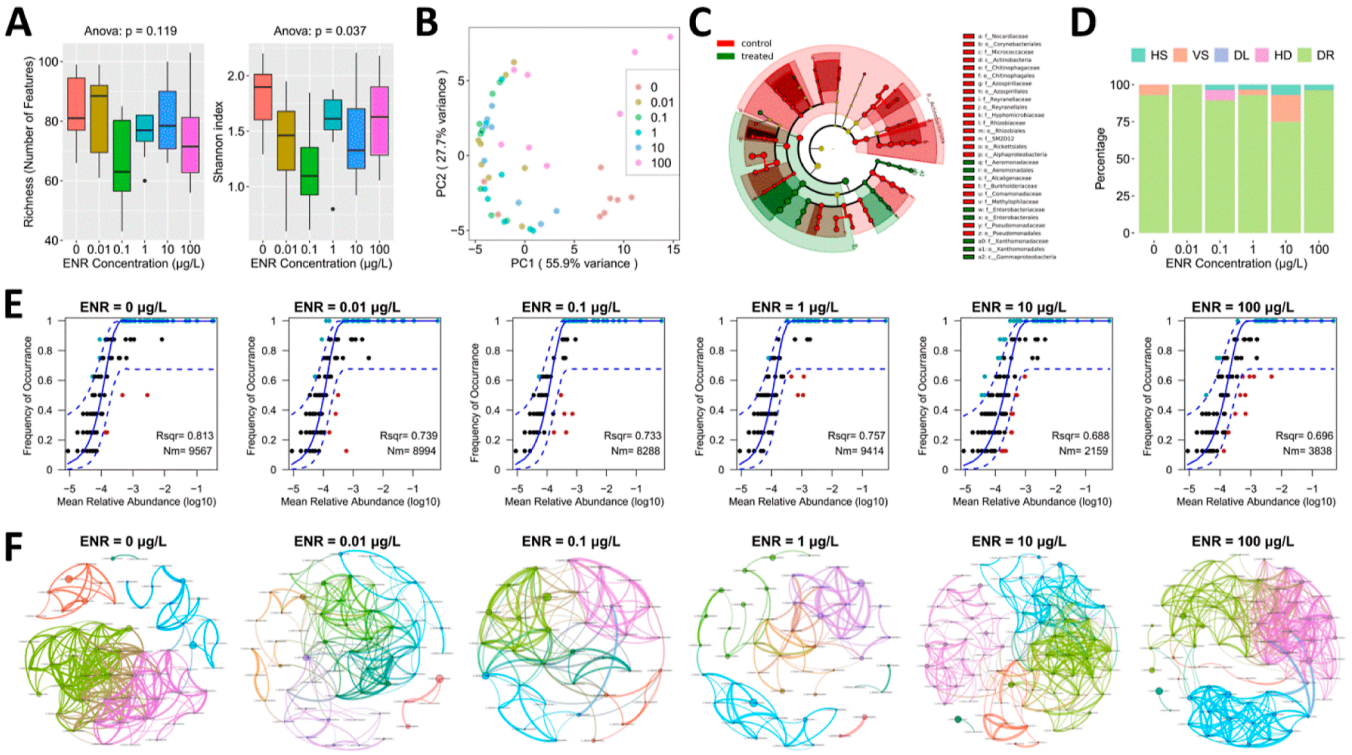

Figure 1. The composition of intestinal microbiota in zebrafish larvae was altered following ENR exposure by 240 hpf. (A) α-Diversity across various ENR treatments. (B) PCoA based on Bray−Curtis distance of all gut samples. (C) LEfSe cladogram of the gut bacterial community obtained from control and ENR-treated groups. (D) Percent turnover in community composition governed primarily by variable selection (VS), homogeneous selection (HS), dispersal limitation (DL), homogenizing dispersal (HD), and drift (DR). (E) Occurrence frequency of different OTUs as a function of mean relative abundance using the neutral model. (F) Co-occurring network of the gut bacterial community based on correlation analysis. Data are from one experiment representative of eight independent experiments. (A−F) $n = 8$ (each treatment group had eight replicates, and each replicate contained 60 zebrafish).

metrics (RCbray) were calculated to estimate the effects of assembly processes in the bacterial communities.[21,22] The OTUs with a max relative abundance of >0.1% were selected for co-occurrence network analysis by using the igraph R package. A pairwise Spearman correlation coefficient of >0.6 or <−0.6 and an adjusted p-value of < 0.05 were used to denote statistical significance. The network was visualized in Gephi. The correlation among the immune parameters, mantel tests between bacterial community structures, and immune parameters were performed in R-4.0.0 using the ggcor 0.9.8 package.

2.6. Quantification of ENR and Quality Assurance/ Quality Control. ENR concentrations in exposure solutions and larvae were determined using HPLC−tandem mass spectrometry (HPLC−MS/MS, Agilent, USA) according to our previous studies.[23] Detailed protocols for the extraction and cleanup, instrumental analysis, and quality assurance and quality control (QA/QC) parameters are provided in the Supporting Information. ENR concentrations in the test solutions are measured to ensure the accuracy of exposure concentrations (Table S1).

2.7. Biochemical Assays. Biochemical assays were conducted with 240 hpf larvae. The changes in the levels of complement 3 (C3), immunoglobulin M (IgM), C-reactive protein (CRP), and lysozyme activity (LYSO) are known classic humoral defense factors of the immune system in fish[24] and have been also tested as sensitive biomarkers to chemical exposure.[25−27] Levels of C3, IgM, CRP, and LYSO were measured using a Fish enzyme-linked immunoassay (ELISA) Kit (Nanjing Jiancheng Bioengineering Institute) using a quantitative sandwich enzyme immunoassay technique. A standard curve was constructed in parallel whenever the samples were tested to determine concentrations. For ELISA analysis, the intra- and interassay coefficients of variance (CVs) were <10 and <12%, respectively.

2.8. qPCR for Immune-Related Genes. Total RNA was extracted from larvae using an RNA prep pure tissue kit (Tiangen Biotech, China). The quality of RNA was confirmed via formaldehyde agarose gel electrophoresis and spectrophotometric analysis. cDNA was generated using the Transcriptor First Strand cDNA synthesis kit (Roche, USA), per manufacturer instructions, and transcripts were amplified using the FastStart Universal SYBR Green Master (ROX) kit (Roche) and the iQ5 Multicolor real-time PCR detection system (Bio-Rad, USA). Gene expression was normalized to the housekeeping gene, β-actin, and corrected based on efficiencies of each gene using the Pfaffl method.[28] Primer pair sequences used for transcript amplification are provided in Table S2.

2.9. qPCR for Antibiotic Resistance Genes. Microbial community genomic DNA was extracted as described in Section 2.4. DNA samples were amplified using the FastStart Universal SYBR Green Master (ROX) kit (Roche). A total of 21 fluoroquinolone resistant genes were detected in the present study. Ct-based relative quantification, with an efficiency correction, was normalized to the housekeeping gene 16S rRNA, according to a previous study.[29] Primers for

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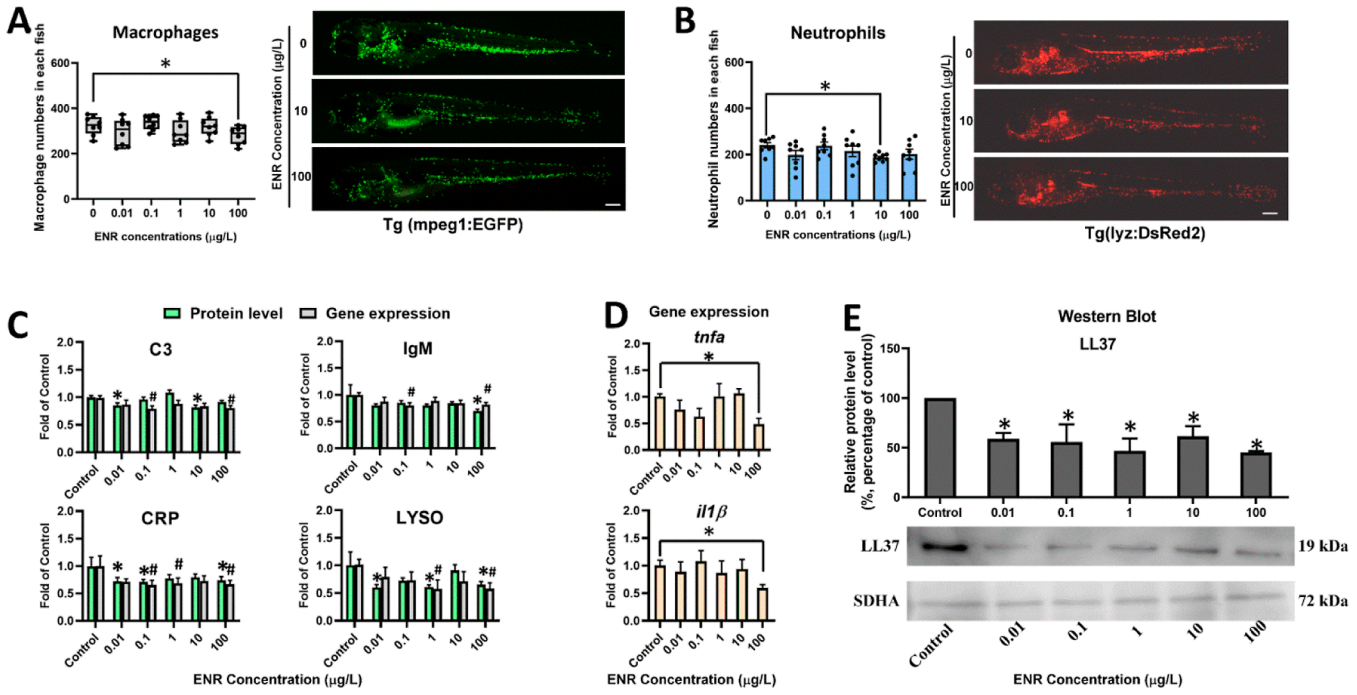

Figure 2. Biomarkers of the immune function were significantly reduced in larval zebrafish following ENR exposure. (A) Number of macrophages in 120 hpf larvae. White bar = 200 μm. (B) Number of neutrophils in 120 hpf larvae. White bar = 200 μm. (C) Protein levels and mRNA gene expression of immune function biomarkers including C3, IgM, CRP, and LYSO in 240 hpf larvae. (D) mRNA gene expression of cytokines *tnfα* and *il1β* in 240 hpf larvae. (E) Western blot analysis of cathelicidin LL37 in 240 hpf larvae. Data are representative of eight independent experiments $^{*}p < 0.05$ and $^{\#}p < 0.05$, according to one-way ANOVA with the LSD test. Error bars indicate the standard error of the mean (s.e.m.); A–B, $n = 8$ (each treatment group had eight replicates, and each replicate contained 10 zebrafish); and C–E, $n = 8$ (each treatment group had eight replicates and, each replicate contained 60 zebrafish).

the 21 antibiotic resistance genes (ARGs) are provided in Table S3.

2.10. Immunoblotting Analysis. Immunoblotting was performed as previously described.[30] The LL37 (cathelicidin) antibody (dilution 1/1000, catalog ID is LS-C331606) was obtained from LifeSpan BioScience (USA). The anti-succinate dehydrogenase complex flavoprotein subunit A (SDHA) antibody (dilution 1/1000, ab137040) was obtained from Abcam (UK). The antibodies used in immunoblots were compatible with zebrafish. Target proteins were normalized with the reference protein SDHA. Digitally captured films were analyzed using ImageJ software.

2.11. Statistical Analysis. SPSS Statistical software (version 18.0; SPSS Inc., Chicago, IL) was used for statistical analysis. All data were assessed for normality and homogeneity of variance using the Kolmogorov–Smirnov one sample test and Levene's test. A one-way ANOVA, followed by a least significance difference (LSD) test, was conducted to assess mean differences between exposure treatments. A $p < 0.05$ was used to denote statistical significance.

3. RESULTS

3.1. ENR Exposure Significantly Altered the Intestinal Microbiota of Zebrafish Larvae. A total of 1,228,688 high-quality bacterial 16S rRNA gene sequences and 229 OTUs were obtained by sequencing 48 samples. Thereafter, the sequence data were rarefied to 14,042 sequences per sample for subsequent diversity analysis. There was no significant difference in species richness among ENR treatments, and the Shannon Index denoted that only 0.1 μg/L ENR treatment showed significant changes in comparison with the control (Figure 1A and Table S4), suggesting that ENR did not significantly influence the α-diversity of the gut bacterial communities. The PCoA ordinations based on the Bray–Curtis distance showed that the bacterial communities of controls were clustered and separated from ENR exposure treatments (Figure 1B). In particular, the 100 μg/L ENR treatment was strongly clustered based on bacterial community profiles, as determined using permutational multivariate analysis of variance (Table S5). The LEfSe biomarker discovery suite was used to compare the abundance of bacterial compositions for the control and ENR-treated groups. Figure 1C depicts cladograms that visualize all detected bacterial compositions from the domain level to the genus level. A total of 56 and 17 differential taxa were detected as potential biomarkers for the control and ENR-treated groups, respectively. A null model and a neutral model were used to estimate the influences on ecological processes of the gut bacterial communities. The null model determined that communities exposed to different treatments were primarily governed by the stochastic assembly, and a greater homogeneous selection was observed in treatments of higher ENR concentrations (Figure 1D). Similar to the results of the null model, the neutral model showed that the reduced community variance was significantly related to the increasing ENR concentrations (p-value = 0.033, linear least square method), suggesting that the selection played a more important role at higher ENR concentrations (Figure 1E). Moreover, the network complexity of gut bacterial communities, in terms of the number of vertices, edges, average degrees, and average clustering coefficients, was largely decreased after low concentrations of ENR exposure but increased at high

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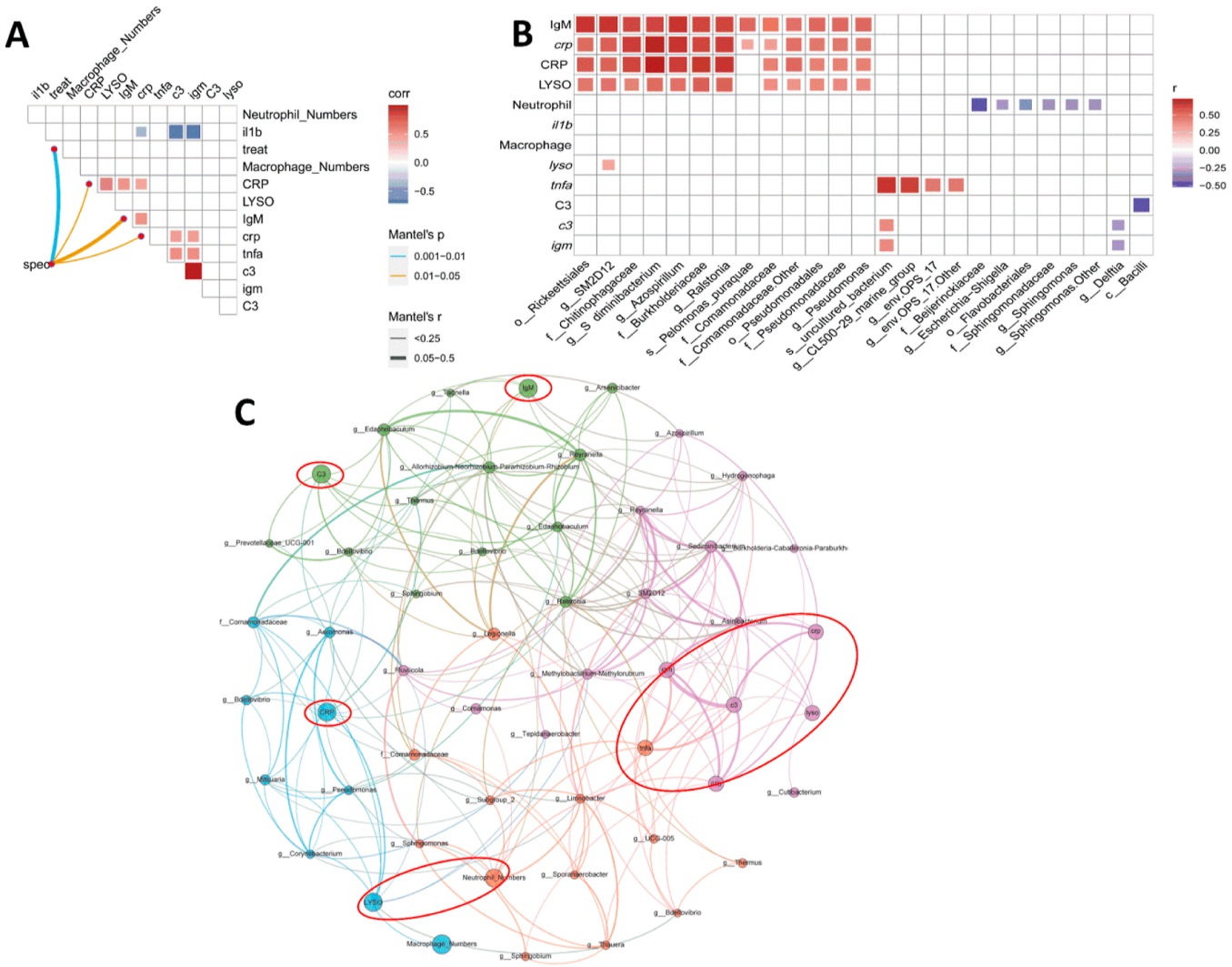

Figure 3. Immunosuppressive biomarkers significantly correlated with the alteration of intestinal flora following ENR exposure. (A) Correlations between the bacterial communities and immunosuppressive biomarkers. Spearman's correlation coefficients between environmental factors with *p*-values > 0.05 denoted by a color gradient. Mantel tests are performed for relating the bacterial community composition (Bray−Curtis distance) with immunosuppressive biomarkers. (B) Pearson's correlation between immune biomarkers and gut microbial abundance. (C) The network analysis demonstrates the co-occurrence patterns between immune biomarkers and gut microbial communities. (A−C) $n = 8$ (each treatment group had eight replicates and each replicate contained 60 zebrafish).

concentrations (Figure 1F and Table S6), which may be due to the different sensitivities of different gut bacteria taxa to ENR exposures.

3.2. ENR Exposure Significantly Induces Immunosuppression of Zebrafish Larvae. The number of macrophages in 120 hpf zebrafish larvae were significantly decreased in 100 μg/L ENR-treated fish ($p < 0.05$, LSD's test), and neutrophil numbers were significantly decreased following exposure to 10 μg/L ENR relative to that of the control ($p < 0.05$, LSD test; Figure 2A,B). Moreover, selected biomarkers of the immune function, including the protein and mRNA expressions of C3, IgM, CRP, and LYSO, were significantly reduced after 10 days of ENR exposure (Figure 2C). Notably, the protein levels of C3, CRP, and LYSO were largely reduced following a low 0.01 μg/L ENR treatment ($p < 0.05$, LSD test). mRNA expressions of cytokines *tnfα* and *il1β* were also significantly decreased following exposure to 100 μg/L ENR ($p < 0.05$, LSD test) (Figure 2D). The expression of cathelicidin LL37 levels in zebrafish was significantly inhibited in all ENR treatments, relative to that of controls (Figure 2E).

3.3. Immunosuppressive Biomarkers Are Significantly Correlated with Alteration of Intestinal Microbiota under ENR Exposure. Immunosuppressive biomarkers tested were significantly correlated with the alteration of gut microbes (Figure 3). Twelve pairs of immunosuppressive biomarkers were significantly correlated (fdr-adjusted $p < 0.05$; Figure 3A). The Mantel test revealed that gut community compositions were significantly correlated with three immunosuppressive biomarkers, that is, *crp*, IgM, and CRP (Figure 3A). The correlations between the immune parameters and specific bacterial taxa were further identified. A total of 22 taxa of bacteria in the zebrafish gut were changed and correlated with immunosuppressive biomarkers after ENR exposure (Figure 3B). Alterations in 13 taxa were positively correlated with the IgM content and mRNA expression levels of *crp*; 12 taxa were positively correlated with CRP and LYSO contents; six taxa were negatively correlated with neutrophil numbers;

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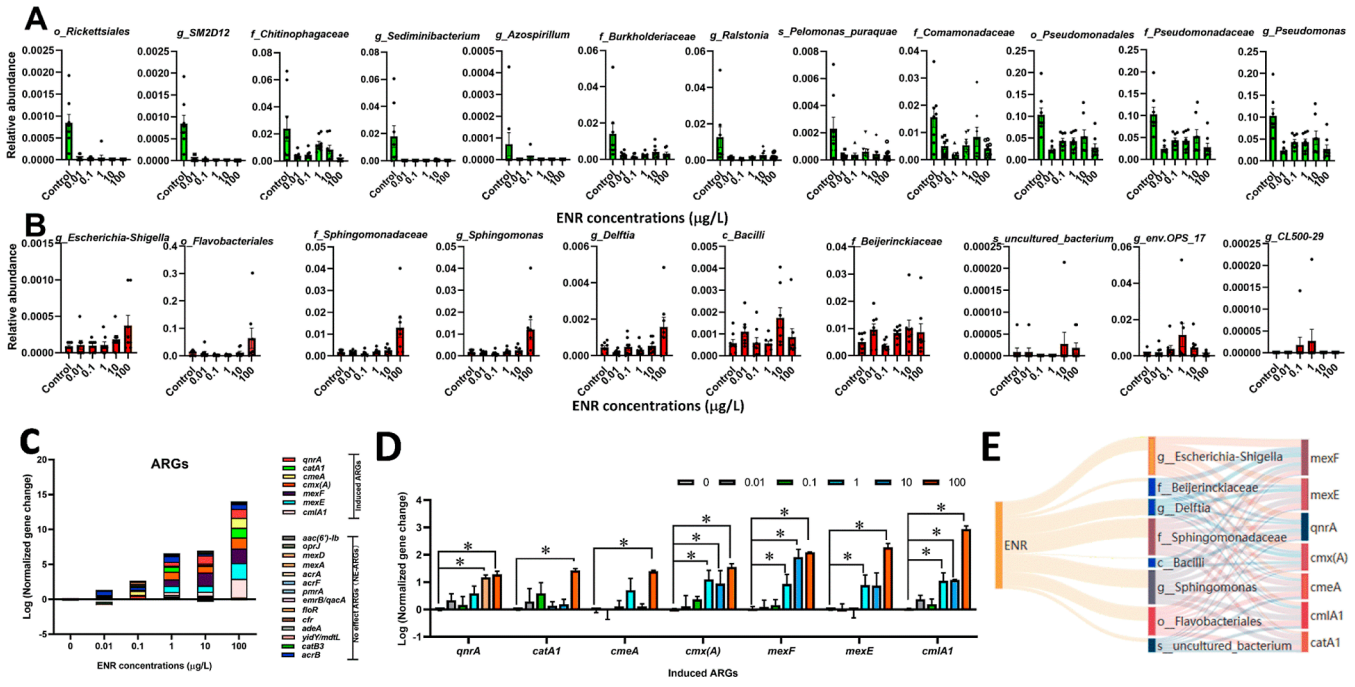

Figure 4. A total of 22 taxa of Bacteria associated with immunosuppressive biomarkers in zebrafish gut were changed after ENR exposure. (A) The relative abundance of 12 taxa bacteria in zebrafish gut in total 22 taxa bacteria were inhibited following ENR exposure. (B) The relative abundance of 10 taxa bacteria in zebrafish gut in total 22 taxa bacteria were induced following ENR exposure. (C) Expression of 21 fluoroquinolone-resistant genes following ENR exposure. (D) Expression profiles of fluoroquinolone-resistant genes induced in ENR treatments. *$p < 0.05$, according to one-way ANOVA with the LSD test. (E) ENR-induced resistant bacteria were significantly correlated with induced fluoroquinolone-resistant genes. Data are representative of eight independent experiments. Error bars indicate the s.e.m. (A–E) $n = 8$ (each treatment group had eight replicates, and each replicate contained 60 zebrafish).

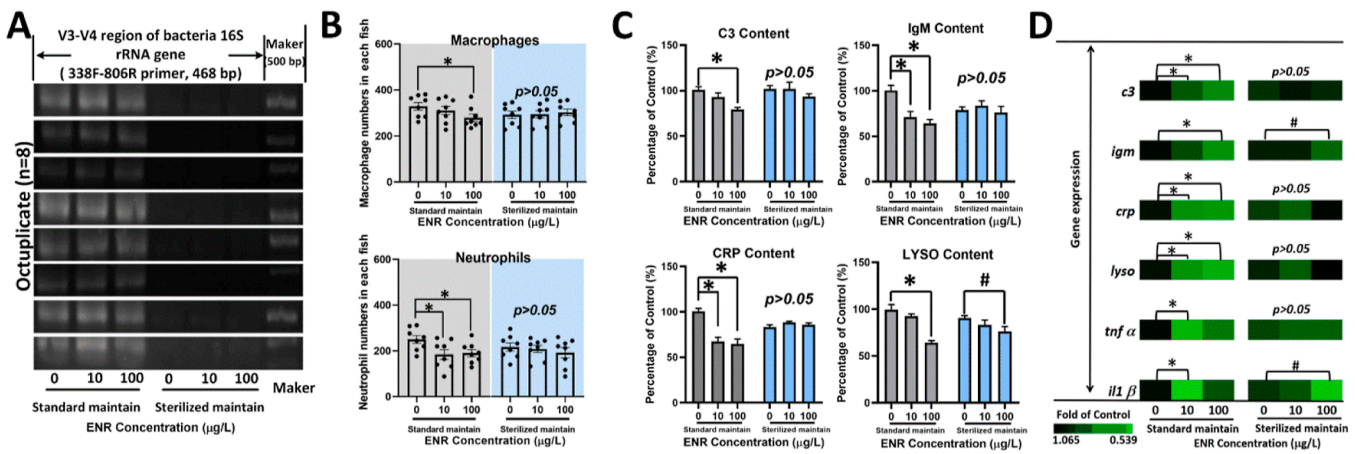

Figure 5. Comparative immunomodulatory effects of ENR exposure between non-sterile and sterile zebrafish. (A) The non-sterile zebrafish had a defined 16S rRNA gene product on a 2% agarose gel, whereas no band, and hence no contamination, was present in the sterile zebrafish. (B) Macrophage and neutrophil numbers in 120 hpf larvae after ENR exposure in non-sterile and sterile zebrafish. (C) Protein levels of immune function biomarkers including C3, IgM, CRP, and LYSO in 240 hpf larvae after ENR exposure in non-sterile and sterile zebrafish. (D) mRNA expression of immune genes including *c3*, *igm*, *crp*, *lyso*, *tnfα*, and *il1β* in 240 hpf larvae after ENR exposure in non-sterile and sterile zebrafish. Data are representative of eight independent experiments. *$p < 0.05$ and #$p < 0.05$, according to one-way ANOVA with the LSD test. Error bars indicate the s.e.m. (A–D) $n = 8$ (each treatment group had eight replicates, and each replicate contained 60 zebrafish).

and four taxa were positively correlated with mRNA gene expression levels of *tnfa* ($p < 0.05$; Figure 3B). The co-occurrence network analysis also indicated a significant correlation between gut OTUs and the immunosuppressive biomarkers (Figure 3C).

3.4. Changes in Bacteria Are Significantly Associated with Key Immunosuppressive Biomarkers under ENR Exposure. According to above results, a total of 22 taxa of bacteria in zebrafish gut were changed and correlated with immunosuppressive biomarkers after ENR exposure (Figure 3B,C). We further analyzed the relative abundance of the 22 taxa of gut bacteria. Our results showed that the relative abundance of 12 taxa bacteria in zebrafish gut, including *o_Rickettsiales, g_SM2D12, f_Chitinophagaceae, g_Sediminibacterium, g_Azospirillum, f_Burkholderiaceae, g_Ralstonia, s_Pelomonas_puraquae, f_Comamonadaceae, o_Pseudomonadales,*

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f_Pseudomonadaceae, and g_Pseudomonas, was inhibited in a dose-dependent manner (Figure 4A). This suggested that ENR exposure significantly inhibited the proliferation of these gut communities, leading to interference with the immune function. Interestingly, another 10 taxa gut bacteria including *g_Escherichia-Shigella, o_Flavobacteriales, f_Sphingomonadaceae, g_Sphingomonas, g_Delftia, c_Bacilli, f_Beijerinckiaceae, s_uncultured_bacterium, g_CL500-29_marine_group*, and *g_env.OPS_17* presented a reverse increase under increasing exposure concentrations of ENR (Figure 4B). This may imply that the 10 taxa bacteria have become ENR-resistant bacteria in the gut. In addition, the expression of 21 fluoroquinolone-resistant genes was quantified in the gut of 240 hpf zebrafish treated with ENR. Seven ARGs, including *qnrA, catA1, cmeA, cmx*(*A*), *mexF, mexE*, and *cmlA1*, were significantly induced at 1, 10, or 100 μg/L ENR (Figures 4C,D, S1). These results confirmed that ENR exposure induced ENR resistant bacteria that were significantly correlated with the induced fluoroquinolone-resistant genes (Figure 4E) and key immunosuppressive biomarkers (Figure 3B,C).

3.5. ENR Exposure Causes Intestinal Microbial-Mediated Effects on Immune Regulation. The immunomodulatory differences following ENR exposure were compared between zebrafish cultured under sterile and non-sterile conditions to verify intestinal microbial-mediated effects of ENR on immune regulation (Figure S2). 16S rRNA gene PCR products and subsequent qPCR expression assessment were used to detect contamination between the non-sterile and sterilized zebrafish cultures. The non-sterile zebrafish had a defined 16S rRNA gene product on a 2% agarose gel, whereas no band or contamination was present in the sterile zebrafish (Figure 5A). Similarly, qPCR results of 16S rRNA gene expression showed a high abundance of bacteria in the non-sterile group, whereas none were detected in the sterilized maintain group (Figure S3).

To further test whether the observed immunotoxicity of ENR was mediated via the impacts on the intestinal flora, we compared the immune interference after ENR exposures in the presence and absence of intestinal flora by using both non-sterile and sterile zebrafish. The numbers of macrophages and neutrophils were significantly different between the non-sterile and sterile zebrafish following ENR treatments (Figure 5B). The number of macrophages was significantly decreased in the 100 μg/L ENR non-sterile treatment ($p < 0.05$, LSD test), and the number of neutrophils was significantly decreased in the 10 and 100 μg/L ENR non-sterile treatment ($p < 0.05$, LSD test). In sterile zebrafish treatments, ENR exposure did not change the number of macrophages or neutrophils ($p > 0.05$, LSD test) and showed no effects on the protein levels for C3, IgM, or CRP (Figure 5C) or mRNA gene expression of *c3*, *crp*, *lyso*, and *tnfα* (Figure 5D). The gene expression *il1β* following ENR exposure was inhibited in both the non-sterile and sterile zebrafish (Figure 5C,D). Overall, these results confirmed that the intestinal microbiota closely mediated immune regulation during ENR treatments.

4. DISCUSSION

Despite numerous studies that have focused on characterizing the functional abnormalities of immunoregulation induced by different antibiotics, the underlying mechanisms and modes of action require more investigations, particularly in environmental organisms that are continuously exposed to environmental antibiotics. Some antibiotics are known to weaken immune defenses via depletion of commensal microbes, alteration of microbial composition, or disruption of the colonization of commensal microbes in the gastrointestinal tract.[27−29] To detail whether and how gut microbiota changes upon antibiotic exposure are linked to the potential influence on the development of the immune system in environmental organisms, the immune responses and intestinal microbiota in early-life-stage zebrafish were comprehensively investigated under ENR treatments at environmentally relevant concentrations.

We found significant differences in bacterial communities, homogeneity, and richness of intestinal microbiota between ENR-exposed and control zebrafish. For example, the 100 μg/L ENR treatment groups were strongly clustered based on their bacterial community profiles, suggesting that the diversity of intestinal microbiota was altered under high ENR administration. A similar relationship was previously reported in oxytetracycline-treated zebrafish, which exhibited significant changes in α- and β-diversity of intestinal microbiota in only the highest-dose exposed group (10,000 μg/L).[31] The results of the neutral model in the present study revealed that the proportion by deterministic assembly increased with ENR concentrations, and the homogeneous selection played a more critical role in higher ENR concentration treated groups, which confirmed that ENR exposure affected the intestinal microbiota of zebrafish. Similar adverse effects were observed in a murine model orally treated with ENR, which modified the composition of the gut microbiota.[32] However, the effects of ENR following exposure to ecological levels to disturbances of the gut microbiota have not been investigated. To our knowledge, this is the first study to reveal adverse gut microbiota effects of ENR exposure in environmental organisms.

The intestinal microbiota is involved in the development and differentiation of the immune system of the host.[33] Cash et al. demonstrated that gastrointestinal tract microbiota can stimulate the secretion of antimicrobial peptides in the intestinal lumen in the mammal.[34] Moreover, the number of the immune cells including intestinal T cells, CD4+ TCR$\alpha\beta$ T helper (Th) cells, and inflammatory T regulatory cells can be influenced by intestinal bacteria changes.[35] Depleting commensal microbes and altering the microbiota composition via antibiotic administration can affect intestinal immune defenses.[36−39] Administration of streptomycin renders mice susceptible to infection with *Salmonella* spp. and amoxicillin-induced microbiota changes, reducing the expression of MHC class I and II genes in the small and large intestines.[40,41] In zebrafish, we found here that ENR caused significant immunosuppression with the evidence of inhibition of macrophage and neutrophil numbers, C3, IgM, CRP, and LYSO contents, cytokines *tnfα* and *il1β* levels, and the cathelicidin LL37 level, which were closely correlated with the gut community disturbance, suggesting that the ENR-induced changes in the microbiota impact the general immune system of zebrafish.

We found that a total of 22 taxa of bacteria in the zebrafish gut were changed and correlated with immunosuppressive biomarkers after ENR exposure. Among them, *Rickettsiales*, *Pseudomonadales*, and *Flavobacteriales* were the main order in the changed gut community, also considered to be the common pathogens.[42] *Rickettsiales* and *Pseudomonadales* were inhibited in a dose-dependent manner under ENR exposure, which was consistent with the pharmacological action of ENR

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for effective *Proteobacteria* inhibition.[43] Kim et al. also found ENR significantly reduced proportions of *Proteobacteria* in human fecal suspensions and revealed the functional gene expression responses of the changed bacterial communities and potential health threats.[44] Interestingly, the proportion of *Flavobacteriales* was increased in the ENR-treated zebrafish gut; meanwhile, one-third of fluoroquinolone resistance genes in the zebrafish gut were also significantly induced. This suggested that *Flavobacteriales* is a class of antibiotic-resistant bacteria. Correlations of induced specific resistant bacteria and resistant genes were also reported for other antibiotics. For example, specific resistance genes were increased in human feces following administration of clindamycin.[45] Tetracycline was reported to induce antibiotic-resistant bacteria in zebrafish.[29] Gut microbial communities maintain a dynamic equilibrium that ensures the functioning of the digestive system and defense of pathology, and a significant reduction or increase in the proportion of gut microbial communities may pose a health risk.[46] In this regard, the inhibited proliferation of *Rickettsiales* and *Pseudomonadales* and increased *Flavobacteriales* upon ENR exposure may partly explain the immune interference observed.

Germ-free or gnotobiotic animal models are valuable for studying the functional interactions of gut microbes in the host health.[47,48] We verified the intestinal microbial-mediated effects of ENR on immune regulation by comparing the immunomodulatory differences in non-sterile and sterile zebrafish following ENR exposure. A significant inhibitory effect was induced in non-sterile zebrafish after ENR treatment, but such an effect was significantly salved in the sterile zebrafish. These results further prove that intestinal microbiota plays a key role in mitigating the effects of ENR treatment on immune interference.

Overall, this study systematically understands whether and how ENR-induced changes in the microbiota impact immunity during early life-stage development in zebrafish. It is the first known study to show a strong correlation between gut community disturbances and multi-level immunosuppressive responses induced by ENR exposure in fish. The comparison of the immunosuppressive profiles of sterile and non-sterile zebrafish further confirms the important roles of intestinal microbiota in immune regulation. It is worth noting that the altered gut microbial community may last and persist even after antibiotic treatment stops, shedding light on the long-term impacts that are much less understood today.[49−51] The significantly induced ARGs in the zebrafish by ENR raise the concern of prolonged and exacerbated disruptive effects of ENR in the natural environments. In addition, the dose-responsive biomarkers related to general immune functions identified might also be applied for assessing the immunotoxicity of other antibiotics in future studies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.1c08712.

Additional experimental details, methods, and results, including quantification of ENR and QA/QC, ENR levels in the test solutions, primers of zebrafish immune-related genes, and ARGs, Tukey's honest significance difference for the Shannon index, PERMANOVA, co-occurrence network characterization, experimental scheme for sterile zebrafish experiments, and qPCR results of the 16S rRNA gene (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Chunmiao Zheng − *State Environmental Protection Key Laboratory of Integrated Surface Water-Groundwater Pollution Control, Guangdong Provincial Key Laboratory of Soil and Groundwater Pollution Control, School of Environmental Science and Engineering, Southern University of Science and Technology, Shenzhen 518055, China;* orcid.org/0000-0001-5839-1305; Email: zhengcm@sustech.edu.cn

Elvis Genbo Xu − *Department of Biology, University of Southern Denmark, Odense 5230, Denmark;* orcid.org/0000-0002-4414-1978; Email: elvis@biology.sdu.dk

Authors

Wenhui Qiu − *School of Public Health and Emergency Management, Southern University of Science and Technology, Shenzhen 518055, China; State Environmental Protection Key Laboratory of Integrated Surface Water-Groundwater Pollution Control, Guangdong Provincial Key Laboratory of Soil and Groundwater Pollution Control, School of Environmental Science and Engineering, Southern University of Science and Technology, Shenzhen 518055, China;* orcid.org/0000-0002-9961-3928

Tang Liu − *State Environmental Protection Key Laboratory of Integrated Surface Water-Groundwater Pollution Control, Guangdong Provincial Key Laboratory of Soil and Groundwater Pollution Control, School of Environmental Science and Engineering, Southern University of Science and Technology, Shenzhen 518055, China*

Xinjie Liu − *State Environmental Protection Key Laboratory of Integrated Surface Water-Groundwater Pollution Control, Guangdong Provincial Key Laboratory of Soil and Groundwater Pollution Control, School of Environmental Science and Engineering, Southern University of Science and Technology, Shenzhen 518055, China*

Honghong Chen − *State Environmental Protection Key Laboratory of Integrated Surface Water-Groundwater Pollution Control, Guangdong Provincial Key Laboratory of Soil and Groundwater Pollution Control, School of Environmental Science and Engineering, Southern University of Science and Technology, Shenzhen 518055, China*

Shusheng Luo − *State Environmental Protection Key Laboratory of Integrated Surface Water-Groundwater Pollution Control, Guangdong Provincial Key Laboratory of Soil and Groundwater Pollution Control, School of Environmental Science and Engineering, Southern University of Science and Technology, Shenzhen 518055, China*

Qiqing Chen − *State Key Laboratory of Estuarine and Coastal Research, East China Normal University, Shanghai 200241, China;* orcid.org/0000-0002-3247-7861

Jason T. Magnuson − *Department of Chemistry, Bioscience and Environmental Engineering, University of Stavanger, Stavanger 4021, Norway;* orcid.org/0000-0001-6841-8014

Daniel Schlenk − *Department of Environmental Sciences, University of California, Riverside, California 92521, United States*

Complete contact information is available at:

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Author Contributions

W.Q., C.Z., and E.G.X. designed the experiments; W.Q., H.C., and X.L. performed the fish experiments; W.Q. and E.G.X. wrote the manuscript; S.L. contributed to chemical analysis; T.L. and Q.C. contributed to diversity analyses of microbial communities, J.T.M. and D.S. contributed to the scientific discussion; and J.T.M., E.G.X., D.S., and C.Z. revised the manuscript.

Notes

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