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Effects of norfloxacin, copper, and their interactions on microbial communities in estuarine sediment

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ABSTRACT

The discharge of antibiotics and metals in estuaries is of great concern since they threaten microbial communities that are critical for maintaining ecosystem function. To understand single and combined effects of norfloxacin (0–20 μg g^{-1}) and copper (40 μg g^{-1}) on microbial ecology in estuaries, we evaluated changes in bacteria population, inhibition rates, and microbial composition in estuarine sediments over a 28-day period. Bacteria population significantly decreased following single and combined exposure to norfloxacin and copper throughout the incubation period, except on Day 28 in treatments exposed to copper, 20 μg g^{-1} norfloxacin, or both. These three treatment groups had lower Shannon diversity and Simpson's indices on Day 28 than other treatments and the controls suggesting recovery in bacteria population did not correspond with recovery in richness and evenness. Furthermore, functional predictions revealed that the effect of time and contaminants were significantly different on some microbial community functions on Day 28, especially the combination of Cu and high concentration NFX, including aerobic chemoheterotrophy, methanol oxidation and methylotrophy. Thus, norfloxacin and copper had significant adverse effects on microbial communities in estuarine sediments; however, the combined effects were variable and depended on exposure duration and antibiotic concentration.

1. Introduction

Estuarine ecosystems are an ecotone of marine, freshwater, and terrestrial environments and are severely threatened by anthropogenic activities worldwide (Kennish, 2005). They are highly dynamic ecosystems since they are subject to the contrasting hydrological, sedimentological, geomorphological, and physicochemical characteristics of continental and oceanic environments. Estuaries maintain planetary health and function by supporting nutrient cycling, carbon sequestration, and unique biodiversity and providing storm protection and water quality regulation (Kaiser et al., 2015). Despite covering less than 0.7% of the Earth's surface, estuaries facilitate a large proportion of global biogeochemical activities, probably because they are primary recipients of terrestrial nutrients, sediments, and organic matter from riverine and groundwater discharge prior to exchange with the open ocean (Yu et al., 2021). Rapid urbanization and industrialization in the Anthropocene have significantly contributed to massive influx of anthropogenic pollutants in estuarine ecosystems threatening their ecological function (Zhuang et al., 2019).

Antibiotics and potentially toxic elements are frequently and abundantly detected in estuarine environments due to riverine discharges, emissions from land-based activities, and aquaculture effluent. Approximately 93,309 tons of antibiotics were used in agriculture in 2017 worldwide, and they are expected to increase to 104,079 tons by 2030 (Tiseo et al., 2020). This is particularly concerning because animals metabolize 10–25% of the administered antibiotics, resulting in large amounts entering wastewater systems and estuaries (Marshall and Levy, 2011). A recent study found that fluoroquinolones and β-lactams were the most dominant classes of antibiotics in the global estuarine environment, with norfloxacin being the most dominant antibiotic in sediment (5.14–444 ng g^{-1} maximum concentrations) and water (0.51–6800 ng L^{-1} maximum concentrations) samples (Zheng et al.,

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2021). Norfloxacin concentrations were one to three orders of magnitude higher in estuaries in China than in other geographic locations, probably because from 2010 to 2018, China consumed 30–50% of the antibiotics produced globally (Schoenmakers, 2020).

Previous studies showed that antibiotics affected microbial community structure and composition in soils (Ma et al., 2012), wastewater (Deng et al., 2012; Zhang et al., 2013), canal (Hoa et al., 2011), and river sediments (Deng et al., 2018), but studies in estuaries remain scarce. Antibiotic exposure can affect the ecosystem function of microbial communities. For example, sulfadiazine exposure decreased the relative abundance of microorganisms associated with carbon cycling in soils (Qiu et al., 2021). Microbial richness and diversity significantly decreased ($p < 0.05$) in soils exposed to norfloxacin (1.0–100 μg g^{-1}) for 7 days, although only exposure to 100 μg g^{-1} norfloxacin continued to decrease richness and diversity after 35 days of exposure (Ma et al., 2012). The results suggested that exposure to 100 μg g^{-1} norfloxacin had long-term and potentially irreversible effects on microbial community function. These long-term effects could affect the ecological function of the soil microbiome since norfloxacin exposure (8 mg L^{-1}) in soil was shown to inhibit litter decomposition and nutrient release while increasing the pH and dissolved oxygen concentration in a freshwater microcosm (Zhao et al., 2021).

While the co-occurrence of antibiotics and heavy metals has been shown to yield complex effects in soils and freshwater sediments, there is a dearth of studies on the combined effects of antibiotics and metals on benthic microbial communities in estuaries. Exposing benthic microbial communities to enrofloxacin shifted bacterial richness and diversity in a salt marsh (Fernandes et al., 2015). A recent study found combined exposure to Cu and enrofloxacin inhibited soil enzyme activity and microbial biomass more than single Cu or enrofloxacin exposure (Yang et al., 2021). Similar observations were made in soils exposed to sulfadimidine and Cu (Wang et al., 2019), enrofloxacin and Cd (J. Wang et al., 2018), and sulfadiazine and Cu (Xu et al., 2016). Carboxyl, hydroxyl, sulfhydryl, and amino groups on antibiotics can sometimes combine with co-occurring metals, thus affecting their bioavailability, biotransformation of antibiotics, biogeochemical cycling of metals, and overall microbial community structure and function (Guo et al., 2020). Our previous studies showed that Cu pollution was prevalent in estuaries and coastal areas in the Eastern Guangdong (Zhuang et al., 2019). Copper is widely used antifouling agents that are used in aquaculture farms.

In this study, we investigated the single and combined effects of norfloxacin and copper on microbial community richness and diversity in a water-estuarine sediment microcosm over time using next-generation sediment DNA sequencing. We hypothesized that co-exposing Cu and norfloxacin had higher comprehensive toxicity than single exposure Cu or norfloxacin. Knowledge of the combined effects of antibiotics and potentially toxic elements on microbial community structure and composition can improve our understanding of the stability and resilience of estuarine ecosystems, which is essential for developing effective strategies for conserving threatened estuarine biodiversity.

2. Materials and methods

2.1. Chemicals

Norfloxacin (CAS No. 70458-96-7, NFX) was purchased from Sangon Biotech Co., Ltd. (Shanghai, China); $CuSO_4$ (CAS No. 7758-98-7) was purchased from Xilong Scientific Co., Ltd. (Shantou, China); HPLC grade methanol was obtained from methanol from Thermo Fisher Scientific and Alfa Aesar (Shanghai, China). Ultrapure water was prepared by a Milli-Q 18 MΩ system (Millipore, Germany). All reagents were purchased from Fisher Chemical (Shanghai, China). Ultrapure water was prepared to resist >18 MΩ cm using a Milli-Q water purification system (Millipore). The NFX stock solution was prepared by dissolving in methanol to a concentration of 25 mg mL^{-1} and stored at 4 °C. The working solutions for spiking during the water-sediment microcosm incubation were prepared by serial dilution of the stock standard using methanol.

2.2. Sediment sampling and characterization

Surface estuary sediments (a 5-kg pool from three replicate grab samples collected at 0–5 cm depth) used for microcosm incubation were collected from Shantou Bay (116.43.21E, 23.19.27N), a semi-enclosed bay located at the confluence of the Meixi River and Rongjiang River in Guangdong, China (Yu et al., 2021). The sediment samples (0–5 cm) were collected using grab sampling during low tide in December 2019. The temperature, salinity, pH, dissolved oxygen, and conductivity were detected *in situ* by an IN-Situ SmarTROLL™ Multiparameter. The sediment sample was stored in dark at 4 °C and was homogenized for 4 h prior to further treatment. The moisture content was 54.54%. The background concentration of NFX was determined using a liquid chromatography tandem mass spectrometer (Thermo TSQ-Endura) after ultrasonic-assisted extraction (detailed procedures are given in Supplementary Information) (Zhou et al., 2011), while the Cu concentration was determined using by a Jena PQ 9000 ICP–OES (Analytik Jena AG, Germany) after microwave digestion as described in the Supplementary Information (Zhuang et al., 2019). The concentrations of NFX and Cu in the sediment samples averaged 17.69 ng g^{-1} dw and 18.40 μg g^{-1} dw, respectively.

2.3. Estuarine sediment microcosm setup

Prior to incubation, sodium hypochlorite followed by sterilized water were used to rinse all microcosm equipment to remove potential microorganism contaminants. The microcosm was set up in a total of 27 beakers (1000 mL), each with 90 g (wet weight) estuarine sediments and 300 mL coastal water, sealed with paraffin film and incubated at 20 °C in the dark for 7 days. Following the 7-day acclimatization, the microcosms were spiked at varying concentrations in triplicate with a combination of NFX (0, 1, 10, and 20 μg g^{-1} dry weight (dw)) and $CuSO_4$ (0 and 40 μg g^{-1} dw) solutions. The experiment was carried out at 20 °C with a light to dark ratio of 8:16. The parameters of the overlying water were monitored, including pH, conductivity, temperature and dissolved oxygen. The overlying water was replaced by 150 mL every day to ensure that the dissolved oxygen was ≥2.5 mg L^{-1}. Sampling of 1 g wet weight (ww) (0.54 g dw) sediment for colony count was conducted every 7 days using dilution plating. Briefly, 1 g ww sediment was added with 9 g deionized water and homogenized. Afterwards, 1 g homogenized mixture was diluted and homogenized again, which was repeated for another two times. Approximately 100 μL of the final diluent were added in each plate. Samples were incubated at 25 °C for 36 h in a Luria-Bertani growth medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl, pH 7.0) before colony count. Each experiment was performed in triplicate.

2.4. DNA extraction and 16S rRNA gene sequencing

Sediment samples were freeze-dried, and the moisture content was 54.09%. The DNA of all samples (2 g dw, each) was extracted using an MN NucleoSpin 96 Soil kit following the manufacturer's instructions. 338F and 806R flanking the V3–V4 regions of the 16S rRNA gene were used as primers for PCR amplification. Sequencing libraries were constructed by a two-step polymerase chain reaction (PCR) method. PCR amplification for target sequencing was conducted in a 10 μL reaction containing 50 ng template, fusion PCR primer, KOD FX Neo Buffer, dNTP and KOD FX Neo enzyme. PCR cycling conditions were 95 °C for 5 min, 25 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 40 s, and a final step at 72 °C for 7 min. Further amplification was performed by Solexa PCR in a 20 μL reaction using 10 μL purified amplification products of

target sequencing, 2.5 μL 2 μM of each primer (MPPI-a and MPPI-b), and 10 μL Q5® High-Fidelity 2X Master Mix. The Solexa PCR cycling conditions were 98 °C for 30 s, 10 cycles of 98 °C for 10 s, 65 °C for 30 s, 72 °C for 30 s, and finally 72 °C for 5 min. Sequence analysis of the 16S rRNA gene in the V3 and V4 regions was conducted. The validated libraries were used for sequencing on the Illumina HiSeq 2500 PE250 platform, and the obtained data were transferred by base calling to sequenced reads. After assembly with FLASH v1.2.11 (Magoč and Salzberg, 2011), all the low-quality contigs were filtered out with Trimmomatic v0.33 and UCHIME v8.1 (Bolger et al., 2014; Edgar, 2013). Annotation of all contigs was carried out with the SILVA database for 16S (Quast et al., 2013).

2.5. Bioinformatics

Bioinformatic analysis was performed on the platform BMKCloud (www.biocloud.net). Diversity indices (Chao, ACE, Shannon and Simpson) were determined by Mothur v1.30 (Schloss et al., 2009). Principal coordinates analysis (PCoA) was conducted to analyze the difference in beta diversity based on the Bray–Curtis algorithm to calculate the distance between samples at the OTU level. The linear discriminant analysis effect size (LEfSe) was applied to discern the distinguished taxa in abundance between treatments (Segata et al., 2011), conducted at levels from phylum to genus with an LDA threshold of 4.0. Picrust2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) based on the KEGG database was used for function prediction at the functional classification of level 3 (Kanehisa et al., 2014); Tax4Fun was also employed for prediction of the functional profiles of amplicons of respective samples according to the 16S rRNA gene data (Aβhauer et al., 2015). Functional Annotation of Prokaryotic Taxa (FAPROTAX) was used to evaluate pollutant effects on the top 30 functions of the microbial community.

2.6. Data analyses

The co-occurrence among bacterial taxa was illustrated by network analysis at the genus level, where Spearman's correlation was applied to assess the connection of pairwise genera with the absolute value of Spearman's correlation coefficient (r) > 0.1 and the p value < 0.05. Spearman rank correlation analysis was applied for the correlation heatmaps based on the top 30 genera in relative abundance in the microbial community with a correlation coefficient $r > 0.1$, and $p < 0.05$ was considered statistically significant. Spearman's correlation was used because it is non-parametric and does not rely on the assumptions of normality. One-way-factorial analysis of variance (ANOVA) and *t*-test was employed to assess the biochemical responses of exposed concentrations and time differences in parameters among treatments, and $p < 0.05$ was considered statistically significant. Prior to ANOVA, assumptions of data normality and homoscedasticity were tested using Shapiro-Wilk and Kolmogorov-Smirnov tests (Table S1 and S2) and Levene's test, respectively (Table S3). The data was transformed by cube rooting to improve its homoscedasticity.

The following equation was used to evaluate the effects of antibiotics and heavy metals on microbes:

$$\text{Inhibition rate (IR)} = \frac{(N_{control} - N_{experiment})}{N_{control}} \times 100\%$$

where $N_{control}$ is the number of microorganisms in the control group and $N_{experiment}$ is the number of microorganisms in the respective treatment group. By comparing the measured inhibition rate and of the co-exposure and the sum of single exposure, the interaction type (antagonism, additive, or synergism) was determined. If the sum of the single exposures is approximately equal to the co-exposure, then the combined effect will be additive, if less then it will be antagonistic, but if higher it is synergistic (Wang et al., 2019b).

3. Results and discussion

3.1. Microbial toxicity of separate and combined norfloxacin and copper

Several studies have shown that acute contamination by metals and antibiotics decreases microbial abundance in the short term due to an increase in mortality, growth inhibition, and a decrease in reproduction and metabolism rates (Ding and He, 2010; Nogales et al., 2011). Microbial communities in a water-estuarine sediment microcosm were exposed to varying concentration ratios of Cu and NFX to determine their effects on bacterial populations and growth over time (Fig. 1). Bacterial populations exhibited time-dependent shifts in the control group, as well as in all treatments except at day 28, where the bacterial population in microcosms was treated with NFX20 was higher than that in the control (Table S4). Additionally, there was no evidence suggesting that exposure to Cu altered the bacterial population at day 28. The decrease in the bacterial population following exposure to NFX alone was slightly concentration dependent on day. Obviously, the decreasing rate of bacterial populations was increased along time when exposed to NFX and/or Cu. However, no concentration dependency was observed when the estuarine microorganisms were co-exposure to NFX and Cu. Similar results were observed in a soil microcosm co-exposed to sulfadimidine (0, 13.9, 55.6, and 222 μg g^{-1}) and Cu (102 μg g^{-1}) (Wang et al., 2019). In contrast, co-exposing enrofloxacin (0, 8.98, 35.9, and 144 μg g^{-1}) and cadmium (45.0 μg g^{-1}) decreased the bacterial population in soil microcosms in a concentration-dependent manner (J. Wang et al., 2018). These results suggest that the effect of antibiotic concentration on bacterial populations during co-exposure in microbial communities varies between different antibiotics and metals. The extent to which the bacterial population decreased in the co-exposure microcosm decreased over time, and no significant difference was observed between the control and co-exposure microcosms at day 28. The results suggested that selections occurred on bacteria tolerant and resistant to NFX at high concentration, which stimulated sediment microbial activities because of formation and proliferation of persistent bacteria and their use of NFX as a carbon source (Fang et al., 2014). On the other hand, the slight increase of NFX20 at day 28 compared with NFX1 and NFX10 was possibly because high concentration NFX killed more bacteria, which served as labile carbon sources. The weak effect of NFX at low concentrations was probably on account of the decrease in bioavailability owing to high sorption of NFX in sediment (Danilova et al., 2020). Nonetheless, the effects of time in bacterial population decreasing were more impactful compared with those of NFX and Cu.

The single and combined toxicity ecological effects of antibiotics and metals in estuaries were assessed in this study by measuring the inhibition rates of NFX and Cu in the microcosm to estuarine microorganisms (Fig. 2). Inhibition rates above 60% were observed following co-exposure to Cu and NFX10 at Day 7 as well as single exposure to NFX10, NFX20, or 40 μg g^{-1} Cu at Day 21. The inhibitory effects of NFX10, NFX20, and Cu increased with time until reaching a maximum on day 21 and it subsequently decreased. However, NFX20 showed no inhibition at day 28, this was probably due to the sorption of some NFX in sediment at early stages, which limited the mobility and availability of NFX (Danilova et al., 2020). The interactions between NFX and Cu in all treatments were antagonistic except when the microorganisms were co-exposed to Cu and NFX20 on Day 28 or Cu and NFX10 on Day 7, where the interactions were synergistic. Synergistic inhibitory effects were previously observed in soil microcosms on Day 14 to Day 28 co-exposed to sulfadimidine (55.6 and 222 μg g^{-1}) and Cu on Day 14 to Day 28 (Wang et al., 2019). A previous study found that NFX could coordinate to Cu(II) through carboxylate and ketone groups to form an NFX-Cu complex (Martins et al., 2016). The formation of metal-antibiotic complexes may reduce the microbial toxicity of Cu and NFX (Liu et al., 2022), and this was more likely to occur in our study since the minimum molar ratio of divalent metal and antibiotic concentrations was approximately ten, while Wang (Wang et al., 2019) used

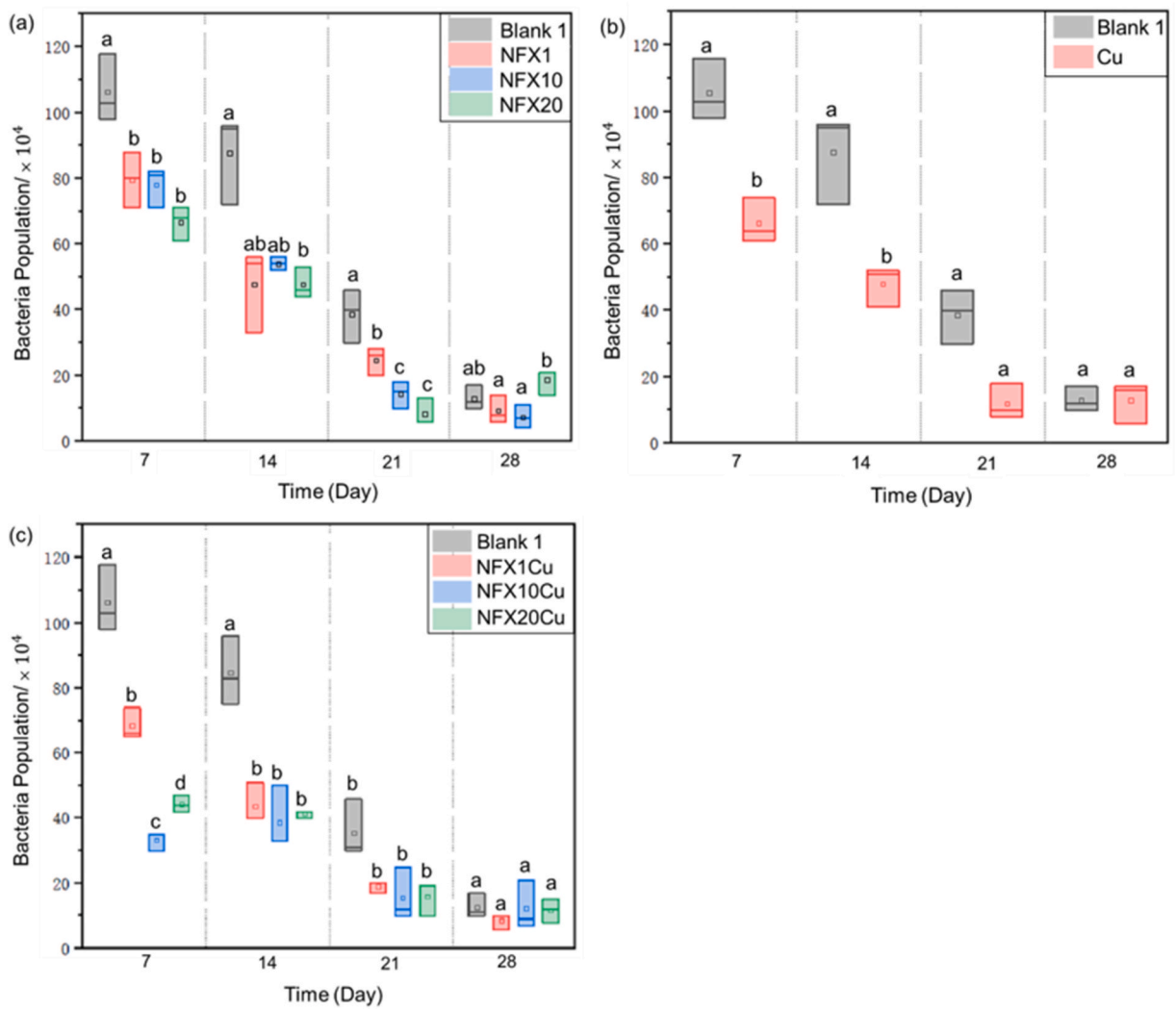

Fig. 1. Bacterial populations in the control group (Blank 1) compared with treated groups exposed to (a) NFX at 1 μg g^{-1}, 10 μg g^{-1} and 20 μg g^{-1}, denoted as NFX1, NFX10 and NFX20, respectively; (b) 40 μg g^{-1} Cu, denoted as Cu; and (c) combinations of 40 μg g^{-1} Cu and 1 μg g^{-1}, 10 μg g^{-1} and 20 μg g^{-1} NFX, denoted as NFX1Cu, NFX10Cu and NFX20Cu, respectively. Different letters above columns denote significant differences ($p < 0.05$) between treatments.

two. Additionally, differences in microbial effects of sulfadimidine and NFX or differences in microbial composition of the soil microcosm in (Wang et al., 2019) and the water-estuarine sediment microcosm in our study could probably account for the differences. Previous studies demonstrated that Cu is not biodegradable and is persistent in estuarine sediments. Cu bioaccumulates in benthic microorganisms over time, resulting in toxicity to microorganisms and disruption of their enzymatic function (L. Wang et al., 2021). On the other hand, NFX can exert adverse effects on microbial community composition, structure, and function by inhibiting bacterial growth, functional diversity and enzymatic activities and altering the biogeochemical cycling of carbon, nitrogen and metals (Chen et al., 2021; F. Wang et al., 2021). Overall, our results suggest that the effect of the interactions of Cu and NFX is time- and antibiotic concentration-dependent.

3.2. Microbial community effects of norfloxacin and copper

3.2.1. Microbial diversities

The effect of metal and antibiotic co-exposure on microbial communities in estuarine sediments over time was determined on the 1st and 28th days using the 16S rRNA gene approach. All treatments (Day 28) and the control (Day 1 and Day 28) had more than 35,000 reads of microbial sequences, and the reads had operational taxonomic units (OTUs) of 1758–1,8717 (Table S5). The ACE, Chao 1, and Shannon diversity indices were stable at approximately 1820 and 1826 OTUs, respectively, and $H' = 9.19$ throughout the incubation time. There was no evidence suggesting that exposure to individual contaminants or their combination significantly shifted microbial diversity, except for the combination of NFX20 and Cu and single NFX20, which had slightly lower alpha diversities than the controls or other treatments (Fig. 3). In contrast, exposure to tetrabromobisphenol A and Cu in river sediments decreased the alpha diversity of bacteria, archaea and fungi following a 60-day incubation (L. Wang et al., 2021). Exposure of microorganisms to single and combined tetracycline and Cu had similar effects on microbial richness (Z. Wang et al., 2018). Overall, NFX and Cu slightly altered the diversity and richness of microbial communities after 28 days of incubation. The plots of principal coordinate analysis (PCoA) showed that samples in most groups were clustered (red circle), except the original group (Blank 1) and the NFX20Cu group (blue circles). The differences between Blank 1 and Blank 2 indicated that time (28 days) influenced the bacterial communities, which is in line with the results reported in Section 3.1. The distance between NFX20Cu and others suggested that the combined treatment of Cu and high concentration NFX was also effective on the bacterial communities (Fig. 4). Therefore, further analysis on microbial community composition was analyzed.

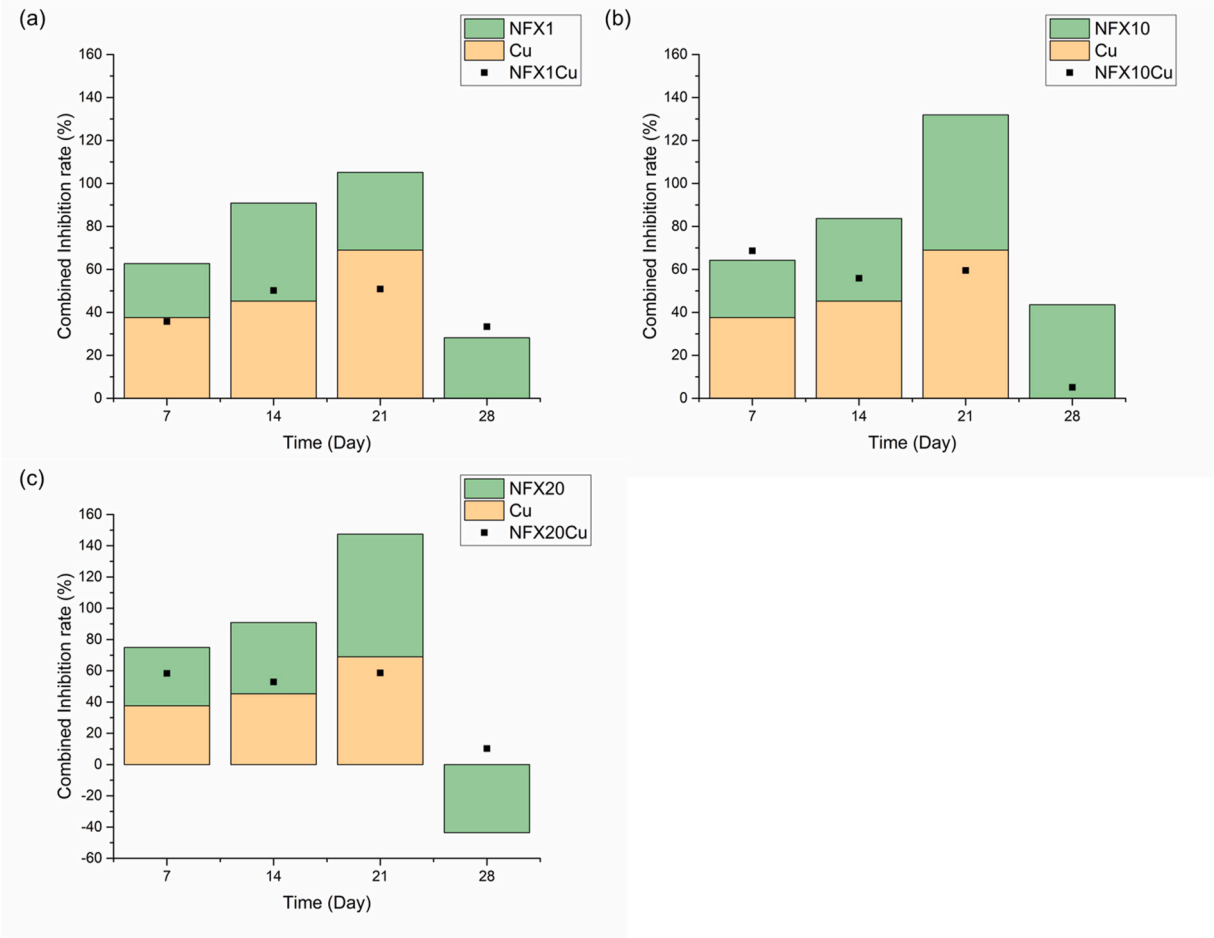

Fig. 2. Inhibition rates to bacteria in sediments treated with 40 μg g^{-1} Cu (orange bar as Cu only) and 1 μg g^{-1}, 10 μg g^{-1} and 20 μg g^{-1} NFX (green bar as NFX only, denoted NFX1, NFX10 and NFX20, respectively) and their combination (black dot, denoted NFX1Cu, NFX10Cu and NFX20Cu, respectively). The stack of orange bar and green bar represents the sum of respective inhibition rate of Cu and NFX. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2.2. Microbial community composition

All OTUs at the phylum level were classified and designated 37 phyla. The top 12 phyla (mean relative abundance >1%) covered over 95.5% of the total amplicons (Fig. 5). *Proteobacteria* was the predominant phylum only in the original sample (31.5% in Blank 1), but *Chloroflexi* took over place in other samples (32.1%–37.6%). An increase in *Chloroflexi* was also observed in sludge exposed to NFX (Zhao et al., 2019). Acidobacteria accounted for a mean proportion of 6.3%, followed by *Actinobacteria* (6.1%), *Bacteroidetes* (5.8%), *Cyanobacteria* (3.1%), *Planctomycetes* (2.8%), *Nitrospirae* (2.5%), *Gemmatimonadetes* (1.9%), *Verrucomicrobia* (1.7%), *Latescibacteria* (1.0%), and *Firmicutes* (1.0%). Likewise, *Chloroflexi, Proteobacteria, Verrucomicrobia, Actinobacteria, Bacteroidetes, Firmicutes, Planctomycetes*, and *Cyanobacteria* were dominant phyla in an aerobic granular sludge membrane bioreactor for the degradation of five pharmaceuticals (norfloxacin, sulfamethoxazole, ibuprofen, naproxen and prednisolone) after 40 days (Wang et al., 2016). *Acidobacteria* have been commonly identified in contaminated estuaries, tidal flats and river sediments (Lu et al., 2017). *Proteobacteria* and *Bacteroidetes* have also been found to be dominant phyla in aquacultural sediments where fluoroquinolones were used (Xiong et al., 2015). Apart from these phyla, *Actinobacteria* and *Chloroflexi* have also been detected as dominant in river sediments polluted with tetrabromobisphenol A and copper. *Proteobacteria*, *Bacteroidetes*, *Acidobacteria* and *Verrucomicrobia* are known for the degradation of intractable organic matter, and their presence results in a decrease in tetrabromobisphenol A in river sediments (L. Wang et al., 2021). Additionally, *Proteobacteria*, *Bacteroidetes*, *Acidobacteria*, *Actinobacteria* and *Nitrospirae* were the most abundant phyla in heavy metal-contaminated environments, such as copper mine tailings containing high concentrations of Cu (Jiang et al., 2021). By comparing Blank 1 and Blank 2, 3 phyla (*Chloroflexi*, *Elusimicrobia* and *Patescibacteria*) were positively related to time (28 days, $r > 0.3$, $p < 0.05$), while 10 phyla were negatively correlated ($r < -0.4$, $p < 0.05$), as *Nitrospirae*, *Gemmatimonadetes*, *Verrucomicrobia*, *Cyanobacteria*, *Epsilonbacteraeota*, *Nitrospinae*, *Bacteroidetes*, *Calditrichaeota*, *Proteobacteria* and *Spirochaetes*. These changes were consistent as indicated in PCoA results that the 28-day period significantly altered the microbial community structure. *Actinobacteria*, *Firmicutes*, *Deinococcus-Thermus* and *Patescibacteria* presented significant positive correlations ($r > 0.54$, $p < 0.01$) with NFX. *Firmicutes* is also known for the degradation of intractable organic matter and may participate in utilizing NFX as a nutrient source; in addition, the phylum *Firmicutes* also hosts plasmid-mediated quinolone resistance genes (Tuo et al., 2018). *Actinobacteria* were related to NFX and Cu ($r > 0.6$, $p < 0.001$). Members of *Actinobacteria* have been found to possess resistance genes to fluoroquinolones such as ciprofloxacin and heavy metals such as Cu (Gallo et al., 2019). In Sava River sediments polluted with macrolide antibiotics and heavy metals, *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Acidobacteria* were the dominant groups, while *Firmicutes* remarkably increased in relative abundance at the heavily contaminated site (Milaković et al., 2019). In the present study, 12 10 phyla were negatively correlated with NFX ($r < -0.54$, $p < 0.05$), including *Gemmatimonadetes*, *Proteobacteria*, *Rokubacteria*,

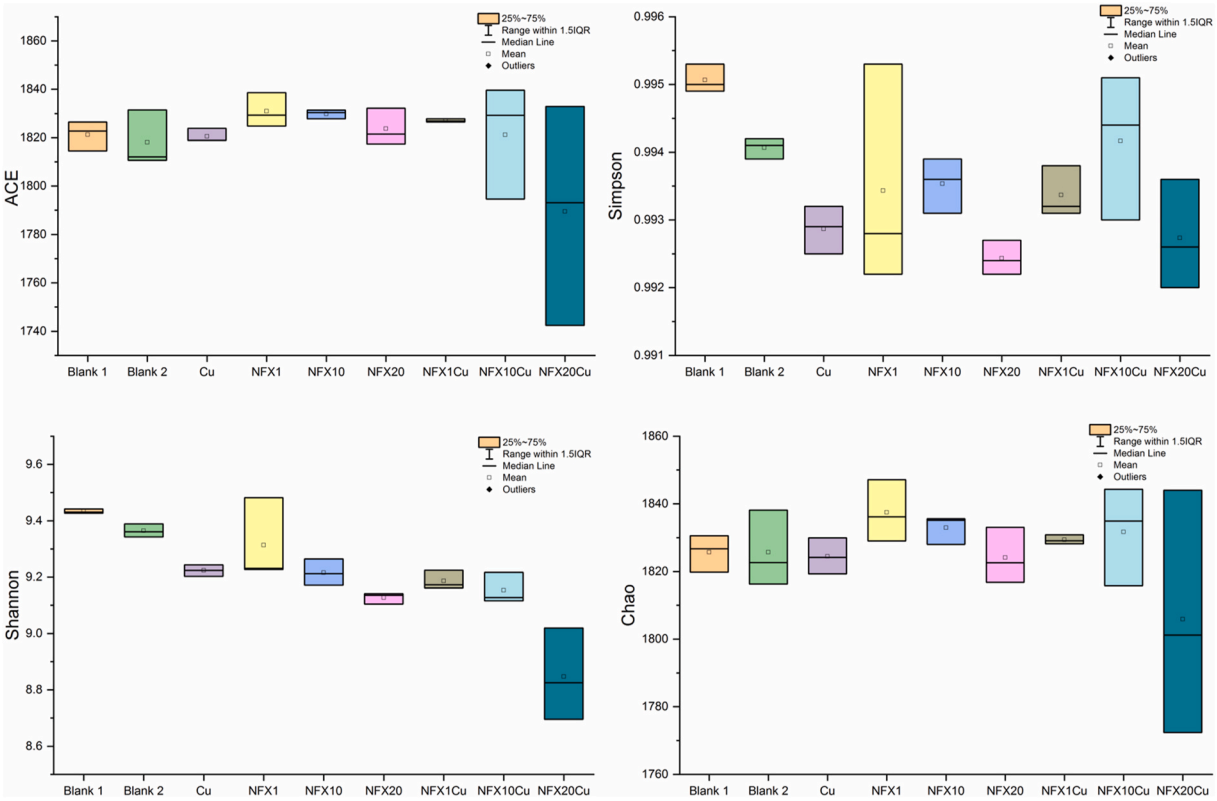

Fig. 3. The α-diversity indices, ACE (a), Simpson (b), Shannon (c) and Chao (d), of the microbial community under different treatments, notations as described in Table S4.

Calditrichaeota, *Planctomycetes*, *Spirochaetes*, *Nitrospirae*, *Zixibacteria*, *BRC1* (*Bacterial Rice Cluster 1*), *Latescibacteria*, and *Nitrospinae* and *Cyanobacteria*, especially *Nitrospirae*, *Zixibacteria*, and *Latescibacteria,* with $r < -0.7$ and $p < 0.0001$. Seven phyla presented negative correlations with Cu contamination ($r < -0.3$ 4 and $p < 0.05$), such as *Spirochaetesuncultured bacterium-k-bacteria*, *Nitrospinae*, *BRC1*, *Elusimicrobia*, *Latescibacteria*, *Nitrospinae*, *WS2*, *uncultured bacterium-k-bacteria* and *Bacteroidetes*, and *Lentisphaerae*, especially *BRC1*, *Nitrospinae* and *uncultured bacterium-k-bacteria* ($r < -0.6$ and $p < 0.001$). Negative correlations between Cu and *Bacteroidetes* were also reported in marine sediment highly polluted with heavy metals (Gillan et al., 2005). Results indicated that selections of tolerant and resistant microcosms to NFX and/or Cu occurred in 28 days.

The FAPROTAX database predicted the top 30 biologically related functions of the microbial community (Fig. 6). The 28-day period (Blank 2 *vs.* Blank 1) largely inhibited aerobic chemoheterotrophy and oxygenic photoautotrophy, while improved respiration of sulfur compounds, sulfate respiration, methanol oxidation and methylotrophy. A comparison between the control (Blank 2) and Cu in functions using the FAPROTAX database was insignificant ($p \geq 0.05$) (Fig. S5), which is in accordance with the inhibition rate of Cu at day 28 (Fig. 2). The sole contamination of high concentration Cu or NFX compared to the control group (Blank 2) showed major hindrance to nitrification, aerobic chemoheterotrophy, methanol oxidation, aerobic nitrite oxidation, methylotrophy, nitrification, and aerobic ammonia oxidation, mainly as well as minor hindrance to aromatic compound degradation, nitrate reduction, oxygenic photoautotrophy, and human pathogens while promoting respiration of sulfur compounds. These results aligned with the changes of the microbial community, as genera *Nitrospira* and *uncultured-bacterium-c-Thermodesulfovibrionia uncultured-bacterium-p-Latescibacteria* and *uncultured-bacterium-f-Rhodobacteraceae* involved in nitrification with the functions of aerobic chemoheterotrophy and aromatic compound degradation were negatively correlated with Cu and NFX. Similarly, the genera *Nitrospira* and *uncultured bacterium c-Thermodesulfovibrionia* are involved in nitrate reduction, while the genus *Nitrospira uncultured bacterium f-Rhodobacteraceae* also participates in aerobic ammonia oxidation, while genus *uncultured-bacterium-c-Thermodesulfovibrionia* related to sulfate reduction of aromatic compound degradation (Queiroz et al., 2020). As a result of Cu and NFX treatments, the decrease in their relative abundance led to the inhibition or promotion of the corresponding functions. Compared to the control (Blank 2), the combination of NFX and Cu showed some similar impacts to that of NFX, with additional obstructions in anoxygenic photoautotrophy, predatory or exoparasitic, nitrogen and nitrate respirations, and promotion in aerobic chemoheterotrophy (Fig. S5). The comparisons of combined contamination with NFX or Cu also indicated that the influence of NFX on the sediment microbial community was more severe than that of Cu, as the differences between NFX and the combination were smaller than those between Cu and their combinations the combined pollution further obstructed the nitrification and aerobic nitrite oxidation. This also implied that NFX might have an antagonistic effect on the inhibition of Cu. Likewise, among the combinations of Cu and various concentrations of NFX, NFX20Cu affected the most, which coincides with the results of α-diversity indices and of PCoA results.

Besides, the 28-day period (Blank 2 *vs.* Blank 1) changed some microbial community functions ($p < 0.05$), as in Fig. S5. Although the 28-day period presented more impactful reductions than NFX and/or Cu did in the bacterial population (Fig. 1), the microbial community structure and its corresponding functions indicated that contaminants of NFX and Cu exhibited significant alterations (Fig. S5). For instance, the 28-day period increased the microbial community functions of methylotrophy, and methanol oxidation and predatory or exoparasitic; however, NFX alone or with Cu displayed adverse effects to methylotrophy. On the other hand, the 28-day period promoted the functions of sulfate respiration, whilst NFX20, NFX1Cu alone or with and NFX10Cu further developed stimulated this function positively. Furthermore, NFX alone

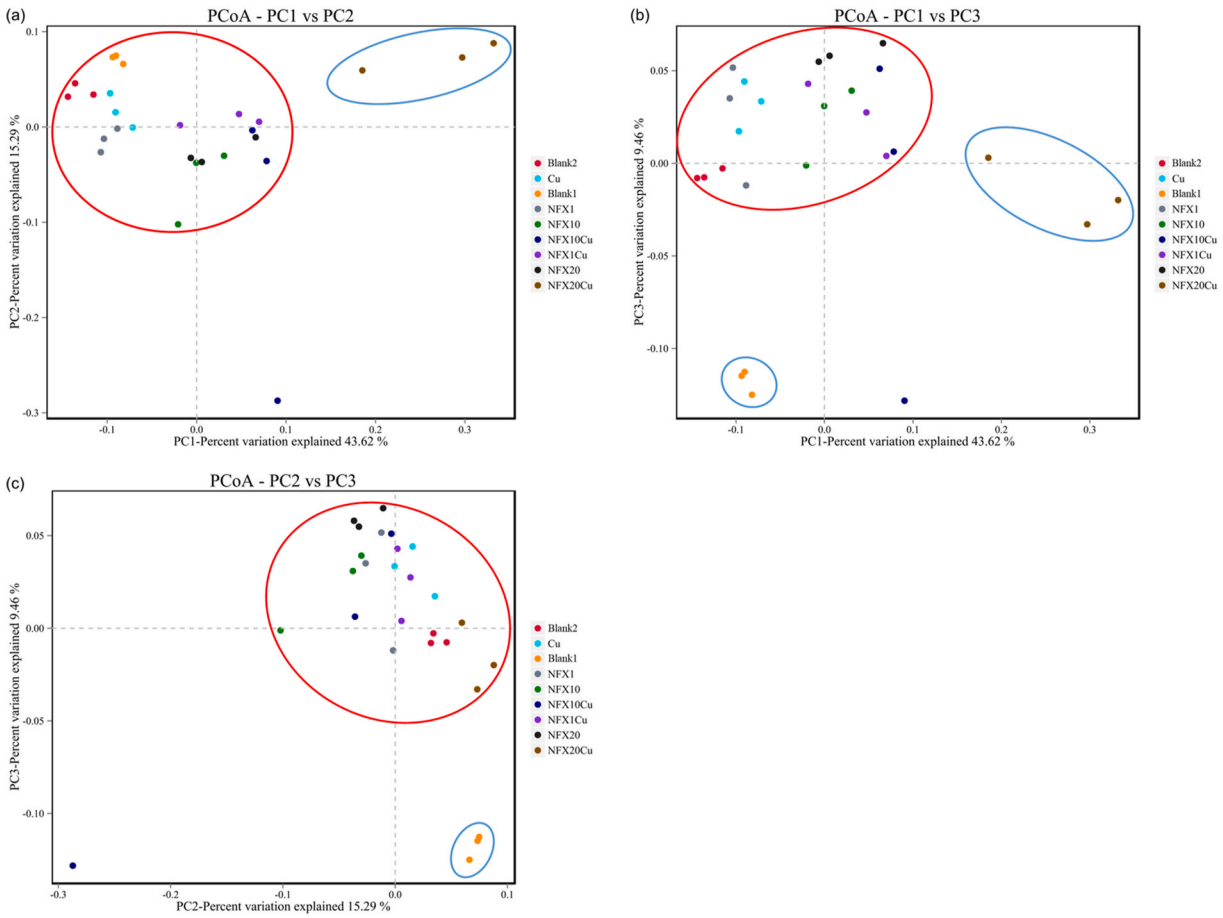

Fig. 4. PCoA plots of sediment samples with different treatments, notations as described in Table S4.

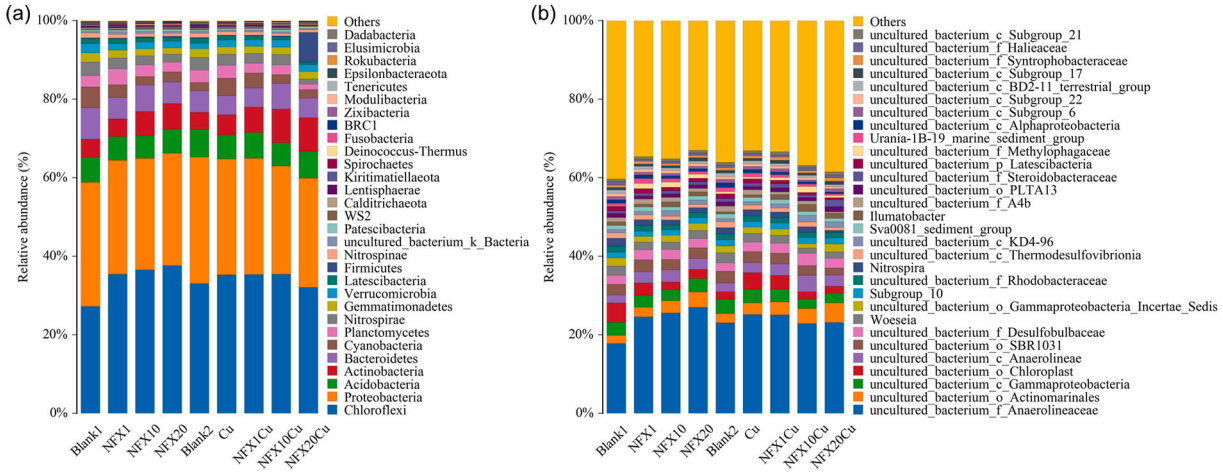

Fig. 5. Taxonomic composition distribution histograms of sediment sample groups with different treatments at the phylum (a) and genus (b) levels, notations as described in Table S4.

or with Cu performed additional inhibition in functions compared to the 28-day period.

3.3. Environmental relevance

The detected NFX concentration in the original sample was 17.69 ng g^{-1}, much higher than the reported results in aquaculture farms in the Jiulong River estuary in Fujian, China (0.7–9.9 ng g^{-1}) (Xi et al., 2015) but lower than that reported in the Beibu Gulf (Zhang et al., 2018). The Cu concentration identified in the collected sediment was 18.4 μg g^{-1}, which shows that the Cu concentration in the estuarine sediments has not changed in the past three years (Zhuang et al., 2019). With an increase in anthropogenic activities along the coastline, Cu and NFX concentrations are expected to increase over the next decade. Our study showed that increasing the Cu concentration from 18.4 μg g^{-1} to 40 μg g^{-1} could inhibit the sediment bacterial population, which may alter the biogeochemical processes that take place in the estuary. The α-diversity indices of OTU results suggested that either Cu or low/mid

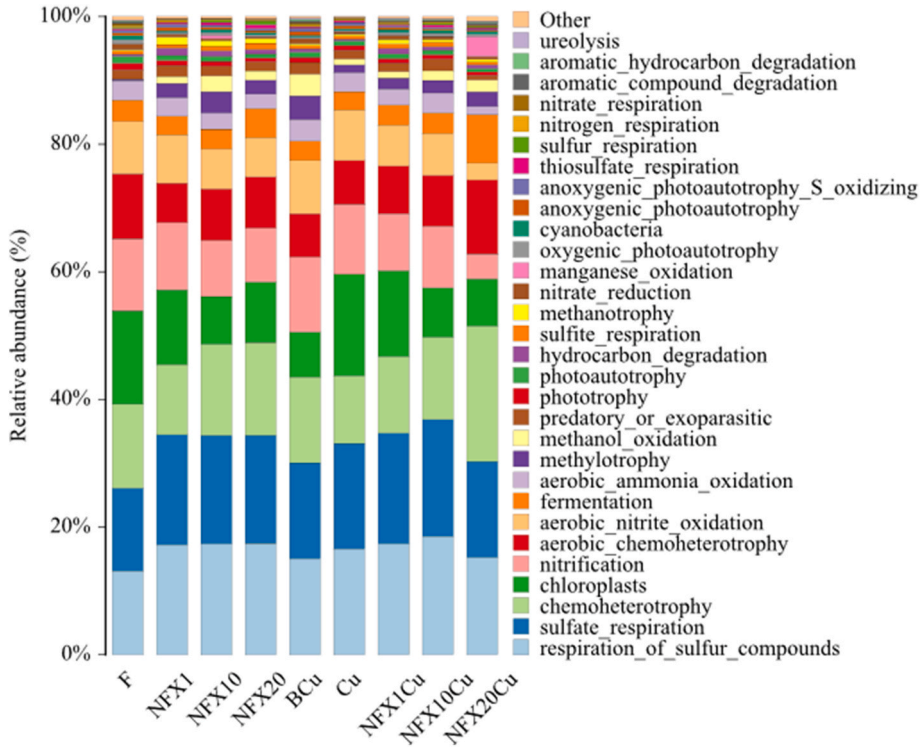

Fig. 6. Functional prediction of sediment samples with different treatments by FAPROTAX.

concentration of NFX showed insignificant disturbance of the diversity and evenness of the sediment microbial community; however, the combined pollution of Cu and high concentration displaying a notable decrease, indicating that this combination had negative effects on the structure of sediment microbial community. The α-diversity indices of the soil bacterial community in a constructed wetland showed significant negative correlations with the concentrations of difloxacin and Cu after 30 days (Huang et al., 2017); the correlation in this study is not as apparent as that reported in the constructed wetland sediments, probably because the bacterial communities in the estuary aquacultural sediment have been more adapted tolerated to diverse metals, antibiotics, and other emerging organic pollutants and rapid environmental changes as they inhabit an ecotone system. The pollution of NFX was much more obvious in affecting the microbial community than that of Cu, probably because the Cu concentration did not vary as much as NFX. Cu and NFX presented mostly antagonistic inhibitory effects; however, high concentrations of NFX promoted inhibition when combined with Cu, although NFX alone at high concentrations improved the bacterial population. These results indicated that high concentrations of NFX stimulated adaptation tolerance and resistance of the microbial community.

4. Conclusion

The contamination of NFX or Cu alone inhibited the bacterial population at an early stage, which became weak at 28 days; however, the 28-day period was more impactful in decreasing bacterial population than the contaminants. The combined pollution of NFX and Cu presented mainly antagonistic effects at the early stage but synergistic at day 28 at low or high NFX concentrations. NFX at high concentrations combined with Cu was the most effective to the microbial community and provoked adaptation selection in resistant and tolerant bacteria of the microbial community at 28 days. Although the microbial community showed some shifts to the contamination of NFX and Cu, the functions were relatively stable, with promotion of respiration of sulfur compounds and sulfate respiration as well as the suppression of aerobic chemoheterotrophy. The effect of time and contaminants exhibited some significant differences on the microbial community structure and functions, especially the combination of Cu and high concentration NFX, including aerobic chemoheterotrophy, methanol oxidation and methylotrophy.

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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2022.113506.

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