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Fate, toxicity and effect of triclocarban on the microbial community in wastewater treatment systems

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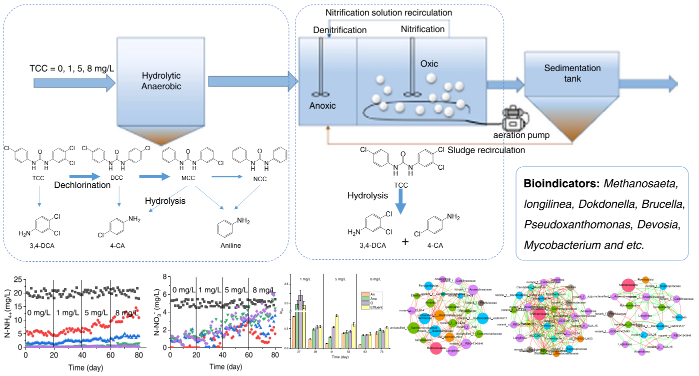
HIGHLIGHTS

- TCC dechlorination and hydrolysis dominated in the HA and A/O sections respectively.
- The toxicity of TCC was partially reduced after treatment, which has an IC_{50} value from 0.09 to 0.54.
- TCC decreased nitrifiers and denitrifiers abundances in activated sludge communities.
- *Methanosaeta*, *Mycobacterium* and other 12 genera are potential TCC bioindicators.

GRAPHICAL ABSTRACT



Toxic effect on N-removal and *Vibrio fischeri*

Reconstruction of the microbial networks

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ABSTRACT

Triclocarban (TCC), one of the typical antimicrobial agents, is a contaminant of emerging concern commonly found in high concentration in water environments. However, the fate and toxicity of TCC in wastewater treatment systems remain poorly understood. Here, we investigated how TCC impacts chemical oxygen demand and inorganic nitrogen transformation in a hydrolytic anaerobic-anoxic/oxic process. In the anaerobic section, the transformation of TCC was dominated by reductive dechlorination and supplemented by two amid bonds hydrolysis. In the anoxic and oxic sections, the hydrolysis of amid bonds dominated. The toxicity was reduced after the treatment (IC_{50} from 0.09 to 0.54). TCC inhibited NH_4^+-N removal in the anaerobic section and led to the NO_3^--N accumulation (2.84–4.13 mg/L) after treatment, with the abundance of N-removal bacteria decreased by

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6%. Furthermore, the original ecological niche was gradually replaced by TCC-resistant/degradative bacteria, formating new microbial modules to resist the TCC stress. Importantly, fourteen genera including *Methanosaeta*, *Longilinea*, *Dokdonella* and *Mycobacterium* as potential bioindicators warning TCC and its intermediates were proposed. Overall, this study provides new insights into the fate of TCC in biological wastewater treatment systems and suggests a great importance for TCC control to ensure the health and resilience of ecosystems.

1. Introduction

Triclocarban (3,4,4′-trichlorocarbanilide, TCC) is one of the most commonly used antimicrobial agents in daily life and is added to various personal care products and textile products (Halden et al., 2005). The antimicrobial activity of TCC mainly stems from its capacity to cause bacterial deformation and hinder microbial metabolism by inhibiting the biosynthesis of methyltransferase (MenG), making it particularly effective against many Gram-positive bacteria (Macsics et al., 2020). Despite of the potential as antibacterial agent, the excessive consumption of TCC causes its spread in the environment and, in turn, threatens both environmental and human health (Halden and Paull, 2005; Iyer et al., 2018; Yun et al., 2020). TCC and its intermediate products (chloroanilines and dehalogenated congeners) can accumulate in aquatic species and pose unprecedented selective pressures, impacting the metabolism and reproduction of microbes and aquatic organisms (Yun et al., 2020). Indeed, TCC may eventually accumulate in humans via the food chain, and the ultimate, well-known consequences are disrupting endocrine hormones (e.g. thyroid hormones), liver and kidney function, and possibly causing prenatal abnormalities (Halden and Paull, 2005; Iyer et al., 2018; Wang et al., 2022a; Wei et al., 2021). Furthermore, the coronaviruse disease 2019 (COVID-19) pandemic greatly impacts the healthcare aspect of many countries, and the indiscriminate usage of healthcare disinfectants in the aftermath of the pandemic may exacerbate the accumulation of antimicrobial agents including TCC in the environment (Chen et al., 2021).

TCC is a polychlorinated substituted contaminant with high lipophilicity and a half-life of 70–340 days in the environment (Lozano et al., 2010). Wastewater treatment systems, in particular municipal, are the main gateway for TCC to enter the environment due to improper handling (Heidler et al., 2006). Although conventional activated sludge process typically achieves 75–98% removal of TCC, the bioconversion rate is only about 21%, which makes the remaining TCC be either disseminated into the environment via effluents or adsorbed to the residual sludge. While some exceptions, the concentrations of TCC in sludge in the United States and China were 0.187–441 mg/kg and 3–43.3 mg/kg (in dry weight), respectively, leaving the intermediate products largely unknown (Yun et al., 2020).

Hydrolytic anaerobic-anoxic/oxic (HA-A/O) process, as a commonly used wastewater treatment process, can achieve detoxification, decarbonization and removal of nitrogen and phosphorus. Exposure to TCC in the range of 0–600 ± 34.3 mg/kg TSS was toxic to the anaerobic digestion system and had a significant impact on the sludge settling qualities(Yun et al., 2017; Yun et al., 2020). The toxicity of TCC had a serious influence on ammonia oxidation in the SBR reactor, denitrification processes, phosphorus release and assimilation, resulting in deteriorated biological treatment system performance (Bian et al., 2020; Wang et al., 2021b, 2022b), implicating, but not proving, that TCC may impact HA-A/O processes. In principle, TCC can undergo biotransformation processes in the environment, yet less likely being mineralized, and two main TCC metabolic pathways are reductive dichlorination and hydrolysis of two amide bonds. The reductive dichlorination produces 4, 4′-dichlorocarbanilide (DCC), 1-(3-Chlorophenyl)– 3-phenylurea (MCC), and N,N′-Diphenylurea (NCC), whereas the hydrolysis of two amide bonds generates 3,4-dichloroaniline (3,4-DCA) and 4-chloroaniline (4-CA) (Yun et al., 2017, 2020). Although being identified, which metabolic pathway of TCC dominates in wastewater biotreatment processes (e.g., HA-A/O) remains unclear. Furthermore, the toxic effect of TCC and its transformation intermediate products on the biological processes as well as the corresponding bioindicators have not been well investigated. To fill the knowledge gaps, this study aims to (i) determine the impact of TCC on the performance of a HA-A/O system, (ii) clarify the migration and transformation pathways of TCC in different sections of the system, and (iii) uncover the response of composition, structure, function, and network interaction of sludge microbial communities to different concentrations of TCC. Our findings provide new insights into the fate and toxic effect of TCC in wastewater biotreatment systems, which is fundamental to the understanding of the water ecological risks of TCC. In the context of TCC omnipresence, our study can benefit the management of TCC propagation by revealing the potential bioindicators.

2. Materials and methods

2.1. Standards and reagents

TCC, DCC, NCC, 3,4-DCA, 4-CA, and aniline (all $\geq$ 97%) were obtained from Aladdin (Shanghai, China). HPLC grade methanol and acetonitrile (>99.9% purity), and MCC (>97% purity) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals with over 99.9% purity were purchased from Tianjin Kemiou Chemical Reagent Co. Ltd., China.

2.2. Experimental device and procedure

A HA-A/O reactor (Fig. 1a), made of polymethyl methacrylate with a working volume of 5.0 L, was employed. The volumetric ratio of anaerobic (AN), anoxic (ANO) and oxic (O) sections is 6:1:3, and the secondary sedimentation tank was vertical type flow cell with a working volume of 0.95 L. The raw sludge was collected from the secondary sedimentation tank of Taiping Wastewater Treatment Plant (Harbin, China) and inoculated into the HA-A/O system. Agitators were installed in the anoxic and oxide zones, and aeration tubes were installed in the oxide zone to ensure that the dissolved oxygen was 3–5 mg/L and that the activated sludge was evenly distributed. The mixed liquor suspended solids (MLSS) concentration in anaerobic, anoxic and oxic section is 4300 mg/L, 3000 mg/L and 3000 mg/L, respectively. The mixed liquor volatile suspended solids (MLVSS) concentration in anaerobic, anoxic and oxic section is 2735 mg/L, 2011 mg/L and 2150 mg/L, respectively. The hydraulic retention time (HRT) of the whole reactor is 24 h, and the sludge retention time (SRT) for each sector is 15 d. The dissolved oxygen concentration of each section was $<$ 0.2 mg/L, 0.2–0.5 mg/L and 2–4 mg/L, respectively. The HA-A/O reactor was fed with a synthetic wastewater (Tabel S1) representative of domestic wastewater in China proposed elsewhere (Peng et al., 2010), ensuring no background antimicrobial agents occurrence.

Chemical oxygen demand (COD), NH_4^+-N, NO_2^--N, and N-NO_3^- were measured once every two days in each section and in the effluent of the HA-A/O reactor, and the removal efficiency of COD and NH_4^+-N was calculated. After steady performance was reached, defined as consistently low effluent COD ($<$ 60 mg/L) and NH_4^+-N ($<$ 0.3 mg/L), TCC was added to the influent in sequential phases at incremental concentrations (no TCC for 0–20 d, 1 mg/L for 21–40 d, 5 mg/L for 41–60 d, and 8 mg/L for 61–80 d) to investigate the impact of TCC on the entire system and each section. On days 23, 24, 27, 42, 43, 45, 64, 65, and 66 of the bioreactor operation, the sludges were sampled and frozen at

− 80 ℃ for 16S rRNA gene sequencing and chemical analysis of TCC and its diverse intermediates.

2.3. Analytical methods

In a previous study, roughly 97 ± 1% of the influent TCC was eliminated from the aqueous phase of an actual WWTP wastewater treatment process on the east coast of the United States (Heidler et al., 2006). This suggested that TCC enters the treatment system to be eliminated mostly via adsorption and biodegradation, therefore it is particularly important to study its content and pathways in the sludge. Freeze-dry of the sludge samples was conducted prior to weighing, based on a well-established procedure (Halden and Paull, 2004). Oasis ® HLB columns (Waters Corporation, Milford, MA, USA) were used to purify the extracted samples. Then, the analytes were desorbed from the HLB column with 15 mL of a 1:1 v/v methanol: acetone solution containing 10 mM acetic acid (Pycke et al., 2014). The solvent in the extracts was then blown dry with a nitrogen blower and redissolved in methanol. Next, the contents of TCC and its intermediates were analyzed by LC-MS/MS (Waters Co., Milford, MA, USA) with a reverse phase C18 column (Waters Co., Milford, MA, USA) in gradient elution mode at a flow rate of 0.15 mL/min. The electrospray ionization source was operated in positive mode with the following parameters: desolvation temperature of 350 °C, gas flow of 850 L/h, and capillary voltage of 3 kV. Both the nebulizer and the collision gas consisted of nitrogen (details shown in Table S3). The LC−MS/MS spectrum of the precursor ion and the daughter ion was available in Fig. S1. Wastewater samples were filtered through a 0.45 μm membrane followed by COD and NH$_4^+$-N determination with Hach Reagentkits (TNT821 for COD, 21194419-CN for NH$_4^+$-N; Hach, CO, USA) and Hach DR3900 Spectrophotometer. NO$_2^-$-N and NO$_3^-$-N were analyzed using a Wayeal IC-6200 ion chromatography (Wayeal Corporation, China), with an Ion Pac AS18 anion column (Dionex Corporation, Sunnyvale, CA).

To determine the toxicity of the effluent samples from all three sections and the whole process, *Vibrio fischeri* was used as a model microorganism to investigate the inhibition of its luminescence activity. On days 27, 35, 42, 52, 63 and 73 of reactor operation, water samples were collected from each section and filtered through a 0.45 μm membrane, with three parallel replicates prepared for each treatment. The lyophilized powder and recovery solution, used in the investigation, were purchased from Zhejiang Tocos Biotechnology Co. China. Microtox® Osmoregulation Solution (OAS, 22% NaCl solution) and diluent (2% NaCl solution) were purchased from Modern Waters (USA). The acute toxic impact (IC$_{50}$) of the effluent samples on *Vibrio fischeri* (15-min exposure) was evaluated by a Microtox® spectrophotometer according to the procedures in the Microtox® System Operation Manual. SPSS 2019 was used to establish statistical significance between pairs using a multiple-range test of least significant difference.

2.4. Microbial community analysis

The sludge microbial communities from different reactor sections were collected and centrifuged at 12,000 rpm for 15 min. Polymerase chain reaction (PCR) was used to amplify the bacterial V3–V4 region of the 16 S rRNA gene (primers: 515 F (GTGCCAGCMGCCGCGGTAA) and 806 R (GGACTACHVGGGTWTCTAAT)); afterward, Illumina sequencing was carried out through Majorbio Bio-Pharm Technology Co., Lt. (Shanghai, China). Majorbio Cloud Platform (www.majorbio.com) was employed for the microbial community analysis, mainly including alpha diversity, taxonomy, Principal coordinate analysis (PCoA), association analysis of environmental factors (RDA), FAPROTAX and microbial network analyses (He et al., 2022a; Qi et al., 2021; Wang et al., 2021a).

3. Results and discussion

3.1. Performance of the HA-A/O process under TCC stress

TCC could reduce the removal of NH$_4^+$-N and lead to the accumulation of NO$_3^-$-N; however, TCC did not affect the removal of COD (Fig. 1). The influent COD fluctuated in the range of 577–648 mg/L, and different TCC influent concentrations exerted a negligible influence on the removal of COD. Most of the COD was removed in the AN section (89.53–90.59%), while only a small part of the COD was consumed in the ANO and O sections. At the influent TCC concentration of 0, 1, 5, and 8 mg/L, the total COD removal efficiencies were 95.30%, 95.23%, 94.87%, and 94.61%, respectively (Fig. 1b). The final COD

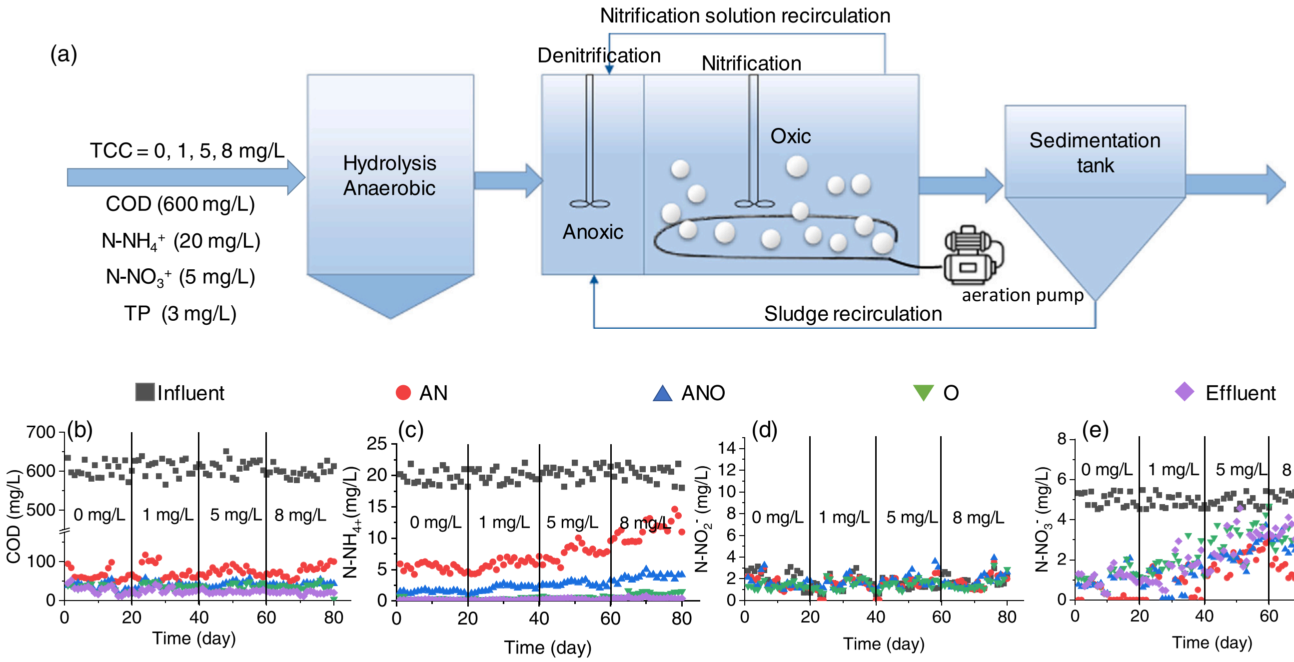

Fig. 1. Schematic diagram of the lab-scale Hydrolytic anaerobic-anoxic/oxic (HA-A/O) process (a). The effects of TCC on pollutant COD (b), NH$_4^+$-N (c), NO$_2^-$-N (d) and NO$_3^-$-N (e) concentration in the influent, anaerobic (AN), anoxic (ANO), oxic (O) and effluent.

concentration was in the range of 10.7–51.1 mg/L, satisfying the national first-class emission standard in China (known as GB 18918–2002). A previous study, however, found that the effluent COD concentration increased with an increase in the initial TCC concentrations in an anaerobic digestion of residual sludge (Wang et al., 2017). The COD removal was not affected by TCC probably for two reasons: the low complexity of the carbon source in the synthetic wastewater (sodium acetate and acetone) and the long HRT (24 h).

A low concentration of TCC at 0.1 mg/L inhibited the denitrification capacity of nitrification and denitrification systems, and 1 mg/L TCC reduced the activity of ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) by 27.8% and 74.7%, respectively (Bian et al., 2020; Wang et al., 2021b, 2022b). However, the effect of 1 mg/L TCC on the NH_4^+-N removal efficiency of the whole process was not obvious (97.76–99.34% after the introduction of TCC). As for each section, TCC at 1 mg/L significantly decreased the NH_4^+-N removal efficiency in the AN section (72.52% vs 69.51%, $P < 0.05$); however, the impact on the accumulation of NO_3^--N in the ANO and O sections was not obvious (Fig. 1 BCE), which may be attributed to the preferential adsorption and biotransformation of TCC in the AN section. When the influent TCC increased to 5 mg/L and 8 mg/L, the NH_4^+-N removal efficiency in the AN section decreased significantly ($p < 0.05$) to 61.12% and 44.95%, respectively. Therefore, the concentration of NO_2^--N in the effluent changed to 1.55 mg/L and 1.65 mg/L; correspondingly, the accumulation of NO_3^--N markedly increased, causing the concentration in the effluent to be 2.84 mg/L and 4.13 mg/L ($p < 0.05$), respectively (Fig. 1bc. The inhibited removal of NH_4^+-N in the AN section and the enhanced accumulation of NO_3^--N in the effluent, taken together, suggested that the inhibition effect of TCC on nitrification and denitrification was much greater than that on COD removal. Consequently, it can be suggested that TCC exerts non-negligible toxicity to microorganisms related to nitrogen metabolism. The impact of TCC on the biological treatment system was significantly reduced when the effluent containing TCC was continuously treated in three units (HA-A/O) as compared to the nitrification, denitrification, or anaerobic digesting processes alone. Specifically, the impacts of TCC on COD, NH_4^+-N, and NO_2^--N in the effluent were eliminated.

3.2. *Transformation pathways of TCC in different sections*

TCC is known to be degraded through two main pathways, namely reductive dechlorination and hydrolysis of two amid bonds. To clarify the degradation and migration patterns of TCC in different sections of the HA-A/O process, its intermediates (dechlorination products: DCC, MCC and NCC; hydrolysis products: 3,4-DCA, 4-CA and aniline) and corresponding concentrations in the sludge were analyzed by LC-MS/MS.

In the AN section, the dechlorination products DCC, MCC and NCC as well as the hydrolysis products 3,4-DCA and 4-CA were all detected, indicating that both hydrolysis and dechlorination of TCC occurred in this unit. Generally, the dehalogenation of TCC requires anaerobic conditions (Kor-Bicakci et al., 2020; Venkatesan et al., 2012). Exemplifying this, the concentration of dechlorination products (DCC and MCC) was obviously higher than that of hydrolysis products (3,4-DCA and 4-CA) (Fig. 2a), indicating that reductive dechlorination was the main degradation pathway of TCC in the AN section. Furthermore, once exposed to higher concentration of TCC, the rate limiting step of the dechlorination process would change. Specifically, the degradation of DCC was the rate limiting step of the process at 1 mg/L TCC, given that DCC was the main intermediate product. When the influent TCC concentration of 5 and 8 mg/L in the sludge, the concentration of hydrolysis products 3,4-DCA and 4-CA reduced from 42.73 ± 21.00 to 38.14 ± 15.75 mg/kg and 3.79 ± 6.42 to 2.16 ± 2.18 mg/kg, respectively. Unlike previous studies (the main dechlorination product is generally DCC) (Liang et al., 2020; Pycke et al., 2014; Sapkota et al., 2007; Venkatesan et al., 2012), the main dechlorination product in the sludge

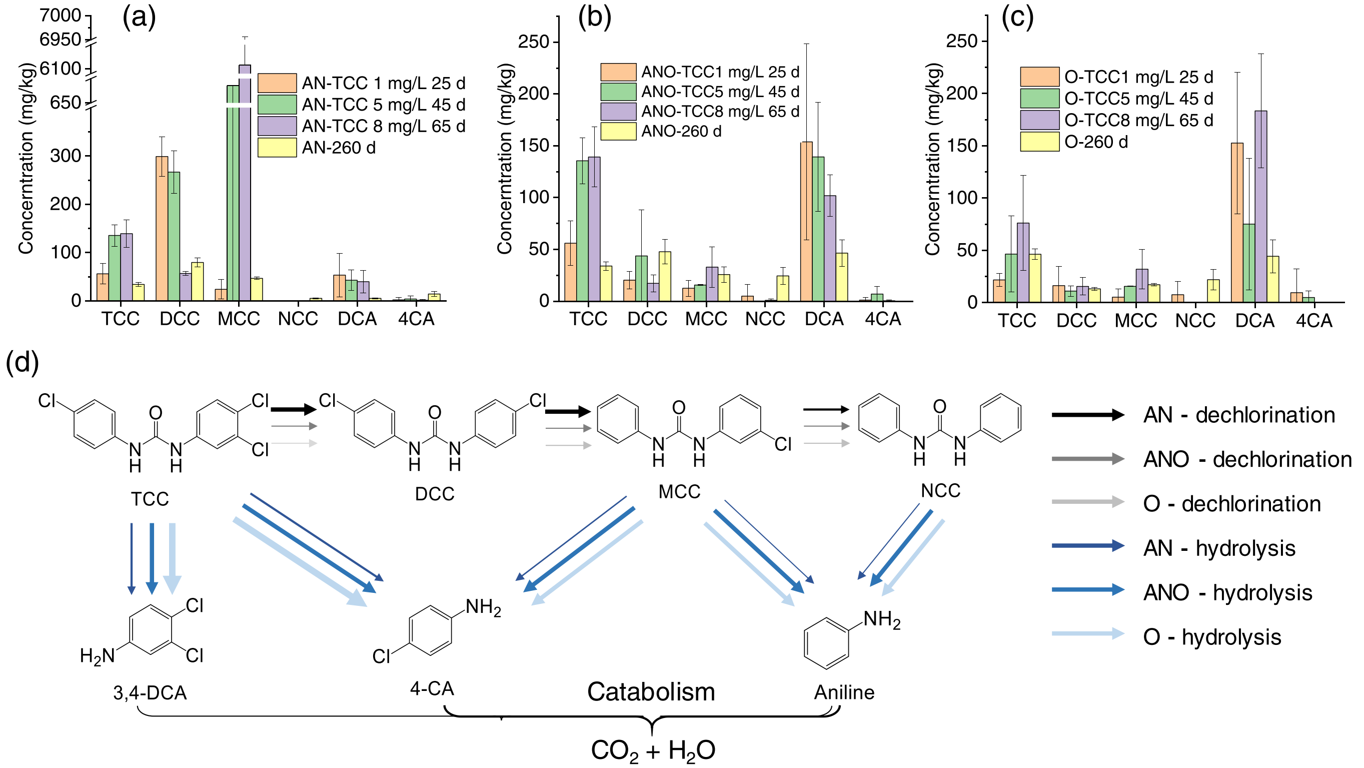

Fig. 2. The concentrations of TCC and its intermidiates products (DCC, MCC, NCC, 3,4-DCA and 4CA) in the AN (a), ANO (b) and O (c) sections in different conditions. The proposed migration and transformation pathways of TCC in different sections (d). The arrow thickness represents the contribution proportion of transformation reactions.

upon 5–8 mg/L TCC influent stress shifted to MCC, and further degradation of MCC became the rate-limiting step. When the concentration of TCC was higher, its hydrolysis products were reduced, indicating that the hydrolysis pathway was weakened by 8 mg/L TCC. Furthermore, a previous study showed that DCC and NCC inhibit the hydrolysis of TCC (Liang et al., 2020). The proportion of dechlorination reaction, as a result, dominated the degradation of TCC in the system. Interestingly, despite the high content of MCC in the sludge (6107.3 ± 848.4 mg/kg), the content of NCC in the anaerobic section was still at a low level (3.06 ± 8.11 mg/kg); therefore, we speculated that the dechlorination of MCC to NCC is a slow reaction (Fig. 2 CE).

In the ANO and O sections, the hydrolysis served as the main degradation pathway of TCC (Fig. 2 BCE). The content of TCC was less in the O section than in the ANO section, which indicated that aerobic conditions are more favorable for the hydrolysis of TCC. In the ANO section, the TCC content in the sludge increased with the TCC content in the influent, while the 3,4-DCA content in the sludge kept decreasing, probably due to the enrichment of microorganisms capable of participating in the catabolism of 3,4-DCA in the activated sludge. In general, the 3,4-DCA content in the O section showed a decreasing trend first then shifted to an increase during the first 65 days of operation at the TCC stress from 1 to 8 mg/L (Fig. 2c). Regarding the biodegradation performance, the activated sludge community showed improved biodegradation capabilities of TCC and 3,4-DCA in the O section, probably due to the long time domestication of functional microbes (65 days operation). No TCC was added to the influent after the 80th day of operation to investigate the changes of TCC and intermediate products in the sludge in the different sections. On the 260th day, the 4-CA catabolism rate in the AN section was slower than that in the ANO and O sections. This observation, together with the existence of residual 3,4-DCA in the ANO and O sludge (26.2–55.4 mg/kg) implied that the catabolic efficiency of 3,4-DCA is lower than that of 4-CA in the activated sludge. Aniline, the hydrolyzed product of NCC, might have been rapidly degraded as it was not detected during the whole process (Fig. 2). Overall, the dechlorination and hydrolysis reactions, in combination with adsorption occurring in the AN section could conceivably reduce the TCC toxicity to the subsequent sections. The main migration and transformation pathways of TCC in each section of the HA-A/O process are summarized in Fig. 2d.

3.3. Ecotoxicity assessment for different concentration TCC stress

TCC is toxic to aquatic organisms, such as horse mackerel and green algae (Yun et al., 2020), and the toxicity of TCC to the system should consider both water and sludge biota. The toxicity of TCC on aquatic organisms after treatment in different sections also urgently needed to be studied. *Vibrio fischeri* luminescence inhibition test was employed to measure the comprehensive toxicity of the effluent from each section and the whole process. IC_{50}, which is defined as 50% of lethal concentration and inversely proportional to toxicity (Jurado et al., 2012), was determined during the test. The IC_{50} of the effluent from different sections after adding TCC was compared; not surprisingly, the comprehensive toxicity of the effluent diminished as the treatment process progressed, indicating that the HA-A/O process obviously reduced the toxicity caused by TCC (Fig. 3a). In addition, the effluent toxicity of the same unit continuously decreased throughout the time under the same conditions, which may be due to the increasing abundance of TCC-detoxifying bacteria in the activated sludge.

The toxicity of a chemical is closely related to its structure, thus Ecological Structure Activity Relationships (ECOSAR) software was employed to estimate the predicted toxicity of TCC and its intermediates. The acute and chronic toxicity of the dechlorination and hydrolysis products of TCC were significantly lower than those of TCC based on the Globally Harmonized System of Classification and Labeling of Chemicals (GHS) (Osha, U., 2013). However, the long-term toxicity of these intermediates to fish, daphnia and green algae was still at toxic or highly toxic levels (Fig. 3b). Although the toxicity of the effluent, mainly considering TCC, was reduced through the HA-A/O treatment, the intermediates still required to be fully mineralized to eliminate their potential environmental and human health impacts.

3.4. Environmental variables shaping the sludge microbial communities

Principal co-ordinates analysis (PCoA) indicated the sludge bacterial communities in the AN section were clustered together but obviously different from the sludge bacterial community structure in the ANO and O sections along the PC1 axis. Furthermore, the sludge communities under low-concentration TCC stress (1 mg/L) and high-concentration TCC stress (5–8 mg/L) were different in each section (Fig. 4a). The return flow of the secondary sedimentation tank sludge resulted in a non-significant differentiation of the ANO and O sludge microbial community structure at the same collection time. Taken together, these results demonstrated that the sludge community structure drastically diverged by oxygen and TCC concentrations.

To explore environmental variables shaping sludge microbial community structure, redundancy analysis (RDA) was performed based on the identified OTUs and measured chemical pollutants properties (Fig. 4b). TCC and its dehalogenation products (MCC and DCC) had a significantly positive correlation with anaerobic sludge communities ($p < 0.005$), indicating that TCC, MCC and DCC were the main drivers shaping the AN sludge communities. Not surprisingly, NH_4^+-N and COD also showed significantly positive correlations with anaerobic sludge communities ($p = 0.001$). For the ANO and O sludge communities, both 3,4-DCA (the hydrolysis product of TCC; $p = 0.005$) and NO_3^--N

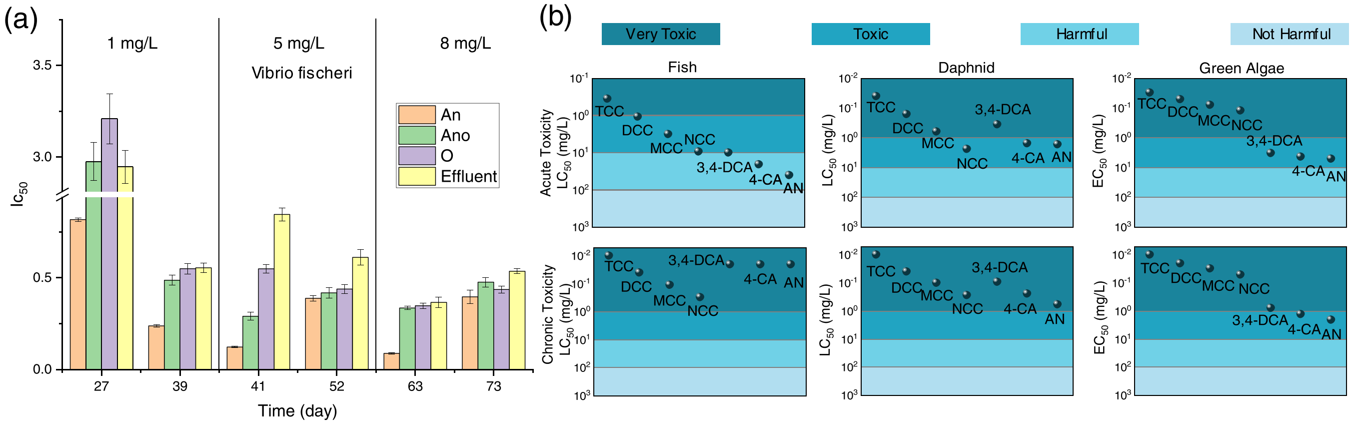

Fig. 3. The comparasion of comprehensive toxicity of different sections of effluent with *Vibrio fischeri* (Microtox®) (a). Biological acute and chronic toxicity evaluation of TCC and its intermediates by ECOSAR (b).

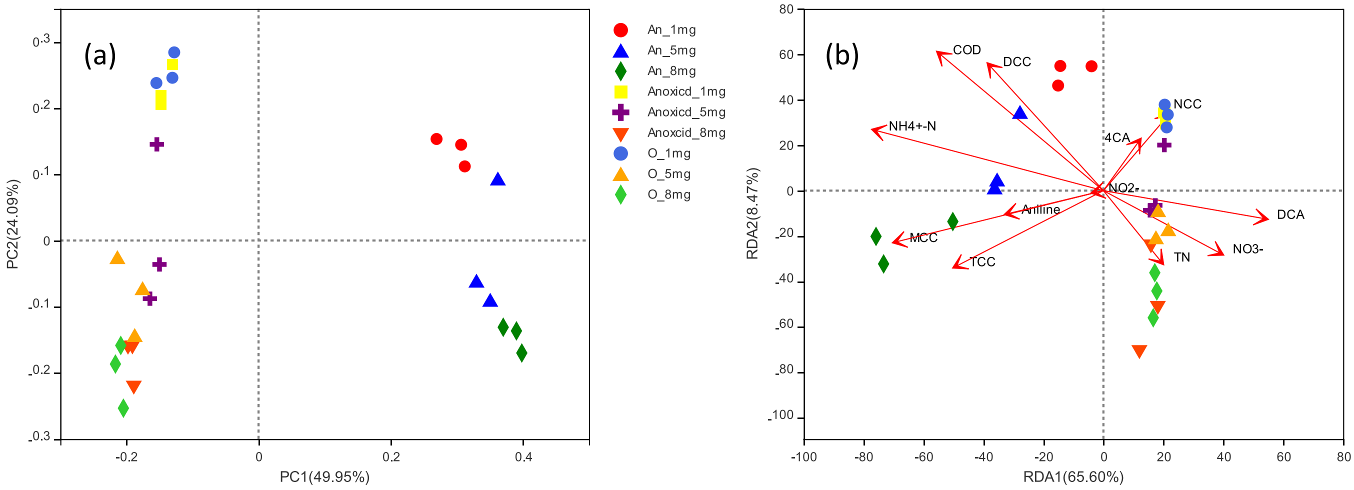

Fig. 4. Effect of different TCC concentration on sludge microbial community structure in different sections. Principal co-ordinates analysis (PCoA) based on Bray-Curtis distance of 16 S rRNA gene sequences (a), and Redundancy analysis (RDA) of 16 S rRNA gene sequences data and explanatory chemical variables (b).

($p = 0.031$) had positive correlation with 5–8 mg/L TCC fed influent. Therefore, 3,4-DCA was the main driver in shaping the ANO and O sludge communities.

3.5. Changes of sludge community taxonomic composition

The relative abundance of bacterial communities at phylum level of each sludge community was shown in Fig. S1. A total of 14 phyla with relative abundance > 1% were identified, including Proteobacteria, Chloroflexi, Bacteroidota, Actinobacteriota, Planctomycetota, Halobacterota and Acidobacteriota. The dominant archaea phylum in the AN section was Halobacterota; moreover, the influx of TCC significantly increased the percentage of Halobacterota, by 7.4%, 13.94% and 23.84% ($p < 0.05$) under 1 mg/L, 5 mg/L and 8 mg/L, respectively. The results were consistent with a previous study in which Halobacterota was involved in anaerobic processes such as methane production in fermentation (Zhu et al., 2020). As expected, the percentage of Chloroflexi increased upon TCC stress in the AN section (17.8–25.3%), the species belonging to which was reported to be involved in dechlorination (Tang et al., 2022; Xu et al., 2022). Conversely, the percentage of Proteobacteria decreased significantly in the AN section, by 25.65%, 16.13% and 11.09% at 1 mg/L, 5 mg/L and 8 mg/L, respectively.

The dominant genera with relative abundance > 1% were further analyzed to uncover the response of functional taxa within sludge communities to the stress of TCC at different sections (Fig. S2). As the TCC influent concentration increased from 1 mg/L to 5 mg/l and eventually reaching 8 mg/L, the abundance of denitrifying bacterial genera in three sections were AN: 13.9%, 6.4% and 4.0%; ANO: 17.9%, 15.0% and 9.9%; O: 5.4%, 4.8% and 4.7%, implying a significantly suppressed denitrifying bacterial activity upon the increased TCC stress (Fig. 5a). *Terrimonas*, a denitrifying bacterium (Li et al., 2021b; Song et al., 2015), was detected in the AN (2.43%) and ANO (1.73%) segments and decreased in proportion to the increased TCC concentrations, and its relative abundance eventually dropped to 1.4% and 1.1%, respectively. The relative abundance of *Ferruginibacter*, a bacterial genus reported to be significantly correlated with denitrification and COD removal (Xie et al., 2017), decreased in the AN (2.0–0.6%) and ANO section (3.7-1.9%). *Dokdonella*, which could involve in nitrite transformation (Wang et al., 2019), also showed a decrease in the relative abundance in the ANO (3.2–0.5%) and O (4.49–0.61%) sections. Therefore, high TCC concentrations potentially inhibited the NH_4^+-N removal and led to NO_3^--N accumulation.

In the AN section, the profile of functional species shifted during the reductive dichlorination of TCC, mainly including *Dechloromonas*, *Mycobacterium*, and *Longilinea*. *Dechloromonas*, a dedicated dechlorinating bacteria reported previously (Yan et al., 2021), only occupied 0.055% of the AN sludge community, implying that other funcitonal microbes participate in the dechlorination process. *Methanosaeta*, an acetophilic methanogen known to participate in the dechlorination of polychlorinated biphenyls (Chang et al., 2019; Zhu et al., 2020), increased its abundance upon TCC stress from 7.3% (1 mg/L) to 13.6% (5 mg/L) and 23.2% (8 mg/L). The total abundance of functional bacteria that may participate in the degradation of benzene-containing compounds also obviously increased in the AN section (19.43–39.65%), comparing to that in the ANO (9.25–15.93%) and O (10.16% and 13.15%) sections. *Mycobacterium* is related to the degradation of benzene series (Pfaller et al., 2021; Zhang et al., 2013), and its abundance in the three sections increased from less than 0.1–2.5% (AN), 4.5% (ANO) and 4.4% (O), respectively. *Longilinea*, which is an acid-producing bacteria participating in hydrolytic fermentation and playing an important role in benzene ring cleavage (He et al., 2022b; Zhu et al., 2018), also showed an increase in the AN Section (3.1% to 5.3%). Some members of norank_f_Anaerolineaceae, which can participate in the degradation of aromatic compounds such as phenol, polycyclic aromatic hydrocarbons (PAHs), xylene and toluene under anaerobic conditions (Li et al., 2021a), were enriched in the AN section, suggesting that it might play a role in the TCC degradation and removal of pollutants. *Terrimonas*, conversely, is more sensitive to TCC, showing a decrease in abundance from 4.12% to 1.25% in the ANO section (Fig. 5b).

In the ANO and O sections, two dominant TCC-degrading bacterial genera *Rhodococcus* and *Pseudomonas* were investigated, which can hydrolyze the two amide bonds first and proceed to catabolize the chloroaniline products (3,4-DCA and 4-CA) (Li et al., 2022; Taweetanawanit et al., 2019). Both *Rhodococcus* and *Pseudomonas* were obviously enriched in the ANO (1.47–3.57%) and O (1.12–3.32%) sections; however, *Sphingomonas*, also a TCC-degrading bacterium (Mulla et al., 2016), only had a small number of detections (Fig. 5c). This observation, in accordance with previous studies, implies that aerobic conditions are more conducive to the enrichment of TCC-degrading bacteria, providing an important basis for the partial detoxification and degradation of TCC in the system, while also matching the TCC transformation products analyses in Section 3.2 (Fig. 2).

3.6. Microbial indicators of TCC and its transformation products

Bioindicators can be used as an early diagnosis of performance changes in wastewater treatment systems and the environment,

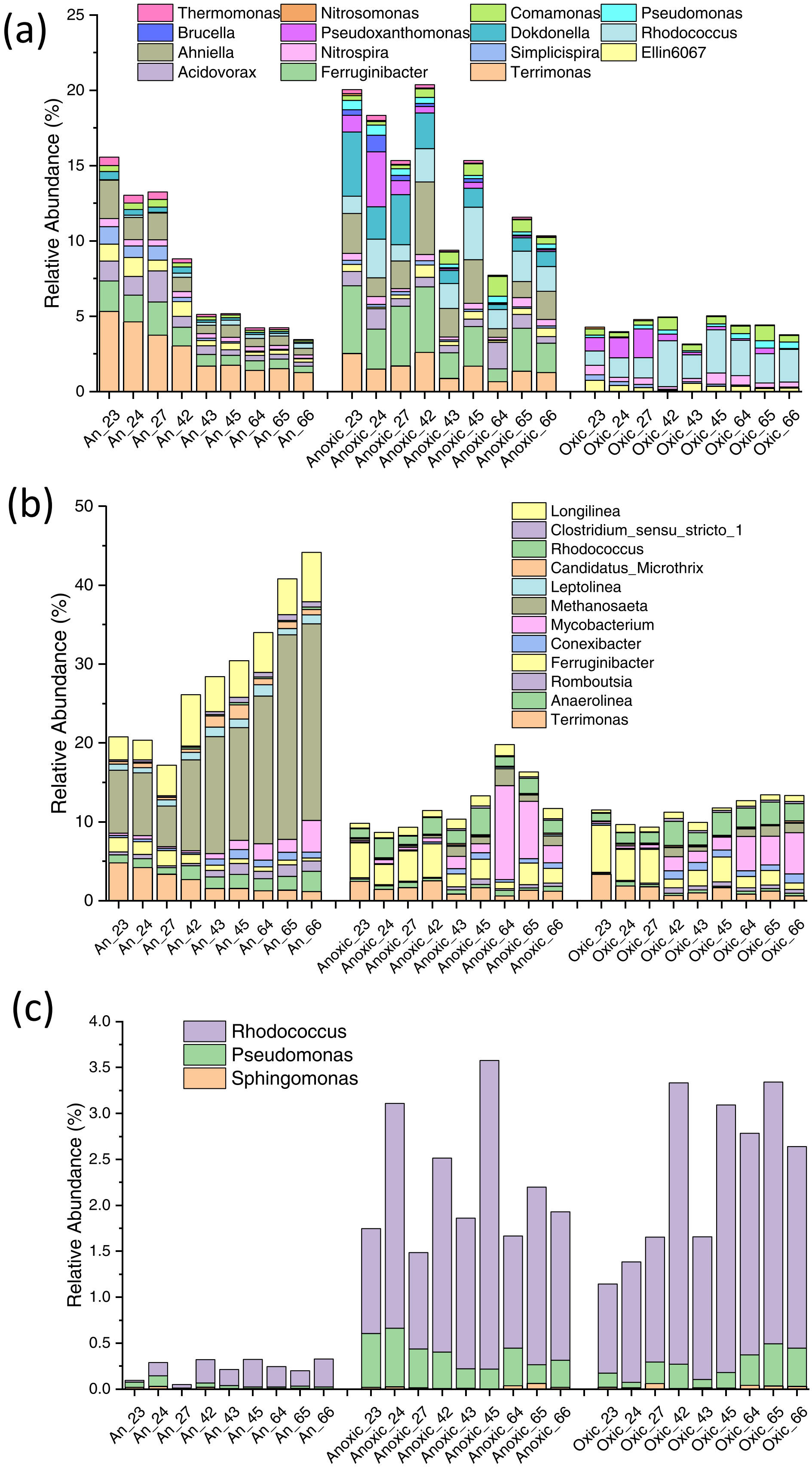

Fig. 5. The dynamic change of functional bacteria abundance in different sections under different TCC concentration. Function bacteria related to nitrogen removal (a), benzene -containing compounds degradation (b) and TCC hydrolysis and chloranilines (3,4-DCA and 4CA) catabolism (C). 23-2723–27 d, 42–45 d, and 64–66 d sludge communities were impacted by 1 mg/L, 5 mg/L, and 8 mg/L TCC, respectively.

considering the microbial community profile change associated with the toxic stresses by TCC and its intermediates. Correlations were calculated between the microbes and concentrations of TCC and its intermediates in the sludge. A total of 14 genera possessed significant correlations with TCC and its intermediates, suggesting their potentials as bioindicators. TCC and its dechlorination products are co-existing and positively correlated in the environment, and exemplifying this, TCC has a strongly positive correlation with DCC and MCC in the system. Nevertheless, the correlation between TCC and its hydrolysis products 3,4-DCA and 4-CA is not clear, mainly because 3,4-DCA and 4-CA are soluble in water and often below the detection limit in the environment. Regarding the microbes, as shown in Fig. 6, *Methanosaeta*, *longilinea*, norank_f_Anaerolineaceae, and norank_f_ A21b showed significantly positive correlations with TCC, DCC and MCC, and negative correlations with NCC and 3,4-DCA ($P < 0.05$). *Dokdonella*, norank_c_SJA-28, *Brucella*, AAP99, *Pseudoxanthomonas*, *Devosia*, *Mycobacterium*, norank_o_PeM15, norank_f_Saprospiraceae and unclassified_f_Comamonadaceae were significantly negatively correlated with TCC, DCC and MCC, yet positively correlated with NCC and 3,4-DCA ($P < 0.05$). In previous studies, bioindicators of TCC, such as *Dokdonella* and *Methanosaeta* found positive correlations and norank_f_Saprospiraceae showed negative correlations with TCC concentrations in different sludge (Tan et al., 2021; Wang et al., 2020a). Therefore, these 14 genera could serve as promising bioindicators for the appearance of TCC and its intermediates in the wastewater treatment process and diverse environments.

3.7. Dynamic changes of molecular ecological networks

Molecular ecological networks analysis was employed to demonstrate the role of TCC in shaping the microbial interactions within sludge communities. The correlations between dominant species (relative abundance > 1%) in different sections were calculated and visualized in the corresponding networks (Fig. 7). Network density, connectivity, and transmissibility characterize the rate of reaction of microbial networks to external perturbations, and highly connected nodes allow collaboration between microbial communities, making the network more resilient to disturbances (De Anda et al., 2018). Specifically, 5 mg/L TCC increased the number of nodes, connections, and averages of the microbial network in the three sections and decreased the modularity index and the percentage of positive correlations (Fig. 7beh). 5 mg/L TCC increased the system complexity, probably due to the increased abundance of TCC-tolerant bacteria altering the original modular structure and competing with the original ecological niches. Furthermore, 8 mg/L TCC increased the modularity index and positive correlations, and decreased the number of nodes, connections, and averages of the microbial network structure in the three sections (Fig. 7cfi). This indicates that 8 mg/L TCC reshaped the sludge microbial network structure. *Methanosaeta* (Chang et al., 2019; Zhu et al., 2020), was one of the core genera in the AN section, and its correlation with denitrifying bacterial genera (*Terrimonas* and Ellin6067) (Liang et al., 2022; Zhao et al., 2019) changed from positive to non-relation, and had more negtive relations with other genera as the TCC concentration increased (Fig. 7abc). In turn, *Methanosaeta* (Wang et al., 2020b) may severely squeeze the ecological niche of nitrogen-transforming genera when increasing TCC concentration. *Mycobacterium*, a benzene series degrading bacterial genus (Pfaller et al., 2021; Zhang et al., 2013), may play an important role as a collector in the ANO and O sections and was positively correlated with the denitrifying bacterial genera. Specifically, *Mycobacterium* showed positive correlations with *Ahniella*, *Devosia*, *Gemmata*, *Terrimonas*, and *Ferruginibacter* in the ANO section (Fig. 7def), and with *Rhodococcus* (a TCC degrader (Li et al., 2022)) and *Gemmata* (Xia et al., 2019) in the O section (Fig. 7hi). The positive correlations between the potential TCC-degrading bacteria and denitrifying bacteria implied a potential co-work, benefiting the denitrifying bacteria to resist the TCC toxicity under ANO and O conditions. High concentrations of TCC improved the average degree and topological features of network density of bacterial communities, indicating that networks were more densely interconnected and resistant to stress. This may be because TCC-resistant bacteria could build contact networks when exposed to TCC, hence potentially increasing the growth and colonization by more TCC-resistant bacteria.

At high TCC concentration of 8 mg/L, a significant cooperative relationship (positive correlation) among the denitrifying bacteria was observed in the three sections, and their shared ecological niches were conducive to resist the toxic stress of TCC. In the AN section, *Terrimonas*, *Conexibacter*, and *Ferruginibacter* showed positive correlations (Fig. 7 BCE). In the ANO section, *Rhodococcus* and *Pseudoxanthomonas* showed a positive correlation; moreover, *Terrimonas*, *Gemmata*, *Ahniella*, and *Ferruginibacter* also displayed a positive correlation (Fig. 7def). In the O section, a positive correlation was found in *Terrimonas* and *Dokdonella*, *Devosia*, *Ferruginibacter* and *Rhodococcus*; *Pseudoxanthomonas* and *Ahniella* also exhibited a positive correlations (Fig. 7ghi). Furthermore, the highest proportion of positive correlations among genera (77.6%) facilitated material exchange and collaboration among denitrifying genera in the O section at 8 mg/L TCC (Fig. 7i). The formed TCC-tolerant modular structure was more resilient for the resistance of TCC stress, ensuring the stability of the modular internal structure of the microbial community.

4. Conclusions

The fate and toxic effect of TCC in the HA-A/O process was investigated. TCC was reductively dechlorinated and hydrolyzed simultaneously in the biological treatment process, but the corresponding pathway contributions varied in the different sections. The TCC stress led to nitrate accumulation in the effluent, but had no influence on COD. The TCC toxicity was partially reduced through the treatment process, releasing residual and diverse intermediate products that require employment of other techniques for effective removal. The information reported here is valuable for understanding the biotransformation pathways and toxic behavior of TCC in wastewater biological treatment processes. These findings benefit research on the impact of TCC on microbial communities and interactions in wastewater treatment systems and provide promising bioindicators for TCC and its diverse intermediates contamination in various environments.

CRediT authorship contribution statement

Hao Wang: Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Hui Yun:** Methodology, Funding acquisition, Writing – review & editing. **Minghan Li:** Methodology, Investigation, Data curation, Writing – review & editing. **Hanlin Cui:** Methodology, Data curation, Writing – review & editing. **Xiaodan Ma:** Methodology, Data curation. **Yanqing Zhang:** Methodology, Data curation. **Xuanyuan Pei:** Methodology, Resources. **Liying Zhang:** Methodology, Resources. **Ke Shi:** Software, Resources. **Zhiling Li:** Writing – review & editing. **Bin Liang:** Conceptualization, Methodology, Resources, Funding acquisition, Writing – original draft, Writing – review & editing, Supervision. **Aijie Wang:** Resources, Funding acquisition, Writing – review & editing, Supervision. **Jizhong Zhou:** Resources, Writing – review & editing.

Environmental implication

Extensive use of antimicrobial TCC results in its omnipresent detection in sewage treatment systems and diverse environments with high concentration level, endangering ecological security and human health. This study found the coexisting TCC dehalogenation and hydrolysis pathways in wastewater biotreatment systems. Traditional biological treatment process partially reduced the TCC biotoxicity, but its dehalogenation and hydrolysis intermediates need further detoxification and mineralization. TCC bioindicators were excavated that could assess its

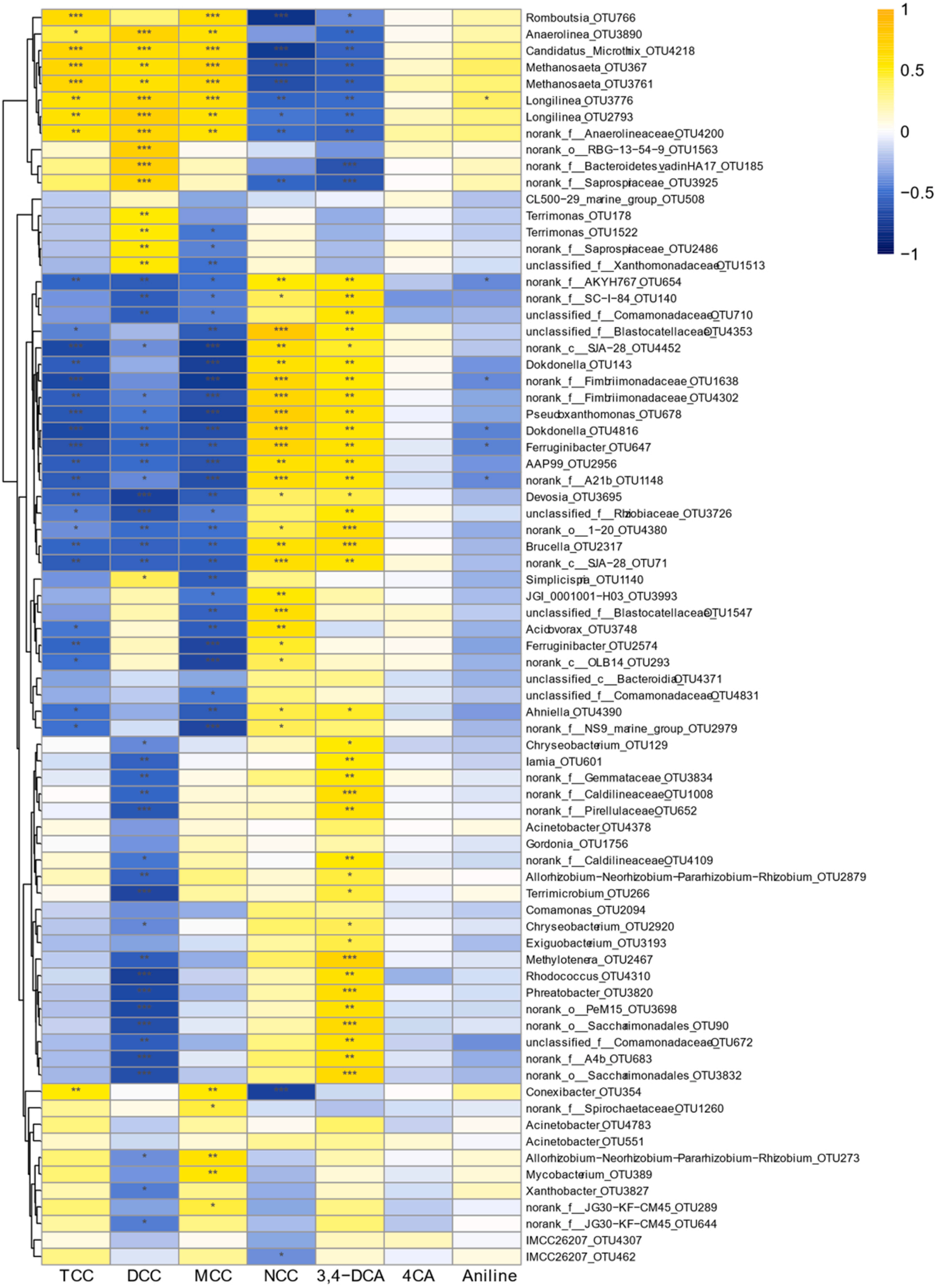

Fig. 6. Spearman correlation analysis of TCC and its transformation products and dominant bacterial taxa in sludge microbial communities. Yellow and blue represents positive and negative correlation, respectively (* $P < 0.05$; ** $P < 0.01$; *** $P < 0·001$).

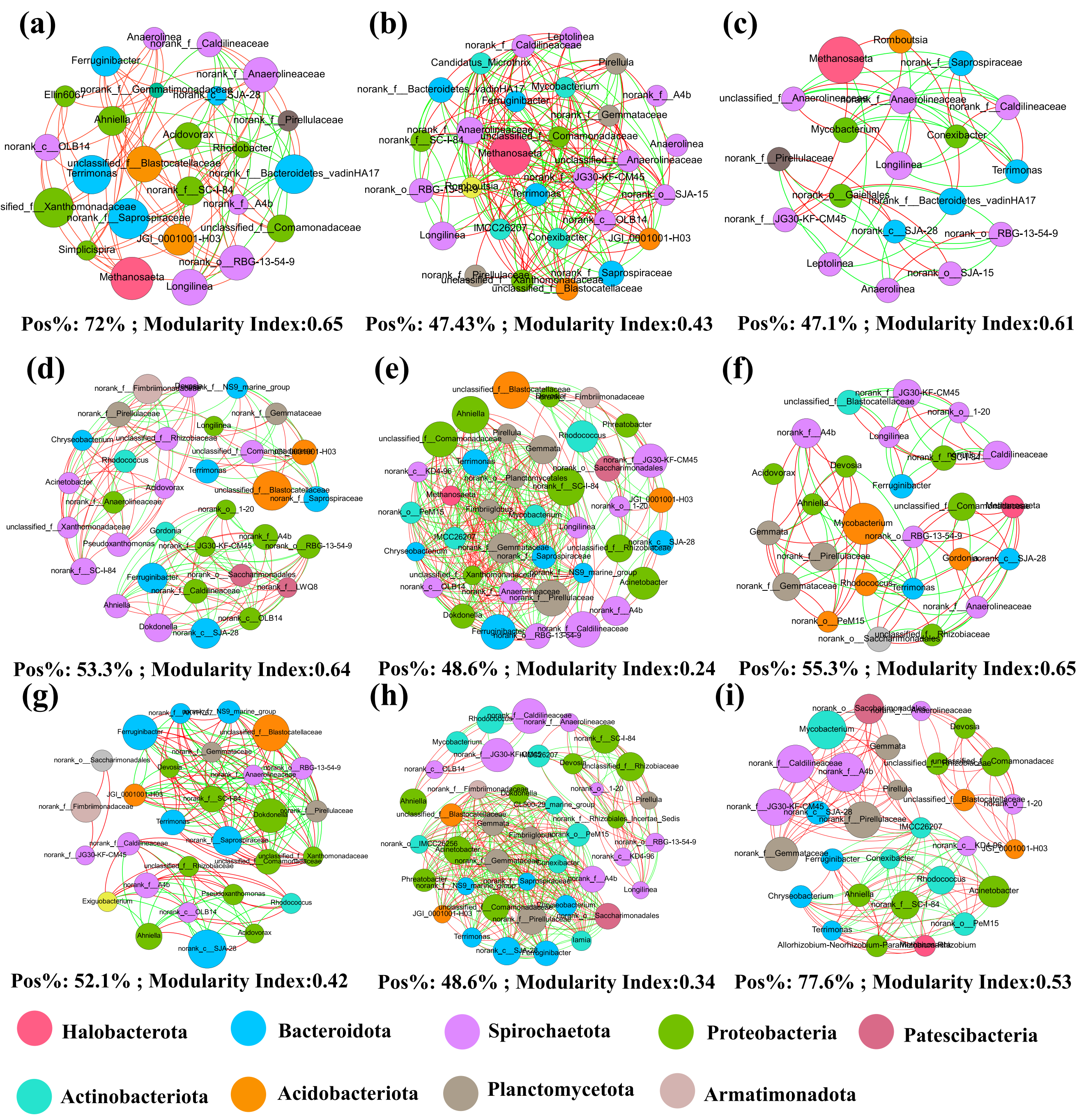

Fig. 7. Molecular ecological networks at genus level in hydrolysis anaerobic (a-c), anoxic (d-f) and oxic (g-i) sections under 1, 5 and 8 mg/L TCC (from left to right) stress. Positive and negative interactions between bacterial taxon are indicated by red and green lines, respectively. Different node colors are used to distinguish the different genera of bacteria. The size of each node represent the genus abundance.

contamination in the environment. This study highlights more detoxification and mineralization technologies involved in risky TCC and its intermediates are needed toward ensuring the sustainable functioning and health of water ecosystems.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2022.129796.

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