

Mixed culture of plants improved nutrients removal in constructed wetlands: response of microbes and root exudates

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Abstract

Root exudates are determined by plant species configuration and affect microbial community, which in turn affect purification efficiency of constructed wetlands (CWs). However, it is not well understood how the plant configuration affects CWs purification efficiency through specific root exudates. Herein, four mixed culture CWs were constructed, CW-G3 with *Iris pseudacorus*, *Iris sibirica*, *Juncus effusus*, and *Hydrocotyle vulgaris* showed the optimal diversity nutrients removal efficiency (TN: 94.2%, TP: 82.9%, COD: 74.7%). Highly increased antioxidant enzymes (peroxidase and catalase) reduced photosynthesis-negative enzyme (malondialdehyde) activity of plants in CW-G3, which ensured oxygen (O₂) and organic carbon (OC) production and successfully released to rhizosphere by well-developed root aeration tissues. Further, plant configurations resulted in diversity microbial communities, CW-G3 enriched higher abundance of genus *Saccharimonadales* and *Flavobacterium*, which benefited nitrogen removal. More importantly, as OC, higher contents of maltose in CW-G3 (6.6 ~ 11.1 folds of that in other three CWs), as well as lauramide, choline, triethylamine and urocanic acid contributed to microbial denitrifying. Differently, higher contents of unsaturated fatty acids linoleic acid and oleic acid in other three CWs inhibited their microbial nitrifying, rather than utilized by bacteria as OC. Co-occurrent network results also proved the interactions between root exudates and functional bacteria. Thereby, plant configuration in CW-G3 provided higher O₂ and OC contents for bacteria, and reduced nitrifying inhibitors, which contributed to higher purifying efficiency. The study promoted the understanding about root exudates' effects on bacteria through plant configurations, and improved the purification efficiency of CWs.

Introduction

CWs, a valuable ecological engineering technology, has been widely applied to treat polluted surface water because of its high nitrogen (N) removal efficiency, low cost and energy consumption (Zhang et al., 2021a; Zhao et al., 2021). In CWs, N was mainly removed by biological process (e.g., 60% – 90% of N removed by microbial nitrification-denitrification, 0.5% – 60% of N removed by plant uptake), as well as physicochemical pathway (e.g., adsorption) (Li et al., 2015; Tan et al., 2020; Wu et al., 2017). In CWs, N removal efficiency by microbial nitrification-denitrification is significantly affected by O₂ and OC contents (Tan et al., 2020). Ammonia is converted into nitrate in nitrification when O₂ is sufficient. Then nitrate is used as the electron acceptor, and OC is utilized as the electron donor in denitrification while O₂ is insufficient (anoxic or anaerobic) to realize the N removal (Jia et al., 2020). Generally, COD/N (chemical oxygen demand) ratio ≥ 4 is suggested to be suitable for microbial proliferation and N removal (Ding et al., 2012). But the ratio of polluted surface water is generally lower than 4 (Wu et al., 2017), which would inhibit the microbial biological process and not conducive to N removal.

As one of the main components of CWs, plants play a critical role in N removal. There are multiple N purification pathways by plants, including adsorption, up-take (Wang et al., 2013). Dense roots can serve as the habitat for microorganisms (Saleem et al., 2016), and radical oxygen loss is also conducive for nitrification (Cardinale et al., 2013). More importantly, 5% – 21% of OC (e.g., amino acids, carbohydrates,

secondary metabolites) fixed by photosynthesis would release to rhizosphere as root exudates, and ultimately be utilized by microorganisms to influence purification efficiency (Huang et al., 2014; Wu et al., 2017). Different plant species differed in growth rates, root structure, root exudates, etc. Therefore, CWs with different plant species (monoculture) showed significantly different nutrients removal efficiency (Cheng et al., 2009; Lu et al., 2012; Vymazal, 2011). But monoculture is not conducive for the stability, sustainability, and productivity of ecosystems due to its lower biodiversity (Bai et al., 2004; Bai et al., 2010). Ecosystems with mixed culture plants could reasonably maintain resilience (Lyu et al., 2020). Moreover, recent studies reported that CWs with mixed culture plants increased diversity nutrients removal efficiency than with monocultured plants (Lyu et al., 2020; Wang et al., 2013; Zheng et al., 2020).

Mechanisms for increased nutrients removal proportion by mixed culture plants have also been studied. Compared monoculture, mixed culture improved belowground and aboveground plant biomass to increase plant N-uptake (Qiu et al., 2011; Wang et al., 2013), as well as positively influenced the sizes, diversity and structure of microbial community to enhance carbon degradation and nutrient cycling (Zhang et al., 2021a; Zhang et al., 2010; Zheng et al., 2020). Mixed culture could also promote photo-protection to increase plant productivity by adjusting photosynthesis (Zhu et al., 2017). However, less studies were focused on roots exudates, and most of these studies were based on selected or mimicked "root exudates" in monoculture mode CWs. For example, OC, soluble sugars and amino acids were mixed as root exudates and added in monocultured CWs to speculate its influence on microbial community and nutrients removal efficiency (Chen et al., 2016; Wu et al., 2017; Zhang et al., 2010). It would leave the actual working exudates undetected and affect the experimental results. Therefore, it is necessary to reveal the mechanisms by detecting actual root exudates. Moreover, the specific root exudates that affect purification efficiency and how to affect have never been reported in mixed culture CWs so far to the best of our knowledge. In addition, plants differ in flowering period, flower color, seasonal variation, and height, rationally configured plants can form a wetland scene with flowers in three seasons and contribute to a livable humanistic environment (Du et al., 2018). However, there are also few studies focused on combining purifying function and aesthetics of CWs.

Hence, present study was aimed to: 1) study the effect of different plant configurations on purification efficiency of CWs; 2) determine the specific root exudates that affected purification efficiency and how to affect. According to the principle of local plant species, purification effects and growth characteristics, 7 commonly used wetland plants in Shanghai were selected in this study (Supplementary material, Table S1). Four tall plants (height > 1 m), two medium-plants (medium-height, 0.5-1 m), and 1 dwarf plant (height < 0.5 m) were selected to form four kinds of CWs with mixed culture plants to treat wasted surface water. Then, the purification performance, leaves enzymes, root structure, microbial communities and root exudates were analyzed to reveal the mechanism. This study would enhance our understanding about the effects of root exudates, improve the diversity nutrients removal efficiency under carbon deficient conditions, and provided a theoretical basis for the selection and allocation of wetland plants.

Methods

CWs design and plants selection

Laboratory-scaled CWs were built with PVC pipes (diameter × height = 0.4 m × 0.5 m) and the working volume was 15 L (Fig. 1). In CWs, quartz sand was added and 4 kinds of plants with similar biomass were planted. In each CW, PVC tubes were respectively arranged at the height of 0.025 m for water sampling and the height of 0.25 m for O₂ sampling. Three layers of substrates were added in CWs. The quartz sand in the upper and bottom layers had the same diameter (6 ~ 8 mm) and quartz sand with a diameter of 2 ~ 4 mm was added as the middle layer. The depth of the bottom layer was 0.05 m, and the upper and middle layers was 0.2 m.

12 kinds of vertical subsurface flow CWs were established and named, 7 CWs with monoculture plants, 4 CWs with mixed culture plants and 1 CW without plant (Supplementary material, Table S1). Three parallel CWs were arranged and the experiments were repeated 3 times. As for each species, the plants with the same size and growth state were selected. According to the fresh weight of each species, 500 g ± 50 g of plants were weighed and planted in CWs.

System operation

The experiment was carried out in Shanghai Ocean University in the east of Shanghai, China. The CW systems were acclimatized to synthetic wastewater for four weeks for the subsequent development of plants and the introduction of microbes. The ambient temperature and the relative humidity were respectively 15°C ~ 35°C and 60% ~ 95% (CW-AQT100, Pranus, China). According to Constructed Wetland Sewage Treatment Engineering Technology of China (HJ2005-2010) and Discharge Standard of Pollutants for Municipal Wastewater Treatment Plant of China (GB18918-2002), synthetic wastewater (COD = 120 mg/L, NH₄⁺-N = 15 mg/L, NO₃⁻-N = 15 mg/L, and TP = 2 mg/L) was prepared by dissolving 50 g glucose, 15.6 g KNO₃, 14 g NH₄Cl, 2.2 g K₂HPO₄, 1.5 g MgSO₄, and 0.5 g CaCl₂ with 250 L of tap water. Hydraulic retention time was four days.

Performance of CWs

Water samples from influent and effluent were collected and analyzed every 24-h to determine the performance of CWs. Dissolved oxygen (DO), ORP, pH, and water temperature were determined with a digital analysis instrument immediately after sampling (HQ40d, HACH, USA). TN (UV spectrophotometric method with alkaline potassium persulfate digestion), NH₄⁺-N (spectrophotometric method with Nessler's reagent), NO₃⁻-N (UV spectrophotometric method), and TP (spectrophotometric method with ammonium molybdate) were analyzed with a spectrophotometer (DR900, HACH, USA). Water samples were filtered (0.45 μm) (Q/YY8188, Xinya, Shanghai) to remove suspended solids. COD was analyzed with the potassium dichromate oxidation method (COD-571, Leici, Shanghai) (Zhao et al., 2021).

Enzymes activity, root structure and root exudates of plants

Superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and malondialdehyde (MDA) of plant leaves were detected before feeding with wastewater and after 4 days treatment, and the difference value

was used to characterize changes in enzymes activity (Yang et al., 2020). Enzymes activity were analyzed by enzyme kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Approximately 0.2 g of fresh leaf was crushed together with 2 mL of 0.1 M $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ (pH 7.0) buffer in an ice water bath. The obtained homogenates were centrifuged for 10 min (4°C , 5000 rpm). The supernatants were collected to spectrophotometrically determine enzyme activities (Supplementary material, detection of enzyme activity).

Plant roots were cut and purified with ultrapure water for 30 s to remove dead roots and quartz sand, then dehydrated with four concentrations of acetone solutions (10%, 40%, 60%, and 80%, each concentration for 1 h). Dehydrated plant roots were dried in a freeze-dryer for more than 12 h (LC-10N-A, Lichen, Shanghai). After plant roots are completely dehydrated and dried, scanning electron microscopy (SEM) on a Quanta 400 FEG (FEI, USA) was used to characterize the root structure, samples were coated with gold prior and treated with 5 kV accelerating voltage (Mahon et al., 2017).

Fresh quartz sand in the rhizosphere (20 g) was collected in a 150-mL Erlenmeyer flask and then 20 mL of distilled water was added. After ultrasonic extraction for 30 min, the supernatant was acquired. After repeating the above operation, the supernatants collected in the first and second times were mixed together (Zhao et al., 2021). After centrifugation and extraction, the collected liquid was evaporated and then blown to dryness with nitrogen. Then, 100 μL of acetonitrile aqueous solution (acetonitrile: water = 1:1, v/v) was added, vortexed, and centrifuged for 15 min (14000 g, 4°C). Finally, 2 μL of the supernatant was acquired for mass spectrometry analysis.

Microbial community analysis

Fresh quartz sand (20 g) in the rhizosphere (15–18 cm) and the non-rhizosphere (35–38 cm) were firstly collected respectively from each CW and placed in a sterile bag. 16S rRNA in V3 ~ V4 region was used, and gene was amplified, purified, quantified and sequenced on an Illumina Miseq 2000 platform (Guo et al., 2021). Quantitative Insights Into Microbial Ecology (QIIME, V1.8.0) was used to analyze high-quality reads. Sequences were clustered into operational taxonomic units (OTUs) at the 97% similarity level through UCLUST. Greengenes Database was applied to classify filtered OTUs with a confidence threshold of 0.80 (Su et al., 2021). Metabolic pathway of microbial community was predicted by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2).

Statistical analysis

Statistical analysis was conducted in SPSS 26.0 software (IBM Corp, USA). The differences were analyzed by One-way ANOVA (ANOVA, $P < 0.05$). Principal co-ordinates analysis (PCoA) was performed by RStudio (V 3.4.1) with packages “vegan”, “ggplot2”, “ggalt”. The distance between the points in the PCoA chart is Euclidean distance, which represents the similarity between the microbial compositions of corresponding two samples. The closer the two points are, the more similar the composition is. Co-occurrent network was also performed by RStudio (V 3.4.1) with packages “Hmisc”, and “igraph”, and network was visualized by the software Gephi (V 0.9.2) based on correlation matrix was conducted using the thresholds (coefficient $r > 0.8$ and significance $P < 0.05$) (Guo et al., 2021).

Results And Discussion

Analysis of nutrients removal performances

The purification efficiency of each CW in different seasons were summarized (Supplementary material, Fig.S1). The nutrients removal efficiency of CW-BK was significantly lower than experimental groups (ANOVA, $P < 0.05$), and CWs with different monoculture species were also performed significantly different in nutrients removal ($P < 0.05$). It was in accordance with previous publications (Lu et al., 2012; Vymazal, 2011), which compared the nutrients removal efficiency of CWs in different plant species, and the results showed that different plants differ in nutrients removal efficiencies. Further, CWs in mixed culture plants resulted in significantly increased nutrients removal efficiency compared monoculture plants ($P < 0.05$) ($\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$, TP and COD increased by 4.6% – 28.7%, 1.2% – 20.3%, 2.5% – 6.4% and 5.9% – 12.7%, respectively). More importantly, CWs with different mixed plant species also showed significantly different nutrients removal efficiency ($P < 0.05$).

To better understanding the differences in purifying function between different mixed culture CWs and figure out the optimal plant configuration, annual average nutrients removal efficiency of each CW in mixed culture was analyzed (Fig. 2A, B and C). CW-G3 and CW-G2 achieved higher removal efficiency of TN (Fig. 2A), CW-G3 and CW-G1 achieved higher removal efficiency of TP (Fig. 2B), while CW-G3 and CW-G2 achieved higher removal efficiency of COD (Fig. 2C). Thus, CW-G3 performed well in all TN, TP and COD removal. Furthermore, the concentrations of N in different forms were detected after 4 days treatment (Fig. 2D, E and F). $\text{NH}_4^+\text{-N}$ is firstly transformed into $\text{NO}_3^-\text{-N}/\text{NO}_2^-\text{-N}$ by nitrifying bacteria with O_2 and then converted into $\text{N}_2/\text{N}_2\text{O}$ by denitrifying bacteria with the aid of electronic donors OC (Tan et al., 2020). $\text{NO}_3^-\text{-N}$ concentrations of CW-G3 were significantly lower than CW-G1 and CW-G4 (Fig. 2D and E), while $\text{NO}_2^-\text{-N}$ concentration of CW-G1 were significantly higher than other three CWs (Fig. 2F) ($P < 0.05$). These results indicated that CW-G3 were sufficient in O_2 and OC to complete nitrifying-denitrifying process (Zhao et al., 2021).

Thereby, mixed culture of plants positively impacted purification efficiency of CWs, and different plant configurations differed in nutrients removal efficiency. Moreover, among the four CWs, CW-G3 showed better diversity nutrients removal efficiency (TN, TP and COD: 94.2%, 82.9% and 74.7%, respectively), which was the optimal plant configuration.

Analysis of plant activity

Plant roots could enhance nitrifying efficiency by releasing O_2 and increase denitrifying efficiency by secreting OC to the rhizosphere. Thus, the healthy roots of plants are critical for N removal in CWs (Yang et al., 2020). The aeration tissues of plant roots were observed through SEM (Supplementary material, Fig. S2). Four species plants in CW-G3 maintained the complete root structures with tightly arranged stomas, large openings, and well-developed aeration tissues (Fig. S2 C). But root structure of several plant species in other three CWs were rot without complete aeration structure (Fig. S2 A, B and D). Kludze

et al., (2013) proved that the well-developed roots aeration tissues could release O₂ to the rhizosphere and improve DO content around rhizosphere. Further, the DO content in rhizosphere effluent of each CW were detected, and CW-G3 was significantly higher than that in other CWs (0.92, 1.42, 1.96 and 1.22 mg/L in CW-G1, CW-G2, CW-G3 and CW-G4, respectively), which was consistent with SEM results.

We also assessed enzyme SOD, POD, CAT and MDA levels of plant leaves (Fig. 3). After 4 days treatment of wastewater, SOD, POD, CAT and MDA activities increased 1.1 ~ 1.8 folds, 1.3 ~ 3.3 folds, 1.2 ~ 20.1 folds and 1.1 ~ 5.8 folds, respectively. Moreover, the increase value of POD and CAT enzyme activities of plants in CW-G3 was significantly higher than that of plants in other CWs (Fig. 3B and C), while its MDA enzyme activity was significantly lower than that of plants in other three CWs (Fig. 3D) ($P < 0.05$). As antioxidant enzymes, SOD, POD and CAT play critical roles in the reactive oxygen species scavenging system (Baxter et al., 2014). Increased antioxidant enzyme activities indicated that wastewater triggered stress responses in plant leaves. MDA is a negative product of stress response under adverse conditions, which is also a hidden danger to photosynthetic ability of plant leaves (Yang et al., 2020). When photosynthetic ability was destroyed, O₂ and OC would not be successfully produced by leaves and released to the rhizosphere, which led to reduced nitrification and denitrification efficiency in the rhizosphere, thus inhibiting N removal process (Racchetti et al., 2017; Salgado et al., 2018). It indicated that plants in CW-G3 could reduce oxidative stress by increasing POD and CAT enzyme activities, thereby reducing the accumulation of MDA to maintain a high photosynthetic capacity and product high level of O₂ and OC. Yang et al., (2020) reported that exposure of nanoplastics in CWs increased MDA level of plant leaves and reduced root activities, which further inhibited N removal efficiency. This is also consisted with our research. Meanwhile, well-developed roots aeration tissues of CW-G3 ensured O₂ and OC release to rhizosphere. Therefore, CW-G3 performed well in nutrients removal.

Analysis of microbial community in rhizosphere and non-rhizosphere

Microbial community was analyzed to demonstrate the variation of N concentrations. Biodiversity indexes Chao1 and Shannon of microbial community in rhizosphere and non-rhizosphere were provided (Supplementary material, Table S2), which reflect the richness and diversity of microbial community, respectively (Fu et al., 2019). Shannon index of the rhizosphere of four experiment groups was significantly higher than of the non-rhizosphere, while no significantly difference was found in the blank group, which may indicate that root exudates benefit the microbial diversity. Both Chao1 and Shannon of CW-G3 were significantly higher than that of other three CWs, indicating that plant configuration in CW-G3 provided a suitable growth environment for microorganisms and positively affected the richness and diversity of the microbial community.

Furthermore, the distribution of microbial communities at phylum level was analyzed (Supplementary material, Fig. S3). Phylum Proteobacteria (26.47% – 57.4%), followed by Actinobacteria (1.2% – 40.4%), Bacteroidetes (2.9% – 22.9%), and Patescibacteria (1.8% – 19.2%) were dominant in four CWs, which have been reported to be widespread in aquatic environments (Pang et al., 2016; Sun et al., 2019b).

Proteobacteria (Cheng et al., 2016), Actinobacteria (Lv et al., 2021) and Bacteroidetes (Jia et al., 2021) could significantly reduce N, thus, CWs performed well in N removal (higher than 89.1%) (Fig. 2). The relative abundance of Patescibacteria in CW-G3 (rhizosphere: 9.0%; non-rhizosphere: 19.2%) was significantly higher than that in other CWs ($P < 0.05$). Zhang et al., (2021c) reported that adding OC in CWs significantly increased the abundance of Patescibacteria, which may suggest that more OC can be utilized by Patescibacteria in CW-G3. At genus level (Fig. 4A and B), there were obvious differences in the microbial communities of each experimental group, which was resulted by different plant configurations. Moreover, the relative abundances of *Saccharimonadales* and *Flavobacterium* in the rhizosphere (8.5% and 17.7%, respectively) and non-rhizosphere (8.7% and 10.0%, respectively) of CW-G3 were significantly higher than those in other CWs. *Saccharimonadales* affiliates to Patescibacteria, which is beneficial to CWs denitrification (Chen et al., 2018; Zhang et al., 2021b). Chen et al., (2022) proved that extra OC added in CWs increased relative abundance of *Saccharimonadales* and improved denitrifying process. This result indicated that more OC was utilized by bacteria in CW-G3, which was consistent with phylum results. *Flavobacterium* was also widely reported to have the nitrate-reducing capability (Pishgar et al., 2019). Therefore, CW-G3 showed the highest N removal efficiency.

Moreover, principal co-ordinates analysis (PCoA) was employed to investigate the differences in microbial composition between two samples (Fig. 4C). The samples were clustered various in the plot since CW-G1 was placed top-right, CW-G2 was placed top-left, CW-G3 and CW-G4 were placed bottom-middle in the figure. The rhizosphere samples and non-rhizosphere samples in each CW were also clustered on two sides of the oval. These results indicated diversity of microbial communities because of root exudated by different plant configurations. Further, degradation/utilization/assimilation pathways of microbial community were analyzed by PICRUSt2 (Fig. 4D), which showed that carbohydrate degradation (6030.59 PWY/Million) was predominant, followed by nucleoside and nucleotide (5090.38 PWY/Million), and amino acid degradation also took a great portion. Carbohydrates and amino acids are low-molecular-weight compounds, which can be easily up-taken and metabolized by microorganisms (Koner et al., 2021). Nucleoside and nucleotide can be utilized by bacteria to favor their proliferation (Bugenyi et al., 2020). The results indicated that plants in CWs provided extra OC (sole carbon glucose was added into CWs) for bacterial function and proliferation.

Analysis of root exudates

Non-targeted metabolic profiling was conducted to analyze the differences in metabolic profiles among different CWs. In total, 1250 and 634 metabolites were detected in positive ion mode (POS) and negative ion mode (NEG), respectively. The metabolites were divided into seven major groups, including organic acids (e.g., salicylic acid, succinic acid), amino acids (e.g., alanine, glycine), carbohydrate (e.g., maltose, sucrose), fatty acids (e.g., linoleic acid, oleic acid), amines (e.g., triethylamine, tributylamine) alkaloids (e.g., xanthine, guanine), lipids (e.g., methyl palmitate), and others.

Moreover, metabolites were mapped to the reference annotation pathway in Kyoto Encyclopedia of Genes and Genomes (KEGG), and metabolic pathways between CW-G3 and other mixed culture CWs were compared, respectively (Fig. 5). Among these pathways, biosynthesis of unsaturated fatty acids

(ath01040) and linoleic acid metabolism (ath00591) were significantly different and present in three compared groups (Fig. 5A, B and C) ($P < 0.05$). Their metabolic pathway was depicted further (Fig. 5D and E), which showed that levels of metabolites linoleic acid, oleic acid and arachidonic acid (Fig. 5D), as well as their fatty acid ester linoleate and arachidonate (Fig. 5E) were significantly different between CW-G3 and other CW. Relative contents of these different regulated metabolites were analyzed (Table 1), all linoleic acid, oleic acid and arachidonic acid in CW-G3 were significantly lower than that in other three CWs ($P < 0.05$). Subbarao et al., (2008) demonstrated that unsaturated fatty acids linoleic acid released by plant roots inhibited microbial nitrifying, so that $\text{NH}_4^+\text{-N}$ is hard to convert into $\text{NO}_3^-\text{-N}$ in soil, while arachidonic acid showed no significantly inhibitory effect on nitrification. Similarly, Souri, (2017) also reported that linoleic acid, oleic acid significantly inhibited nitrification process, in which higher concentrations have led to less nitrite production. Thereby, lower N removal efficiency of other three CWs (Fig. 2A) indicated that higher concentrations of linoleic acid, oleic acid inhibited microbial nitrifying, rather than utilized by microbes as OC.

The other significantly different metabolic pathways included glycerophospholipid metabolism (ath00564), purine metabolism (ath00230), histidine metabolism (ath00340), tropane, piperidine and pyridine alkaloid biosynthesis (ath00960) (KEGG pathways were shown in Supplementary material, Table S3). Relative contents of significantly different metabolites were shown in Table 1. Contents of maltose in CW-G3 were 6.6 ~ 11.1 folds of that in other three CWs, which have been widely reported to be used as OC in wastewater purification system to improve N removal efficiency (Wang et al., 2016). Contents of lauramide, choline, triethylamine and urocanic acid in CW-G3 were also significantly higher than compared CW ($P < 0.05$). All these metabolites have been proved to be utilized by microorganisms as OC (Merkova et al., 2018; Gao et al., 2018; Fujii et al., 2014; Wargo, 2013), which indicated that plants in CW-G3 released higher contents of OC than in other CWs. Thereby, with lower nitrifying inhibitors and higher organic C, plant configuration in CW-G3 showed better N removal efficiency (Fig. 2A).

Table 1
Ratios of significantly different metabolites between compared CW groups.

Name	Ratio (RX-G3 / RX-G1)	Ratio (RX-G3 / RX-G2)	Ratio (RX-G3 / RX-G4)
Arachidonic acid	0.466	0.838	0.369
Oleic acid	0.440	0.471	0.220
Linoleic acid	0.267	0.681	0.182
Maltose	7.216	11.100	6.645
Lauramide	1.329	2.520	-
Choline	2.764	-	-
Triethylamine	3.063	-	-
Urocanic acid	-	3.416	-

Notes: "-" represent the metabolites did not detected in CW. Ratio represent relative concentration, ratio > 1: metabolite contents of CW-G3 higher than of compared CW; ratio < 1: metabolite contents of CW-G3 lower than of compared CW.

Co-occurrence network between bacteria and root exudates

The co-occurrence network between bacteria and eight differently regulated root exudates was analyzed (Fig. 6), which was composed of 31 nodes and 98 edges (linkages). Network diameter, average path length and modularity index was 4, 2.077 and 0.467 (> 0.4), respectively, indicated that the real-world network has a module structure (Ren et al., 2020). Unsaturated fatty acids arachidonic acid, oleic acid and linoleic acid were negatively correlated with bacteria (e.g., *Pseudorhodoplanes*, *Geobacter*, *Nitrospira*, *Areomonas*, *Amaricoccus*, *Saccharimonadales* and *Flavobacterium*), which means that the higher contents of these unsaturated fatty acids inhibited bacterial functional performance. *Pseudorhodoplanes* has been proven to be able to fix N and release plant growth promoting regulators (Şeker et al., 2017). Nitrogenase-encoding genes have been shown in nearly all *Geobacter* species (Jing et al., 2022). *Nitrospira* is a widely studied nitrifying bacteria, *Areomonas* and *Amaricoccus* are also take part in nitrification and help for N removal (Daims et al., 2015; Koch et al., 2019). Therefore, the N removal efficiency of other three CWs was lower than that of CW-G3 due to its high contents of N removal inhibitors (acids arachidonic acid, oleic acid and linoleic acid), which inhibited bacterial function.

Nevertheless, maltose, urocanic acid, lauramide, choline and triethylamine were positively correlated with bacteria (e.g., *Thauera*, *Simplicispira*, *Pseudorhodoplanes*, *Thiothrix*, *Chloroflexus*, *Saccharimonadales* and *Flavobacterium*), which means that the higher contents of these OC benefited bacterial functional performance. *Thauera* (Luo et al., 2020), *Simplicispira* (Siddiqi et al., 2020) and *Thiothrix* (Xia et al., 2019) were widely found in wastewater treatment systems as denitrifying bacteria that facilitated N removal. *Chloroflexus* (Sun et al., 2019a) degraded histidine coupled to the reduction of NO_3^- -N enhanced N removal in wastewater treatment systems. *Saccharimonadales* (Zhang et al., 2021b) and *Flavobacterium*

(Pishgar et al., 2019) are denitrifying bacteria and their relative abundances were significantly higher in CW-G3 than that in other three CWs. These results indicated that higher contents of maltose, urocanic acid, lauramide, choline and triethylamine in CW-G3 could be utilized as OC by microbes, which was beneficial for N removal. Secretion of unsaturated fatty acids oleic acid, linoleic acid and arachidonic acid inhibited function of nitrifying bacteria, while maltose, urocanic acid, lauramide, choline and triethylamine enhanced function of denitrifying bacteria. Thereby, CW-G3 performed well in N removal than other three CWs.

Conclusion

This is the first study to determine the impacts of specific root exudates on N removal efficiency in mixed culture mode CWs by a real assay. Mixed culture of plants improved diversity nutrients removal efficiency of CWs, and among four mixed culture CWs, CW-G3 was the optimal plant configuration because of its high TN, TP and COD removal efficiency. Highly increased antioxidant enzymes POD and CAT decreased the levels of photosynthesis inhibitor MDA in plant leaves, couple with well-developed root aeration tissues ensured O₂ and OC production and release to rhizosphere in CW-G3, which was benefit for bacterial nitrifying-denitrifying. Further, CW-G3 enriched higher abundances of genus *Saccharimonadales* and *Flavobacterium* than other three CWs, both were conducive for N removal. More importantly, higher contents of maltose, urocanic acid, lauramide, choline and triethylamine were detected in CW-G3 than that of other three CWs, which enhanced functional performance of denitrifying bacteria by utilized as OC. Differently, higher contents of unsaturated fatty acids arachidonic acid, oleic acid and linoleic acid inhibited were detected in other three CWs than in CW-G3, which inhibited functional performance of nitrifying bacteria in other three CWs. Therefore, plant configuration of CW-G3 ensured leaves photosynthesis and well-developed root structure to maintain production of O₂ and OC and release to rhizosphere successfully. Part of the OC like maltose increased denitrifying process in CW-G3, part of OC like oleic acid and linoleic acid inhibited nitrifying process in other three CWs, thus CW-G3 performed well in N removal. This study promoted our understanding about root exudates' effects on functional bacteria in CWs.

Declarations

Availability of data and materials All data generated or analyzed during this study are included in this published article.

Authorship Contribution Statement Zhufang Wang: Ideas, Methodology, Data, Writing of the manuscript. Yinjiang Zhang: Ideas, Methodology. Xiao Li, Jinkun Li and Xin Hou: Data. Zhimiao Zhao: Writing-review, Supervision and Conceptualization. All authors contributed critically to the drafts and gave final approval for publication.

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Consent to participate All authors have participated in the process, read and agreed to the published version of the manuscript.

Consent for publication All authors have read and agreed to the published version of the manuscript.

Competing interests 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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Figures

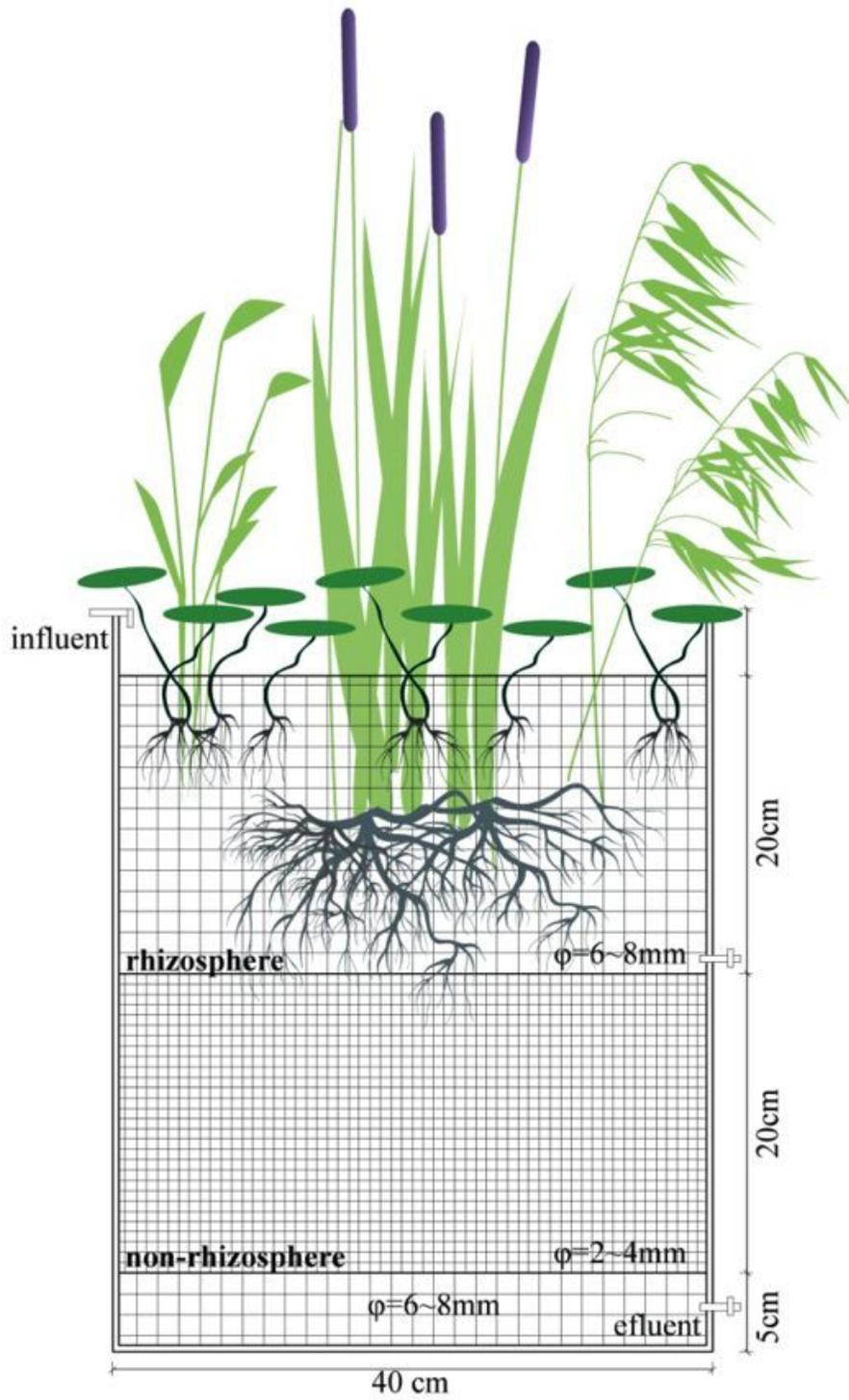


Figure 1

Schematic diagram of constructed wetland.

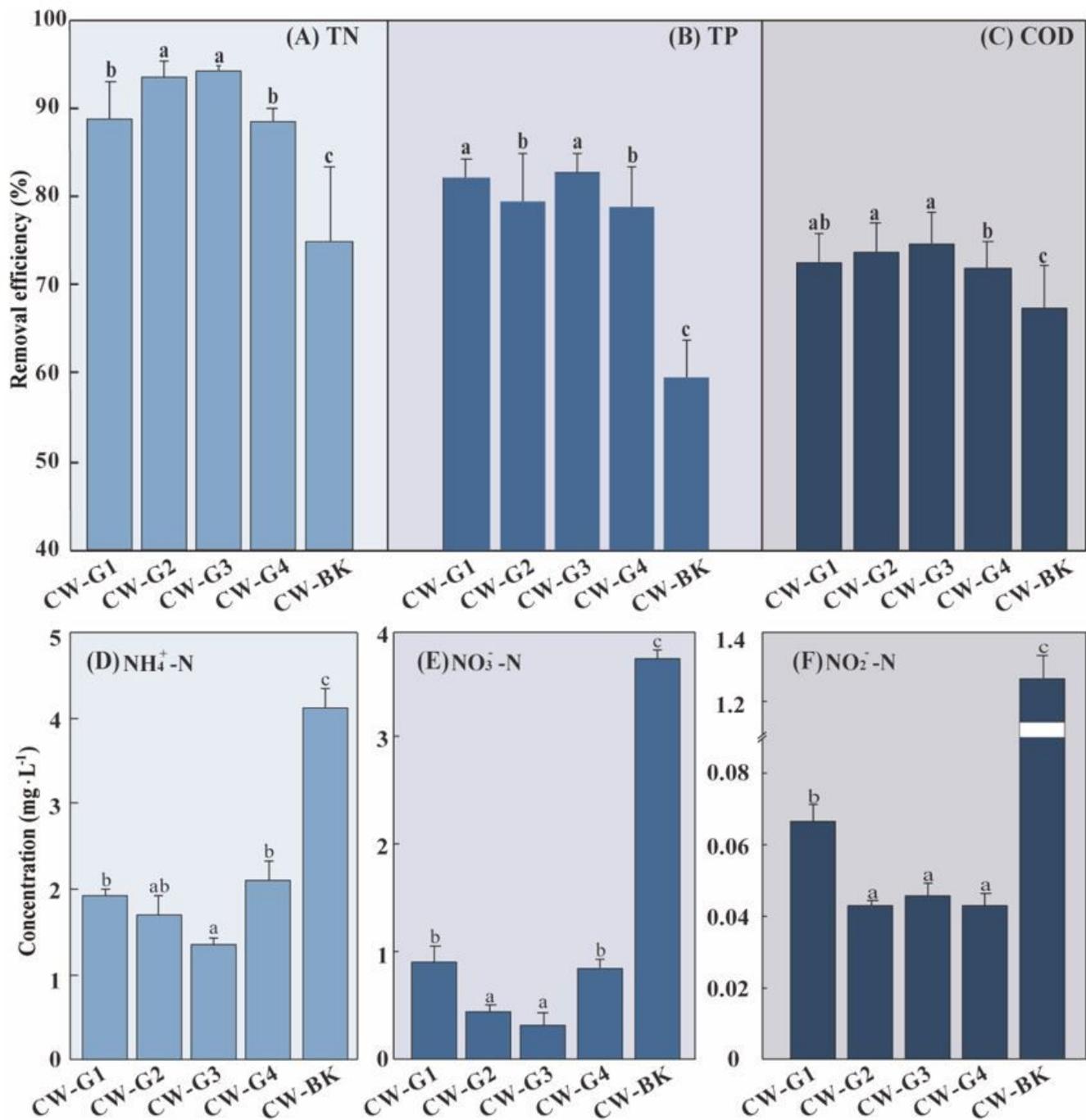


Figure 2

Nutrients removal efficiency of CWs, (A) TN; (B) TP; (C) COD and concentration of N in different forms at the 4-day, (D) NH₄⁺-N; (E) NO₃⁻-N; (F) NO₂⁻-N. Different letters in the same category indicated the significant difference, the same below ($P < 0.05$).

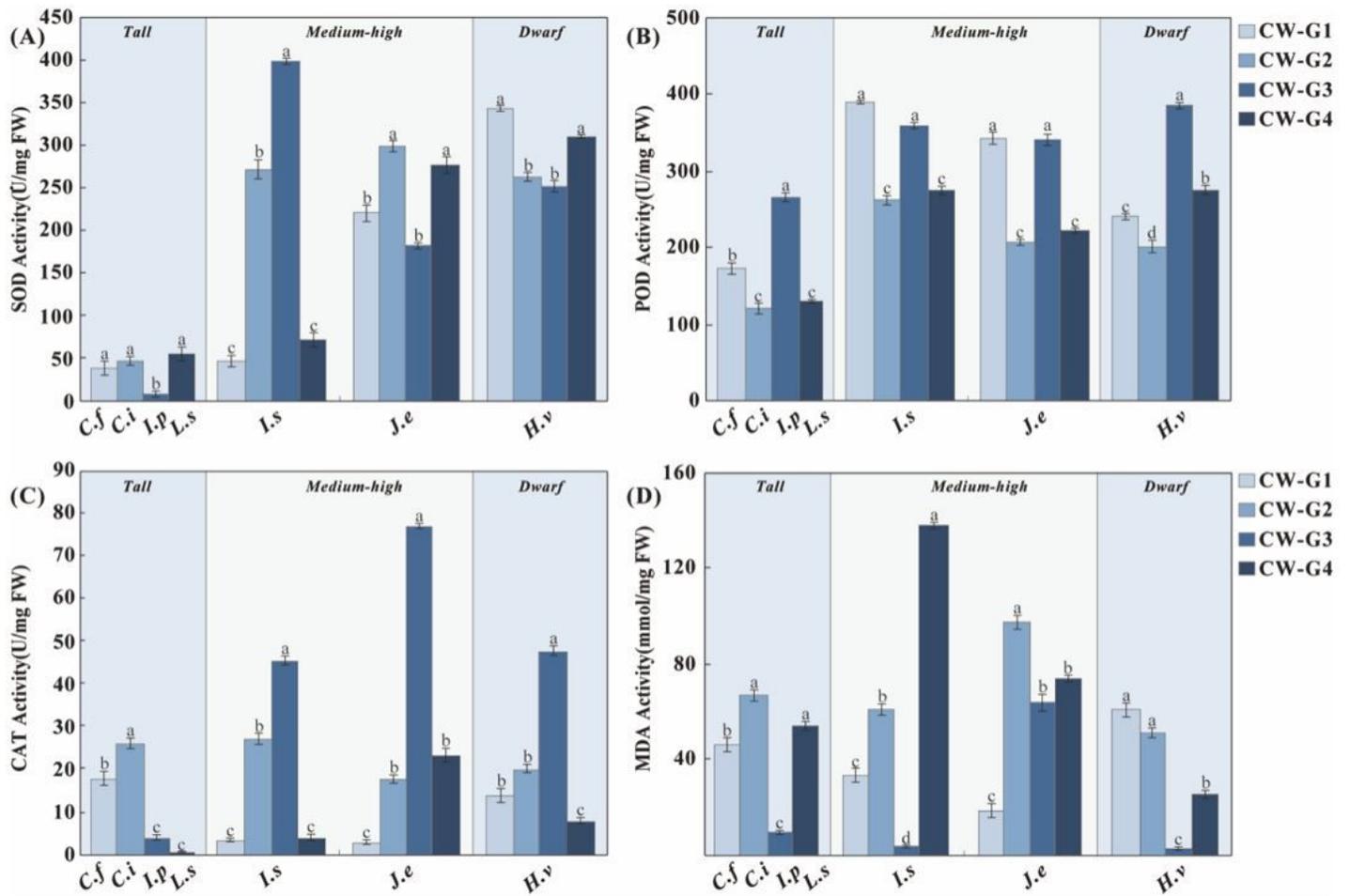


Figure 3

Bar charts of increased enzyme activities after 4 days treatment. (A) SOD; (B) POD; (C) CAT; (D) MDA. Tall plants (C.f: *Canna indica* var. *Flava*; C.i: *Canna indica*; I.p: *Iris pseudacorus*; L.s: *Lythrum salicaria*); Medium-size plants (I.s: *Iris sibirica*; J.e: *Juncus effuses*); Dwarf plant (*Hydrocotyle vulgaris*).

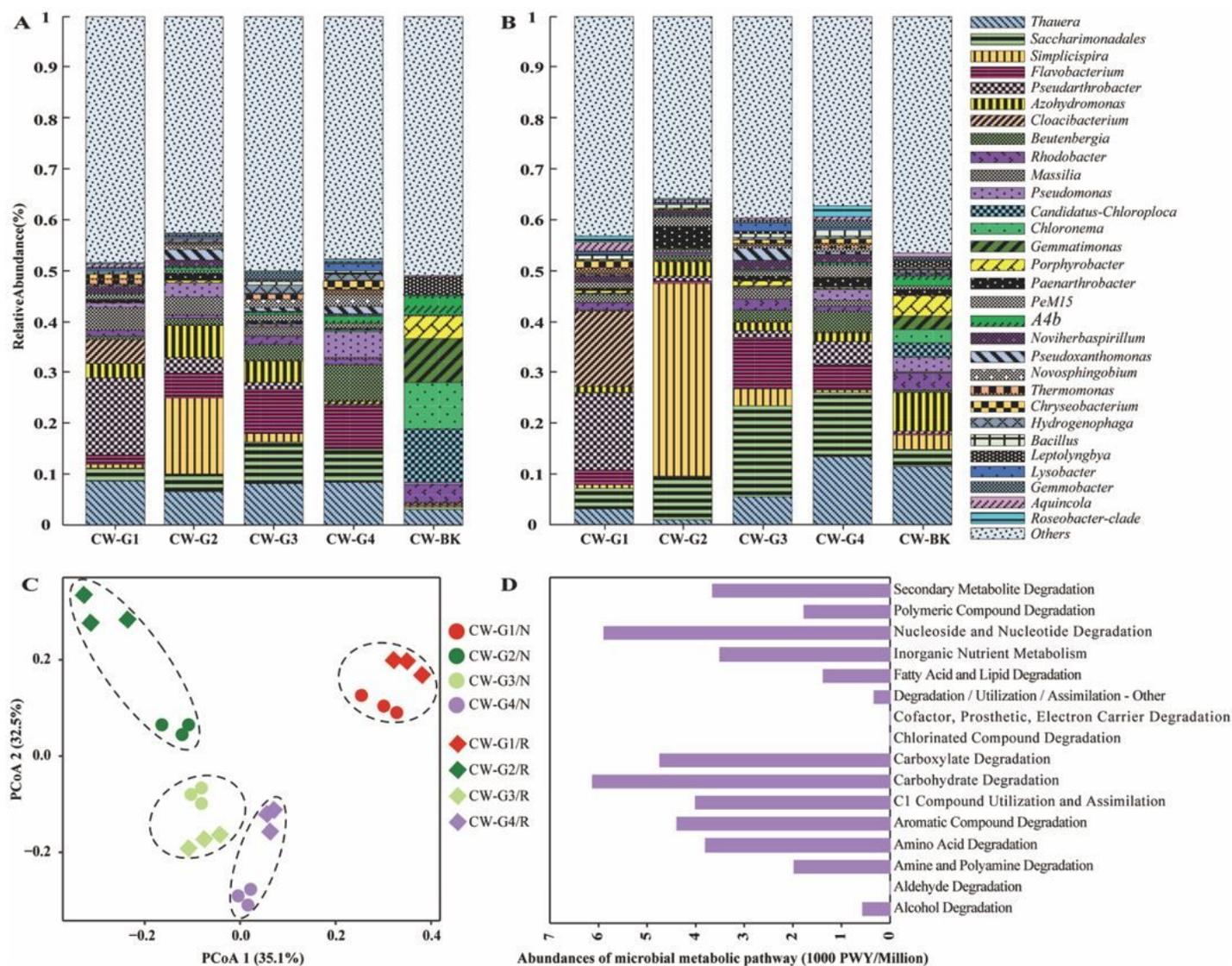


Figure 4

Microbial community distributions of OTUs at genus levels in the samples of rhizosphere (A) and non-rhizosphere (B). C: Principal co-ordinates analysis, N represents non-rhizosphere, R represents rhizosphere. D: Degradation/utilization/assimilation pathways of microbial community.

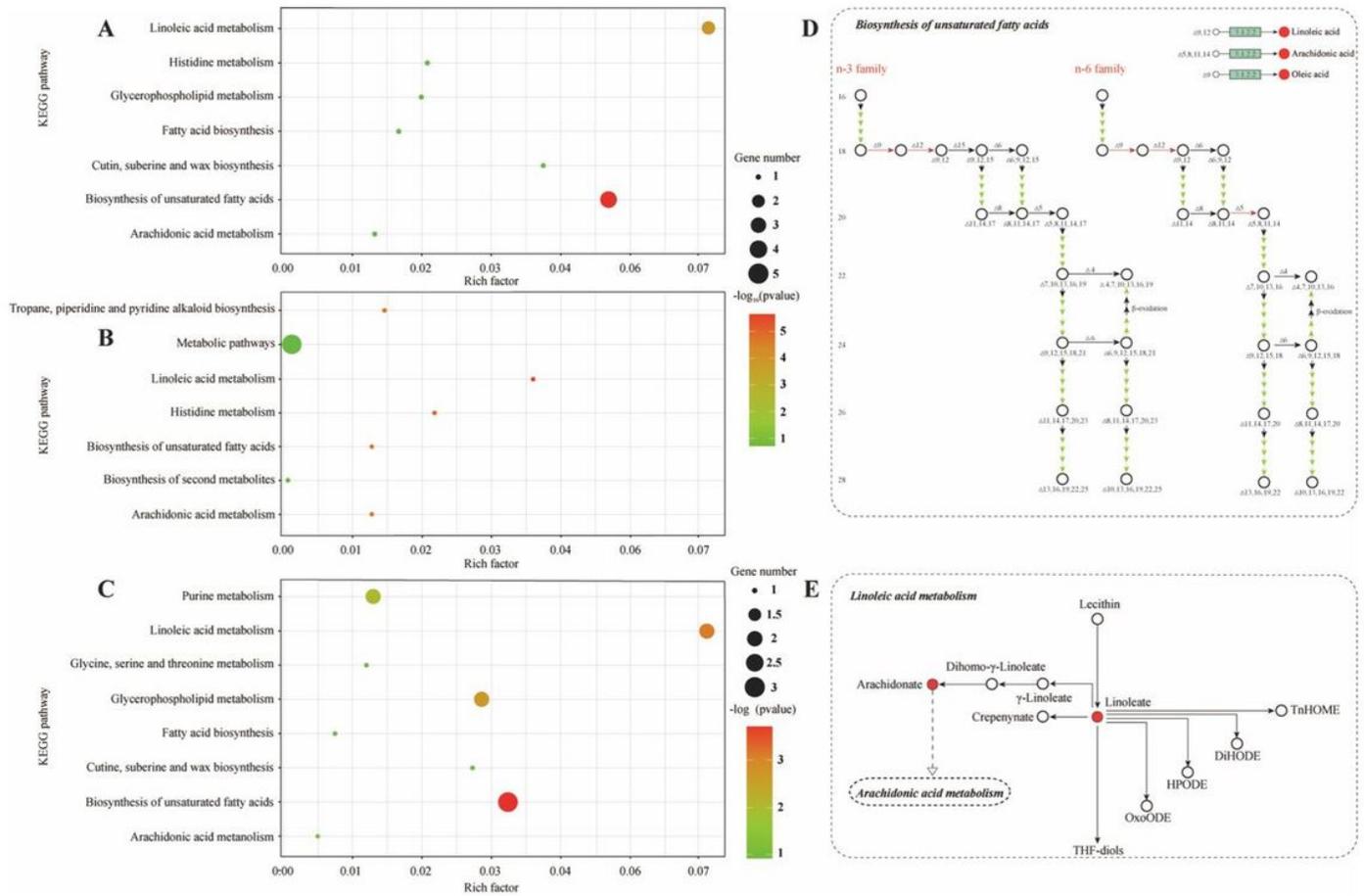


Figure 5

Bubble plot of the significantly different KEGG pathway of root exudates and KEGG pathway figure. A: CW-G3 Vs CW-G1; B: CW-G3 Vs CW-G2; C: CW-G3 Vs CW-G4. D and E: KEGG pathway-biosynthesis of unsaturated fatty acids and linoleic acid metabolism, respectively, and red dots represent significantly regulated metabolites.

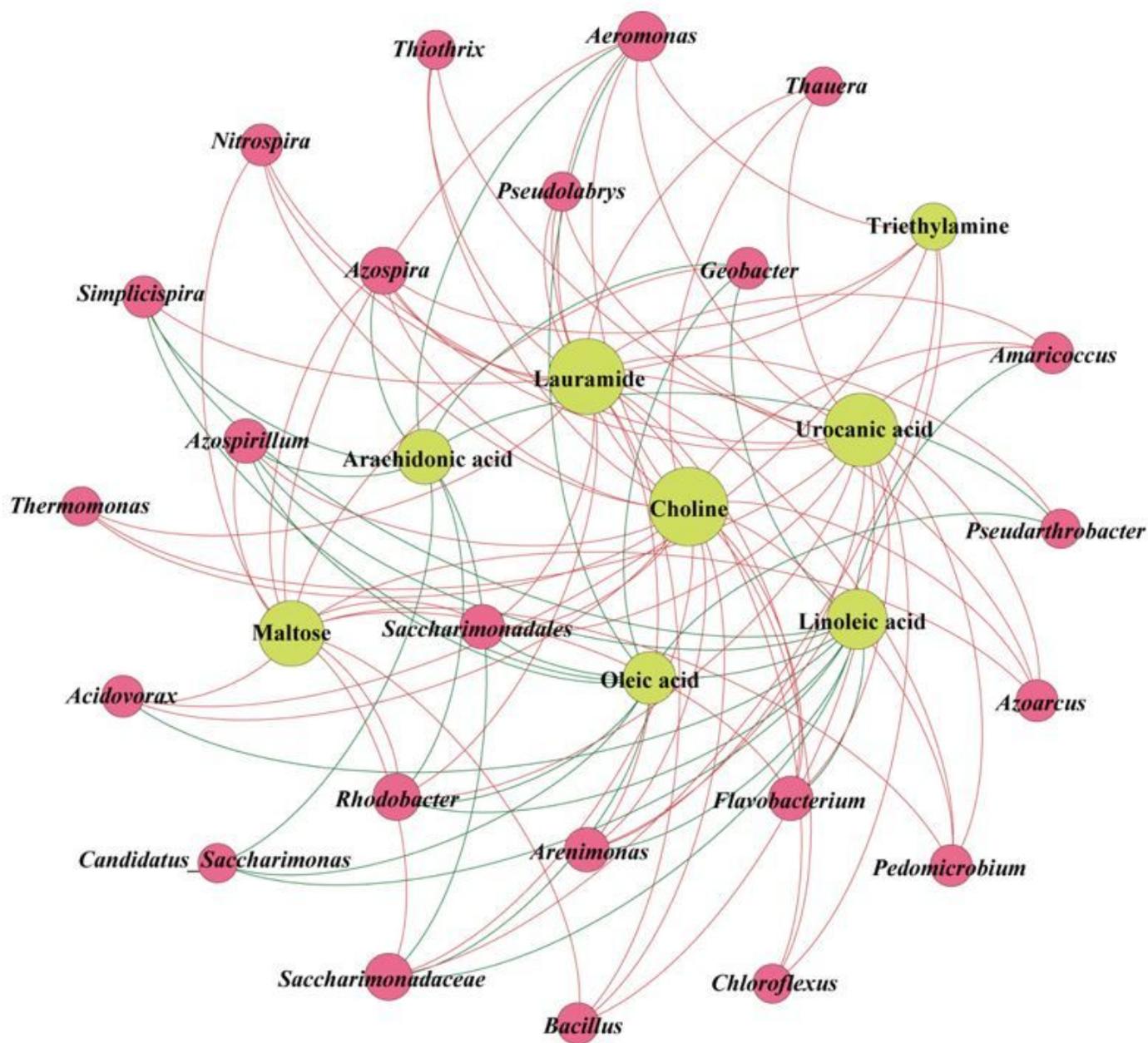


Figure 6

Co-occurrent network between bacteria and root exudates. Red lines represent positive correlation, green lines represent negative correlation. Green dots: root exudates; red dots: bacteria.

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