

# The impacts of cadmium exposure on epiphytic bacterial communities and water quality in mesocosmic wetlands.

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## Research Article

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# Abstract

The damaging magnitudes of heavy metal pollution on freshwater ecosystems are well known, though research on the specific impacts of cadmium (Cd) on water quality and microbial communities in epiphytic biofilms is lacking. In this study, high-throughput sequencing and Scanning Electron Microscopy (SEM) were used to explore the effects of Cd on water quality and bacterial morphology, biodiversity, interactions, and ecological functions in epiphytic biofilms affixed to submerged plants that were natural and synthetic (*Vallisneria natans*, *Potamogeton maackianus*, and artificial macrophytes) in constructed wetlands. The results showed that Cd exposure significantly reduced the ability of natural and artificial plants to remove total nitrogen (TN) (5.7-50%), total phosphorus (TP) (12.5-97.1%), and chemical oxygen demand (COD) (9.45-21.8%), and affected the morphology of epiphytic bacteria. The bacterial  $\beta$ -diversity indices were significantly affected by Cd exposure, whereas bacterial  $\alpha$ -diversity revealed a reverse trend. Notwithstanding Cd application induced the fluctuation (increase/decrease) in bacterial composition dynamics, but the relative abundance of Proteobacteria and Cyanobacteria spiked from 11.3-57.2% and 6.08- 94.35 %, respectively, in natural and artificial plants. Besides, Cd loading disturbed all bacterial network structures, with interactions particularly vulnerable in *P. maackianus*. Our study demonstrated that Cd exposure alters the bacterial diversity, food web structure, and predicted metabolic functions such as metabolism, translation, cell motility, signal transduction, membrane transport, and biodegradation of xenobiotics in epiphytic bacterial biofilms. These findings highlight the detrimental effects of Cd on epiphytic bacterial communities and nutrient removal in constructed wetlands.

## 1. Introduction

Heavy metals (e.g., Cu, Hg, Cd, and Pb) are widespread harmful pollutants that pose a severe worldwide health risk in aquatic ecosystems (Castillo Lora et al., 2019; Ren et al., 2019) due to their high toxicity and persistent biomagnification in food webs (Lu et al., 2018; Yang et al., 2014). For example, Chang et al. (2022a) and Huang al.(2020) revealed that Pb, Cu, and Cd affect the biofilm microbial communities. Besides, Zn, Cd, and Pb exerted a serious threat to the aquatic biofilms (Bere and Tundisi, 2012a; Geng et al., 2019; Kalita et al., 2019).

The key natural and anthropogenic sources of cadmium are volcanoes, rock weathering, forest fires, Ni-Cd batteries, PVC stabilizers, synthetic pigments, phosphate fertilizers, coatings, and metals smelting(Burger, 2008; Patar et al., 2016). The cadmium (Cd) levels were reported up to 4500  $\mu\text{g}/\text{L}$  in heavy metal polluted water bodies, whereas the Cd regulation standard is 30  $\mu\text{g}/\text{L}$  in China (Feng et al., 2010). Furthermore, it may range from 0.005- 0.1 mg/L in tropical freshwater periphytic communities (Bere and Tundisi, 2012a), 900–1100  $\mu\text{g}/\text{L}$  in marine sediments (ICdA, 2012), and up to 1.48 mg/kg in soils(Lenntech, 2019) and 200 mg/L in phosphorus fertilizers (Cook, M.E., 1995).

The Cd discharge into the aquatic ecosystem may produce a high bioaccumulation propensity for living biota such as plants, microbes, invertebrates, fishes, etc. (Martins et al., 2004). For example, Cd ions

impact photosynthesis by impairing chlorophyll synthesis(Li et al., 2008), alters antioxidant activity and enhance production of reactive oxygen species (ROS) that disrupt cell organelles and microbial structure(Gong et al., 2018; Singh et al., 2006), hamper the uptake and translocation of essential elements resulting in growth reduction(Hassan et al., 2008), and impair plasma membrane integrity by increasing lipid peroxidation (Tian et al., 2011). Consequently, broad public concerns have been raised recently about the hazardous effects of Cd on aquatic biota.

Constructed wetlands (CWs) have gained popularity as economical and environmentally acceptable restoration methods that offer the best substrate for microbial development and treatment(Ohore et al., 2022). The adsorption, absorption, and removal of pollutants by wetlands depend heavily on submerged macrophytes, substrates (both natural and artificial), and microbial biofilms, which are all critical parts of CWs(Kataki et al., 2021).

For example, due to their fixed submersed lifestyle, submerged plants can alter ecological food chain equilibrium and act as pollution bioindicators in aquatic systems(Rezania et al., 2016). Submerged macrophytes play a crucial role in bioremediating inorganic and organic contaminants in wastewater by directly up taking various nutrients from the water column and provide the ideal substrata for epiphytic biofilms (Qiu et al., 2021). Conversely, heavy metals such as Cd and Pb in wetlands could considerably influence the aquatic systems, and its phytoremediation potential is shaped by heavy metal concentration, pH, Eh, TSS, sulfide, and OM contents, etc. (Beni and Esmaeili, 2019). Henceforth, assessing the possible harm that Cd may cause to the wetland ecology is critical.

Extracellular polymeric substance (EPS) matrix encased bacteria, algae, fungi, protozoa, and other metazoans forming the complex microbial communities known as epiphytic biofilms, which develop on the surfaces of macrophytes (Manirakiza et al., 2022; Wijewardene et al., 2022). The preservation of ecosystem structure, interactions, and biogeochemical cycling are just a few of the several roles these microbial communities in epiphytic biofilms play in aquatic habitats (Wijewardene et al., 2022; Zhang et al., 2020). The assemblage of the wetland biofilm communities changes in response to contaminants, impacting their microbiological metabolic processes(Gong et al., 2018). Nevertheless, the biofilm EPS matrix offers greater tolerance compared to planktonic microbes. For instance, polyanionic groups can adsorb heavy metals on biofilm EPS matrix by ion exchange and complexation and surface precipitation(D'Acunto et al., 2016; Li and Yu, 2014), protecting bacteria from harmful compounds(Hu et al., 2019). The ecological menaces of heavy metals in plants and animals and their buildup in wetland water bodies and sediments have been the subject of previous field research(Ajmal et al., 1987; Demirak et al., 2006; Yusuf et al., 2019).

Notwithstanding the presence of heavy metals stress and accumulation in submersed plants, animals, water, and sediments have received much attention (Mu et al., 2018), however, the effects of Cd exposure on epiphytic biofilms and water quality in constructed wetlands have not been thoroughly studied and are still poorly understood. Prior reports focused on heavy metals toxicity and buildup in various aquatic plants, animals (e.g., *Rana chensinensis* tadpoles), and periphytic biofilms (Bere and Tundisi, 2012b; Mu

et al., 2018). Handful reports explored the impact of heavy metal stresses on bacterial community structure in epiphytic biofilms. For example, Fan et al. (2021) found that most metal (loid) concentrations (apart from Hg and Cr) were greater in epiphytic biofilms than those in submerged macrophytes (*Potamogeton lucens* L. and *Myriophyllum verticillatum* L.). Besides, Geng et al. (2022) explored the bacterial community structure in epiphytic biofilm on *Potamogeton crispus* L. and its contribution to heavy metal (Cu and Cr) accumulation in an urban industrial area in Hangzhou. The findings indicated that the epiphytic biofilm had a beneficial effect on metal bioaccumulation and this ability increased with the hydrodynamic forces. However, Huang et al. (2020) and Chang et al. (2022b) showed that heavy metals (e.g.,  $\text{Cu}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Cd}^{2+}$ ) inhibited the bacterial abundance, diversity, and function (e.g. nitrogen and sulfur cycling) in epiphytic biofilm on *V. natans* and *Myriophyllum spicatum*. Notably, the impact of single heavy metal stress was more damaging compared to the impact of various heavy metals, and the combined stress did not always result in synergistic effects. Recent studies by Zhang et al. (2022) and Yan et al. (2019) showed that exposure to nitrogen compounds (e.g., nitrate and ammonium) and heavy metals had altered the food web structure and bacterial ecological functions. As far as the authors know, no study has examined how Cd exposure impacts wetlands' capacity to remove nutrients and the morphology, structure, interactions, and metabolic processes of the bacterial communities that live there. Hence, the study aimed to: 1) examine the impact of Cd exposure on wetlands' capacity to remove nutrients, 2) examine the effects of Cd exposure on bacterial community morphology and structure in epiphytic biofilms, and 3) examine the effects of Cd loading on potential metabolic functions of bacterial epiphytic biofilm communities. This work shed important insight into the effects of Cd on the shape, variety, and composition of bacterial communities, interactions, and ecological activities in epiphytic biofilms, as well as the capability of artificial wetlands to remove nutrients.

## 2. Materials and methods

### 2.1. Materials and chemicals

Submersed macrophytes *Vallisneria natans* and *Potamogeton maackianus* were collected from a lake in Gaochun District (Nanjing, China) and pre-cultured for two days before acclimatization. The soil was collected from the back hill of Hohai University in Gulou District (Nanjing, China) and pre-treated by removing stones or blocks and plant debris. Besides, it was tested for Cd level and transferred to the plastic tanks or buckets. Nutrient solutions, encompassing Potassium dihydrogen phosphate (3 mg/L), Potassium nitrate (15 mg/L), Ammonium sulfate (5 mg/L), and Sucrose (30 mg/L), were added to the overlying water to boost submersed plants and epiphytic bacteria growth.

### 2.2. Constructed wetland setup, experimental plan, and sampling.

About 500 g of healthy submersed macrophytes (*V. natans* and *P. maackianus*) with the same stages of growth (20-30 cm height, 9-8 cm length, and 2.5-2.4 g weight for each plant) and synthetic plants were planted in 12 plastic tanks (67 x 46 x 27 cm each). The individual plastic tanks held 90 L and 10 cm thick

soil (Yan et al., 2018) at a density of 519 g/m<sup>3</sup>. Notably, this experiment used new and well-sealed polyethylene (PE) tanks in order to prevent heavy metals leaching.

Prior Cd loading, *P. maackianus*, *V. natans*, and synthetic plants were acclimated for 7 days. The stock solution of Cd was prepared by dissolving Cd<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>·6H<sub>2</sub>O in dechlorinated tap water to a final concentration of 2240 µg Cd/L. The treatment solutions were then prepared by diluting the stock solution to 500 µg Cd/L, 1000 µg Cd/L, and 2000 µg Cd/L. At the end of the experiment, the treatments were explored for available metabolites to characterize the impact of heavy metals on the submerged plants.

The experiments were carried out to determine the ecotoxicological influence of Cd on water quality and epiphytic microorganisms in constructed wetlands. The experiments were carried out to determine the ecotoxicological influence of Cd on water quality and epiphytic microorganisms in constructed wetlands. The concentrations of Cd added to the overlaying water harboring aquatic plants (*V. natans*: VN, Artificial plant: AP, and *P. maackianus*: P) were 0 mg/L (control group with 0 mg/L Cd concentration: AP0, VN0, and P0), 0.5 mg/L (treatment group with 0.5 mg/L Cd concentration: AP0.5, VN0.5, and P0.5), and 2 mg/L (treatment group with 2 mg/L Cd concentration: AP2, VN2, and P2). There were duplicate samples used for each treatment group. The experiments were carried out in the greenhouse at Hohai University's Key Laboratory of Integrated Management and Resource Development of Shallow Lakes (32°03'24.7"N 118°45'32.4"E) for 1 month (August-September).

### 2.3 SEM investigation for bacterial morphology

Artificial plants and similar growth young leaves of *V. natans* and *P. maackianus* were indiscriminately selected for scanning electron microscope (SEM) (Hitachi, Japan, S-3400N) analysis according to our recent report (Chen et al., 2023).

### 2.4 Determination of water quality

The physico-chemical parameters, including pH, electrical conductivity (EC), dissolved oxygen (DO), TP, TN, and COD, were assessed twice weekly employing the ammonium molybdate and alkaline potassium persulfate digestion methods using AutoAnalyzer3 (SEAL, Germany), while COD was determined by the dichromatic oxidability method. Besides, Cd concentrations were quantified for 30 days in overlying water, leaves, and sediments post cadmium exposure using an inductively coupled plasma-mass spectrometer (ICP-MS; Thermo Fisher Scientific, USA).

### 2.5 Biofilm Collection, DNA Extraction, and 16S rRNA Illumina Sequencing

Bacterial biofilms were extracted from 40g of *V. natans* and *P. maackianus* fresh leaves and artificial plants according to the methods described in Manirakiza et al. (2022). The biofilm DNA was extracted using E.Z.N.A.® soil DNA Kits (Omega Bio-Tek, Norcross, GA, U.S.A.) per the manufacturer's instructions. Using 1% agarose gel electrophoresis and the NanoDrop One fluorometer, DNA samples were evaluated qualitatively and quantitatively (ThermoFisher Scientific, MA, USA). The BioRad S1000 was used for PCR

amplification (Bio-Rad Laboratory, CA, USA). For Illumina sequencing, three aliquots of DNA samples from three different biological replicates were mixed. To examine bacterial communities, 16S rRNA gene primers (515 F, 5'-GTGCCAGCMGCCGCTAA-3', and 806 R, 5'-GGACTACNVGGGTWTCTAA-3') were employed. The Illumina sequencing procedures were performed according to our recent study (Chen et al., 2023).

USEARCH software (v8.0.1517, <http://www.drive5.com/usearch/>) was used to perform the sequencing analysis. Bacterial sequences with  $\geq 97\%$  similarity were allocated to various taxonomic levels (OTUs, genus, and phylum) utilizing a greedy algorithm in UPARSE (version 7.1 <http://drive5.com/uparse/>). By using UCHIME, the chimeric sequences were identified and edited. The Silva (SSU123) 16S rRNA database was used as a reference for the RDP classification method, which examined the 16S rRNA gene sequences. The sequence reads have been deposited in the NCBI SRA (under BioProject accession number: **PRJNA 943567**).

## 2.8 Statistical Analysis

The R environment (<https://www.r-project.org/>) and Majorbio Cloud platform ([www.majorbio.com](http://www.majorbio.com)) were employed for data processing and visualization. The 'vegan' package served to compute the alpha ( $\alpha$ ) and beta ( $\beta$ ) diversities, which were then visualized by 'ggplot2' package in R. The PERMANOVA (Permutational Multivariate Analysis of Variance) test was adopted to inspect the  $\beta$ -diversity between the bacteria community structure (OTUs) in control and treatment groups using the taxonomy-based (Bray-Curtis) distance. Circos and complex heatmaps were generated using R 4.1 (R Core Team, 2015), while bar graphs were created using Microsoft Excel (version. 2016) and Origin software (version 9.65). SPSS software (version 23.0) was used to perform a one-way analysis of variance (ANOVA) on physicochemical parameters. The network analysis was performed using the corr.test function of the "psychology" package in R (version 3.5.3) and then shown in Gephi. (version 0.9.3). Functional metabolic pathways of bacterial communities were predicted using PICRUSt (Langille et al., 2013) and compared based on Fisher's exact test using STAMP bioinformatic tool.

## 3. Results and discussions

### 3.1 Cadmium accumulation kinetics in constructed wetlands

Several recalcitrant pollutants, including heavy metals, are found in aquatic ecosystems. Though, our understanding of what happens to Cd in a wetland ecosystem that supports submerged macrophytes is limited. The concentration and removal rates of Cd were measured following a 30-day exposure of water, sediment, and submersed macrophyte leaves (*V. natans* and *P. maackianus*) to the metal. There was a decrease in Cd concentration in water (93.8–98.3%) (**Fig. S1 and Table S2-S3**) and submersed plant leaves (*P. maackianus* = 8.09–21.9%, *V. natans* = 16.03–26.17%, and Artificial plant = 0%), while sediment samples showed Cd accumulation (+ 55.5–83.3%) (**Fig. S1 and Table S2-S3**). Notably, the highest

decrease for Cd in the leaves was for *V. natans*, whereas artificial plants revealed the highest Cd concentration in sediments. This study suggests that submerged plants and their epiphytic bacteria reduced Cd from the overlying water after 30 days of treatment, whereas sediments are considered as sinks or repositories of various pollutants (Guo and Yang, 2016). The high Cd uptake by *V. natans* in this study may be due to its potential to remediate recalcitrant pollutants in constructed wetlands, while artificial plants lack innate features facilitating them to filter pollutants into the sediments, consistent with previous reports (Chen et al., 2023; Ohore et al., 2021a). Conversely, Qiu et al. (2021) revealed that artificial plants had better performance in nutrients removal (e.g., TN, TP, NH<sub>4</sub>-N, and COD) than *Myriophyllum Spicatum*. Thus, the difference in Cd removal rate may be attributed to Cd concentrations and aquatic plant types.

Figure 1

### 3.2 Cadmium impeded the growth of epiphytic biofilm

Cd had an impact on the development and colonization of epiphytic bacteria. Figure 1 displays the findings of the qualitative analysis of confocal laser scanning microscopy (CLSM) data. Different outcomes for epiphytic bacteria exposed to Cd and control groups were seen when SEM micrographs were visualized: different microbial groups, including algal cells (diatoms), cocci/cocci-like and bacilli/bacilli-like microorganisms, were displayed in SEM micrographs from artificial plants, *V. natans*, and *P. maackianus*. Cadmium considerably decreased the microbial aggregates (Fig. 1). It is worth mentioning that epiphytic biofilm microbial assembly on the submersed plant leaf surfaces declined as Cd concentration spiked. The decline in microbial aggregates on submerged plants with increased Cd concentration may be attributed to the Cd toxicity and allelopathic effect (natural plants) on bacteria growth and colonization (He et al., 2021; Huang et al., 2020). Additionally, bacterial and algal cells were mostly encapsulated in an EPS structure that has been densely produced on artificial plants (AP) and *P. maackianus* (P) with 0.5mg/L Cd concentration, consistent with prior reports (Chen et al., 2023; Ohore et al., 2021b). The presence of more epiphytic microbes on artificial plants than on *V. natans* may be due to the artificial plants' inability to discharge allelochemicals, which impede microbes on the plastic surface.

Surprisingly, the bacterial cells (e.g., bacilli/bacilli-like) in VN0.5, AP1, and AP2 become elongated, while AP0.5 and P0.5 exhibited a high EPS production. These findings suggest that cell elongation and EPS production could be attributed to a bacterial defense mechanism against Cd toxicity and Cd-resistance genes dissemination, in agreement with previous studies (Chen et al., 2023; Pu et al., 2021). A reversal mechanism or resistance of the microbes against Cd stress could be the reason for this observation. Notwithstanding the hardness of the diatom silica cell wall, there was a rupture of the algal (diatoms) cell wall on *V. natans* (VN1 and VN2) and *P. maackianus* (P1&P2).

Figure 1

### 3.3 Cadmium application lowered the capacity of wetlands to remove nutrients.

Generally, this study demonstrated a significant decrease in nutrient removal capacity in the Cd treatment groups compared to the Control groups ( $p < 0.05$ ) (Fig. 2). In week 1, the average removal rate recorded after 30 days increased from 6.9 to 25.5% for TN, but it fell from 5.7 to 50% in the subsequent three weeks (Fig. 2B). Besides, the average TP removal rate increased from 16 to 30%, while in the following three weeks, the removal rate decreased from 12.5 to 97.1% (Fig. 2A). Moreover, the average COD removal rate increased from 4.2 to 14.28% in weeks 1 and 4, whereas it dropped from 9.45 to 21.8% in the second and third weeks (Fig. 2E). Notably, the TN, TP, and COD removal rates also decreased with increasing Cd concentrations. The decrease in TP removal efficacy can be attributed to the limitation in phosphorus removal, which is a result of decreased enzyme activities caused by Cd (Chen et al., 2014). The declined removal capacities of TN and COD are likely due to the effects of Cd stress on the epiphytic biofilm and the microbial communities in the wetland's mesocosm, which are responsible for nutrient metabolism and degradation (Li et al., 2018; Yang et al., 2019), and this finding aligns with those of Ohore et al. (2021b). Whereas the increased COD removal rate with exposure time is possibly due to Cd inactivation in the mesocosmic wetland.

Cd application significantly increased the DO and EC with exposure time, while decreasing the pH in the water column ( $p < 0.05$ ) (Fig. 2C-D &F). The increase of DO may possibly due to the oxygen release by submersed plant photosynthesis (Pedersen et al., 2013). The spike in EC values may be due to nutrient retention in the water column (Ohore et al., 2021b). The high Cd concentration can exert a deleterious impact on the submerged plant- epiphytic biofilm systems, which are involved in the sequestration of ions from the surrounding water column. Whereas the increase in pH may be ascribed to CO<sub>2</sub> emission from the rhizosphere of submerged macrophytes to water, as a result of reduced biofilm and increased chlorophyll contents (Ohore et al., 2021b). The findings of this study collectively indicate that Cd significantly affected the water quality and hindered the nutrient removal capacity of wetlands.

Figure 2

## 3.4 Cd exposure effect on epiphytic bacteria diversity

To investigate the impact of Cd exposure on bacterial diversity in the leaves of *V. natan's*, *Potamogeton maackianus*, and artificial plants, Illumina sequencing was utilized, and a uniform number of 1988 clean 16S raw sequences were obtained in all samples. **Table S1** shows the Shannon, Simpson, Ace, Chao, and Coverage values for all bacteria samples. These alpha diversity indices (Fig. 3A) revealed no significant difference ( $p > 0.05$ ) between the treatment groups (VN0.5, VN1, VN2, P0.5, P1, P2, AP0.5, AP1, and AP2) regardless of Cd exposure, consistent with a previous study (Mu et al., 2018). However, most  $\alpha$ -diversity indices were significantly different ( $p < 0.05$ ) in epiphytic biofilms and intestinal microbiota under Cd exposure (Huang et al., 2020; Mu et al., 2018). The findings indicate that the epiphytic biofilm may have sustained its microbial diversity profile to counteract the impact of Cd stress and/or the present metal stress may not have been substantial enough to cause notable alterations in the alpha diversity. This could potentially be attributed to factors such as Cd concentration, exposure duration, and host species.

The principal coordinate analysis (PCoA) for the bacterial community explained approximately 50.63% of the overall variation. The analysis demonstrated that there were significant statistical differences in the dissimilarity of bacterial communities across the samples ( $p < 0.05$ ) (Fig. 3B). These results suggest that Cd pressure drives important roles in the structure of bacterial communities.

Figure 3

### 3.5 The impact of Cd exposure on the composition of epiphytic bacteria

The impact of Cd exposure on bacterial composition was examined. (Fig. 4). The relative abundance of epiphytic bacterial species (OTUs) in the control group (SM\_Cd\_0mg/L) declined as Cd concentration rises (Fig. 5A). The results showed that the control group had a greater number of bacterial OTUs compared to the Cd treatment groups (SM\_Cd\_0.5mg/L, SM\_Cd\_1mg/L, and SM\_Cd\_2mg/L) (Fig. 4A). This suggests that the high Cd concentrations had a detrimental impact on the epiphytic bacterial communities of *V. natans*, *P. maackianus*, and artificial plants, which is consistent with recent studies highlighting the impact of different heavy metal concentrations on microbial composition in tadpole gut (Mu et al., 2018). Throughout the experiment, the 10 most abundant epiphytic bacterial phyla, on average, were selected for relative abundance analysis (Fig. 4B). The four dominant phyla in all samples were Bacteroidota (51.8%), Gemmatimonadota (49.9%), Acidobacteria (49.01%), and Actinobacteria (48.05%) (Fig. 4B), corroborating previous investigations (Huang et al., 2020; Mu et al., 2018).

Notably, Proteobacteria (14.4-50.52%), Firmicutes (4.5–68.3%), Cyanobacteria (16.4–46.1%), and Actinobacteria (71.5–93.6%) decreased under Cd exposure in *P. maackianus* treatment groups compared to the control group. In contrast, Gemmatimonadota, Acidobacteria, Myxococcota, and Chloroflexi showed a reverse trend (Fig. 4B). Furthermore, Gemmatimonadota (28.8-96.16%), Acidobacteria (62.5–75%), Verrucomicrobia (58.3–77.3%), and Chloroflexi (50%) decreased under Cd exposure in groups treated with artificial plants in comparison to the control group, whereas Bacteroidota, Proteobacteria, and Firmicutes spiked under Cd exposure in the same substrate. On the other hand, Proteobacteria (11.3–51.8%), Cyanobacteria (6.08–94.35%), Actinobacteria (55.9–86.6%), and Verrucomicrobia (37.8-87.01%) decreased under Cd application in *V. natans* treatment groups relative to the control, while Bacteroidota, Gemmatimonadota, and Firmicutes increased under Cd exposure in the same submersed plant. Huang et al.(2020) reported that Cd exposure instigated marked changes in the bacterial composition of *V. natans* and Nile tilapia. The increase/decrease or discrepancy in bacterial community composition under Cd loading noted in this study may be due to the heavy metal concentration and substrate type.

The Firmicutes and Bacteroidota relative abundances were significantly higher in *V. natans* ( $p < 0.05$ ) compared to *P. maackianus*, however, Actinobacteria and Chloroflexi in *P. maackianus* revealed a reverse trend (Fig. S2A). Additionally, Gemmatimonadota, Acidobacteria, and Myxococcota were significantly higher in *P. maackianus* ( $p < 0.05$ ) relative to artificial plants, while Firmicutes, Verrucomicrobia, and Cyanobacteria were significantly ( $p < 0.05$ ) higher in artificial plants than *P. maackianus* (Fig. S2B). Conversely, Verrucomicrobiota, Actinobacteria, Chloroflexi, and Proteobacteria, were of higher significant

( $p < 0.05$ ) occurring phyla in artificial plants than *V. natans*, though Gemmatimonadota, Acidobacteria, Myxococcota, and Bacteroidota exhibited significant dominance ( $p < 0.05$ ) in *V. natans* relative to artificial plants (Fig. S2C). The variation in bacterial genera under Cd application is depicted in Fig. 4C. The dominant genera, namely *g\_\_Aquabacterium*, *g\_\_Flavobacterium*, *g\_\_Rhizobium*, *g\_\_Rhodobacter*, *g\_\_Comamonas*, *g\_\_Gemmatimonas*, *g\_\_Pseudomonas*, *g\_\_Ideonella*, and *g\_\_Porphyrobacter*, accounted for 23.6%, 15.2%, 10.4%, 9.6%, 8.1%, 6.7%, 5.1%, 4.3%, and 3.8% of the bacterial genera in all samples, respectively (Fig. 4C). Notably, there is an increase in relative abundance of *g\_\_Nostoc* (31.9–33.1%), *g\_\_Gemmatimonas* (31.7%), *g\_\_Rhizobium* (27.1%), and *g\_\_Delftia* (33.02–33.1%) in VN, AP, and P, whereas *g\_\_Rhizobium* (23.4%), *g\_\_Comamonas* (21.4–22.89%), *g\_\_Leptothrix* (24.35–24.6%), and *g\_\_Gemmatimonas* (18.1%) decreased in AP and P. Bacterial communities inhabiting harsh ecological niches may survive only by acquiring resistance genes (e.g., heavy metal resistance genes), ensuring their survival and leading to an increase in their relative abundance. Conversely, the decrease in the relative abundance of bacterial phyla and genera might be attributed to the detrimental impact of high Cd concentrations on epiphytic bacterial communities (Mu et al., 2018). This could explain the observed variations in bacterial abundance. Firmicutes comprise pathogen-causing diseases (Fan et al., 2018), while Cyanobacteria sustain bacterial growth (Jiang et al., 2019). Chloroflexi is a slow-growing oligotrophy (Eo and Park, 2016; Will et al., 2010), whereas Actinobacteria and Acidobacteria show high metabolic versatility (Alvarez et al., 2017) and actively thrive in stressed conditions (Fierer et al., 2007). As a result, these bacterial phyla were linked to the ability to withstand heavy metal exposure and thrived.

Figure 4

### 3.6 Cd alters the bacterial network structure

To reveal the structure and interaction of the underlying bacterial communities in both control groups (AP0, PM0, and VN0) and Cd treatment groups (AP\_Cd, PM\_Cd, and VN\_Cd), co-occurrence networks were constructed using the top 48 dominating epiphytic bacterial OTUs. (Fig. 5). A total of 391 edges were identified by 48 nodes (OTUs), 412 edges by 48 nodes, 469 edges by 48 nodes, and 423 edges by 48 nodes in CT, AP\_Cd, PM\_Cd, and VN\_Cd, respectively (Fig. 5A-D).

The impact of Cd exposure on bacterial interactions was investigated by computing topological properties. The modularity and average degree were 2.77 and 17.16 in the AP\_Cd group, 1.11 and 19.54 in the PM\_Cd group, and 4.3 and 17.62 in the VN\_Cd group. According to Scheffer et al., 2012, modularity represents the system's capacity to resist environmental influences, whereas the average degree indicates the strength of the connection between a node and its neighboring nodes. Epiphytic bacterial interactions were more intense and susceptible in *P. maackianus* than in *V. natans* and artificial macrophyte biofilms, as shown by a lower average degree and higher modularity in the AP\_Cd and VN\_Cd groups (Fig. 5B-C) than the PM\_Cd group (Fig. 5D) (Montoya et al., 2006). In addition, a high modularity values in VN\_Cd and AP\_Cd groups than PM\_Cd group revealed that AP\_Cd and VN\_Cd networks have higher modular class ( $> 0.4$ ) (Zhang et al., 2019). This study demonstrates that the networks of *V. natans* and artificial plants were more stable and complex than the network of *P. maackianus*. Notably, there were more edges in VN\_Cd than AP\_Cd, suggesting that bacterial communities in artificial plants inhibited the bacterial

community stability than in natural submersed plants. These findings denote the improvement of bacterial community structure on *V. natans* than artificial plants under Cd loading, consistent with a recent study by Mu et al. (2021). Despite the existing research on the effects of heavy metals on natural macrophytes, studies on their impact on epiphytes in the plastisphere remain insufficient, necessitating further research. All networks' nodes (OTUs) were connected with four bacterial phyla. Among these, three (Proteobacteria, Bacteroidota, and Cyanobacteria) were prevalent, making up a significant proportion of 95.84% of nodes (Fig. 5A-D).

Based on the average degree, *Flavobacterium* and *Sphingorhabdus* (Bacteroidota), *Acinetobacter*, *Aeromonas*, *Hydrogenophaga* (Proteobacteria), and *Exiguobacterium* (Firmicutes) were the keystone taxa in *V. natans* and *P. maaackianus*, while *Novosphingobium*, *Sphingomonas*, *Comamonadaceae*, and *Phenylobacterium* were keystone taxa in artificial plants. The bacterial keystone genera in submersed macrophytes and artificial plants are important for plastic degradation and colonization (e.g., *Exiguobacterium*, *Novosphingobium*, *Sphingobacteria*, and *Flavobacterium*) (Dussud et al., 2018; Oberbeckmann and Labrenz, 2020; Yang et al., 2015), heavy metal biosorption (e.g., *Aeromonas*) (Qurbani and Rafiqi, 2022), bioremediation of xenobiotic compounds (e.g., *Acinetobacter* and *Phenylobacterium*) (Shelly et al., 2020). These findings indicate that the epiphytic bacteria can survive in environments contaminated with xenobiotics, such as heavy metals, hydrocarbons, and herbicides, and may have developed heavy metal resistance mechanisms to survive. Overall, the co-occurrence networks analysis displayed more stable and complex interactions among microorganisms on *V. natans* and artificial plants than *P. maackianus*, and those interactions include cooperation, competition, symbiosis, and parasitism among organisms in biofilms.

Figure 5

## 3.7 Cd altered bacterial metabolic pathways in epiphytic biofilm

KEGG annotation was used to investigate how Cd influences the metabolic pathways of the bacterial community in the epiphytic biofilms (level 2) (Fig. 6). The findings of this study suggest that the bacterial community's primary functional trait was metabolism, with prevalent metabolic pathways linked to energy metabolism, carbohydrate metabolism, amino acid metabolism, cofactors, and vitamins metabolism, signal transduction, membrane transport, translation, nucleotide metabolism, lipid metabolism, and xenobiotic biodegradation in both artificial and natural submersed macrophytes (Fig. 6). It should be noted that control samples showed high percentage values of metabolic pathways compared with treatment samples, consistent with our recent study (Chen et al., 2023).

According to the findings, Cd concentration decreased along several metabolic pathways in epiphytic bacterial populations, presumably as a result of Cd's toxic effects or selective pressure. However, Cd loading in VN1 and P2 triggered a spike in 16 metabolic pathways, which may be due to Cd diffusion impediment by EPS and resistant bacterial strains formation (e.g., Cd-resistant bacteria) (Abbas et al., 2018; Gebreyohannes et al., 2019; Hui et al., 2022).

From an evolutionary point of view, bacteria exposed to heavy metals spontaneously evolved numerous toxic metal homeostasis systems such as uptake, efflux, metallochaperones, detoxification, and sequestration, allowing bacteria to resist or remediate toxic heavy metals, especially Cd (Hui et al., 2022; Kim et al., 2018). These homeostatic mechanisms will eventually safeguard their survival, which explains the observed spike in the metabolic pathways.

Comparing the top 18 metabolic pathways, 11 in *P. maackianus*, 7 in *V. natans*, and 7 in artificial plants exhibited a significant decline ( $p < 0.05$ , **Fig. S3A-C**) when Cd concentration increased from 0-2mg/L. Noticeably, the metabolism (e.g., carbohydrate, lipid, amino acid, energy, amino acids, nucleotides, cofactor and vitamins, terpenoids and polyketides), xenobiotic biodegradation, membrane transport, signal transduction, translation, and cell motility (**Fig. S3A-C**) were significantly lower in heavy metal treatment groups than the control. The predicted functional analysis revealed that the epiphytic biofilm ecosystem's bacterial metabolic function was reduced, indicating the negative effect of Cd stress. For instance, the deterioration in cell powerhouse ATP generation under Cd stress may disturb the bacterial communities in the epiphytic biofilm of *P. maackianus*, *V. natans*, and artificial plants. Additionally, the decline in the translation process inhibits protein synthesis induced by Cd stress. These findings shed new light on the adverse effects of heavy metals on aquatic ecology.

Figure 6

## 4. Conclusion

In this work, the impacts of Cd were examined in relation to the bacterial communities that attach to *P. maackianus*, *V. natans*, and artificial plants in constructed wetlands. The findings indicated a reduction in TN, TP, and COD in Cd-treated groups compared to the control group. Under Cd exposure, the bacterial morphology was disrupted, while no significant alterations were noted in the  $\alpha$ -diversity indices. Despite a decrease in the relative abundance of most bacterial phyla, the relative abundance of phyla Bacteroidota, Myxococcota, and some genera (*Nostoc*, *Gemmatimonas*, *Rhizobium*, and *Delftia*) in Cyanobacteria, Gemmatimonadota, and Proteobacteria spiked in natural and artificial submersed plants. The study demonstrated that Cd significantly impacted the morphology, composition, interactions, and metabolic processes of epiphytic biofilm bacterial communities, resulting in a disturbance in food chain structures and metabolic pathways, particularly in the metabolism. Therefore, this study offers a thorough understanding of the impact of Cd on the bacterial communities in epiphytic biofilms and water quality.

## Declarations

### Declaration of 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.

The authors declare the following financial interests/personal relationships, which may be considered as potential competing interests:

**Ethics approval:** Not applicable

**Consent to participate:** Not applicable

**Consent for publication:** Not applicable.

### **Authors Contributions**

**Deqiang C.:** Conceptualization, Investigation, Methodology, Writing- original draft, Writing - review & editing.

**Adarkwa A. L.:** Conceptualization, Investigation, Methodology, Writing- original draft, Writing - review & editing.

**Manirakiza B.:** Conceptualization, Supervision, Writing - review & editing, Software

**Samwini A. M., Addo FG, Adomako W.B.,Bofah-Buoh R...:** Writing - review & editing, Methodology, Investigation.

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### **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

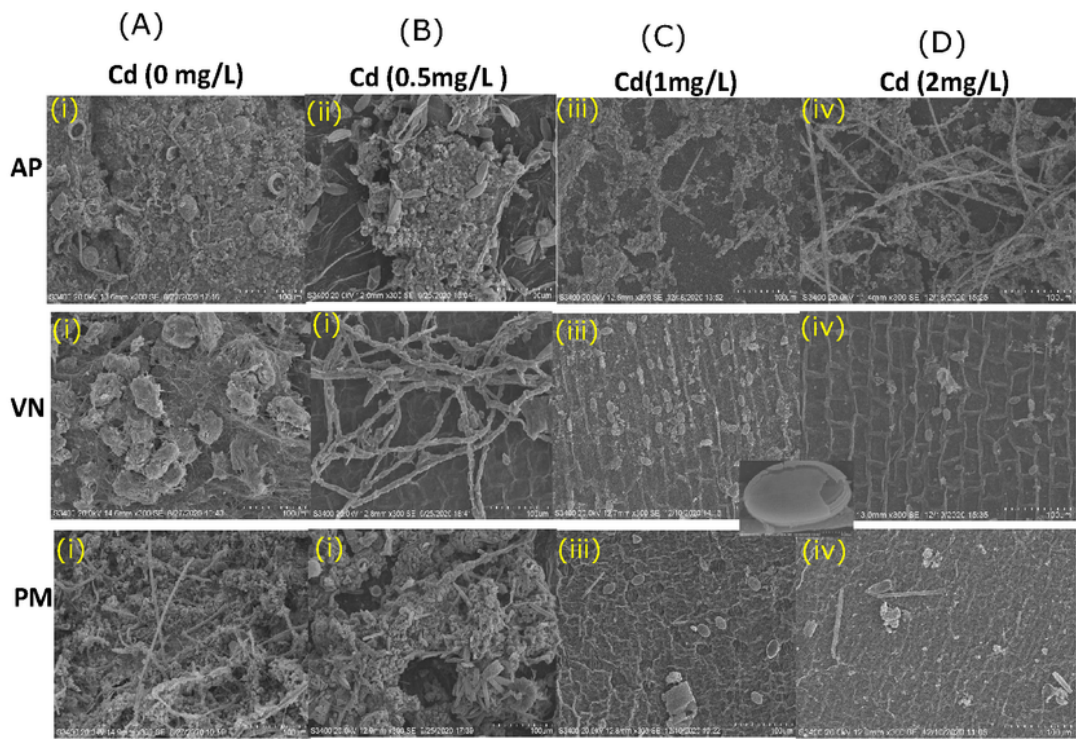


Figure 1

Scanning electron microscopy of artificial plants (A), *V. natans*(B), *Potamogeton maackianus* (C) treated with 0 mg/L, 0.5 mg/L, 1 mg/L (C), and 2 mg/L of Cadmium at day 31. Algae, bacillus/bacillus-like, and cocci/cocci-like bacteria were visualized in SEM images.

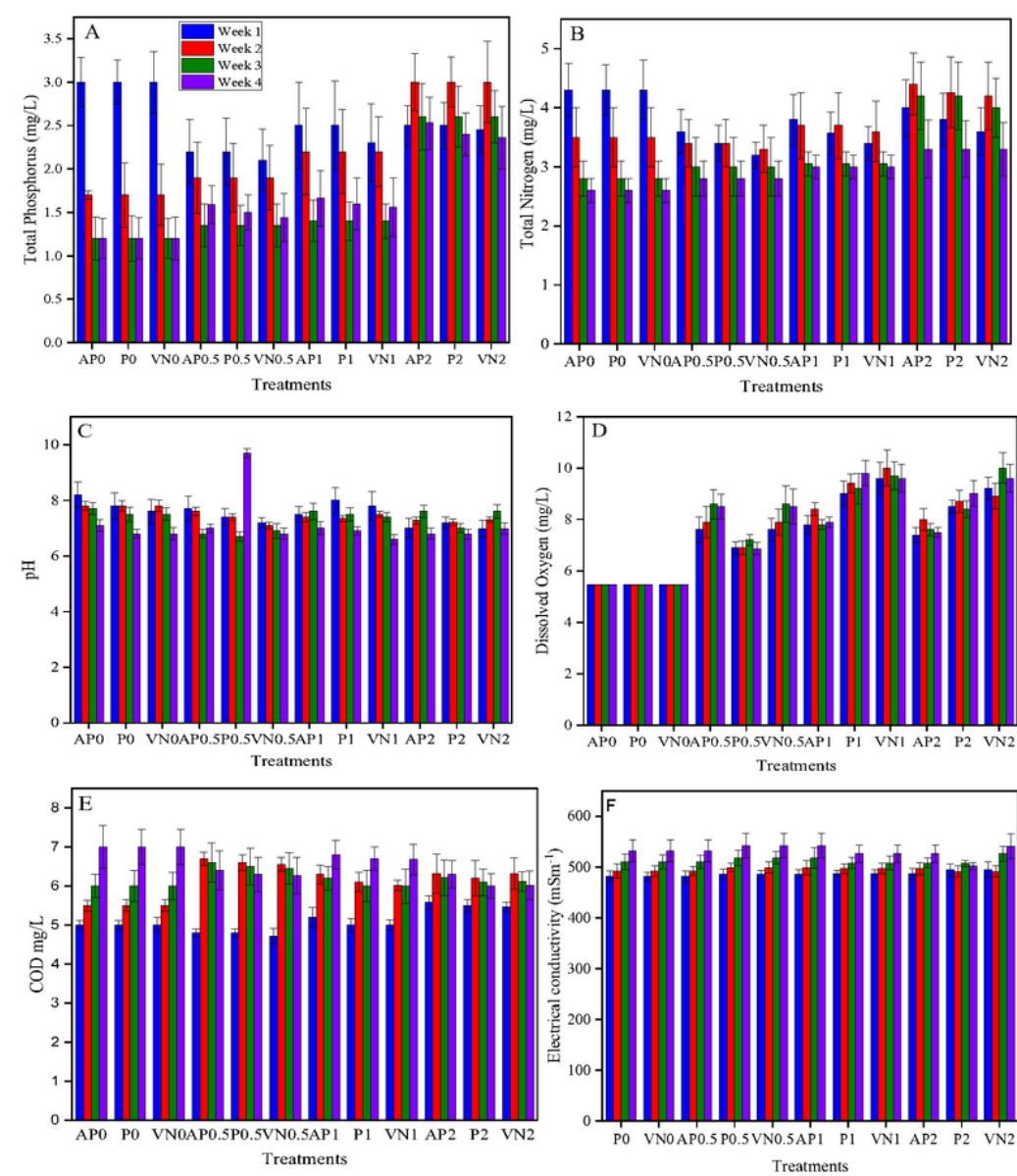
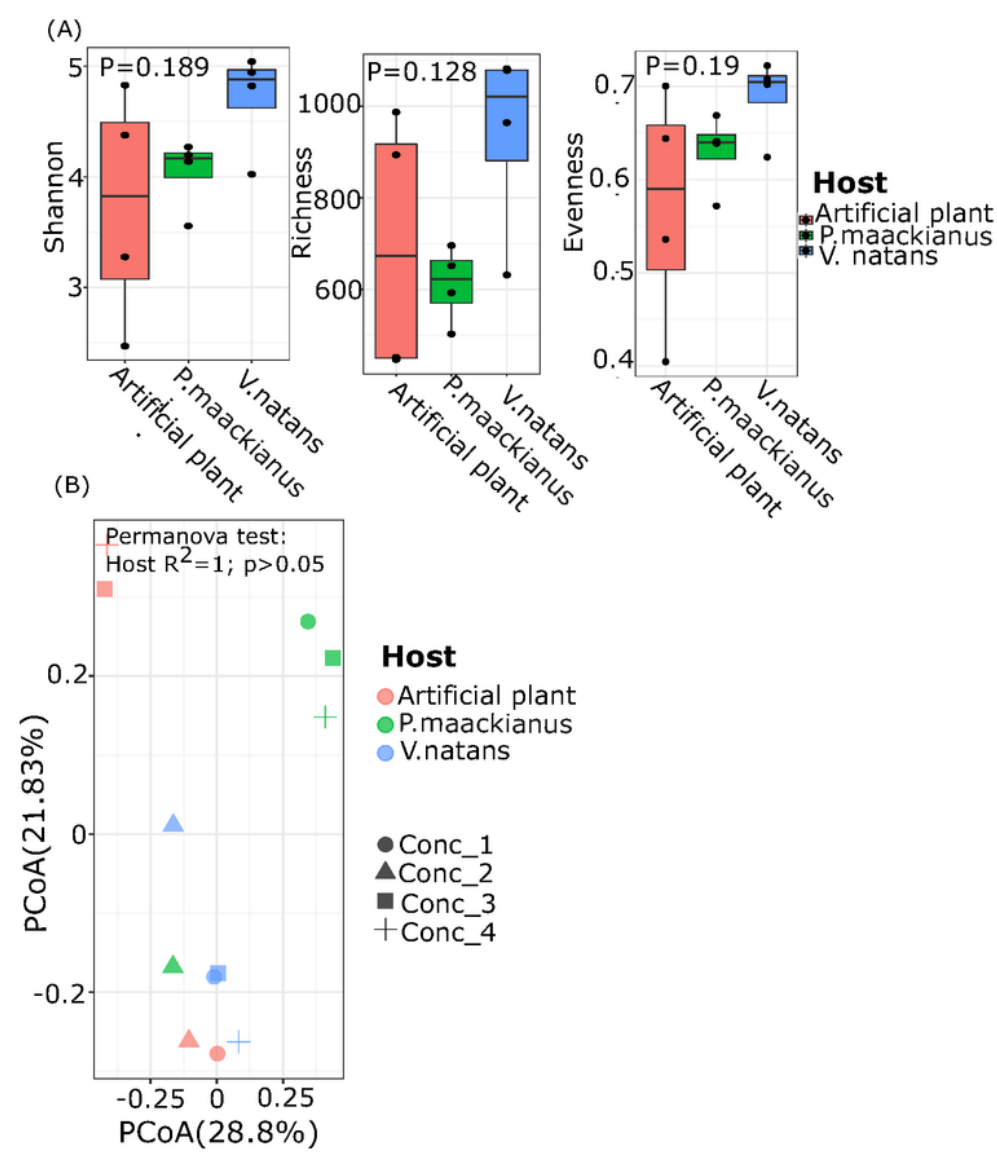


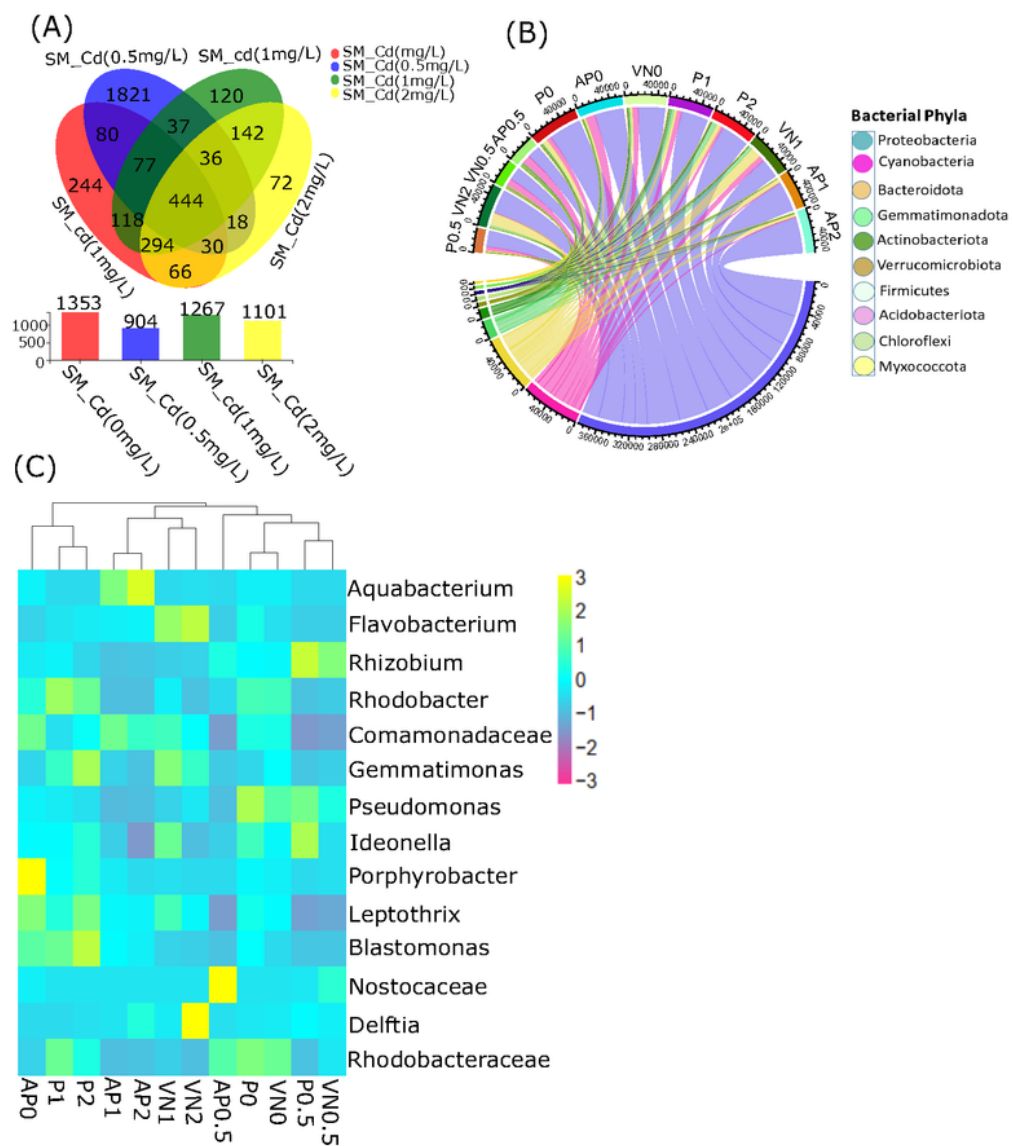
Figure 2

Physical (EC, DO, pH) and chemical (TN,TP, and COD) (A-F) parameters variation in the wetland dominated by *V. natans*, *Potamogeton maackianus*, and artificial plants under Cd (0 mg/L, 0.5mg/L, 1 mg/L, and 2 mg/L) stress.



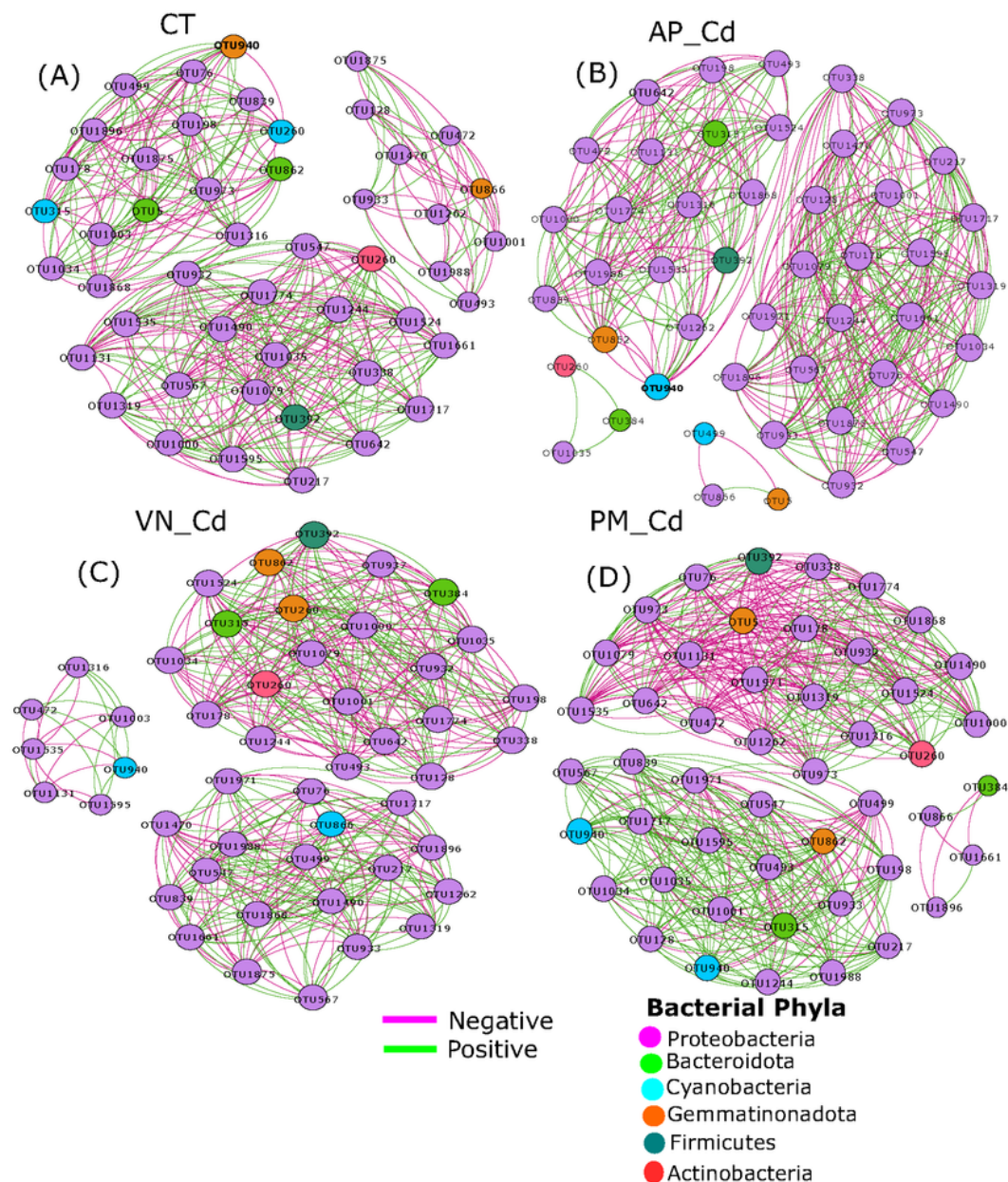
**Figure 3**

Effect of Cadmium exposure on epiphytic bacterial diversity



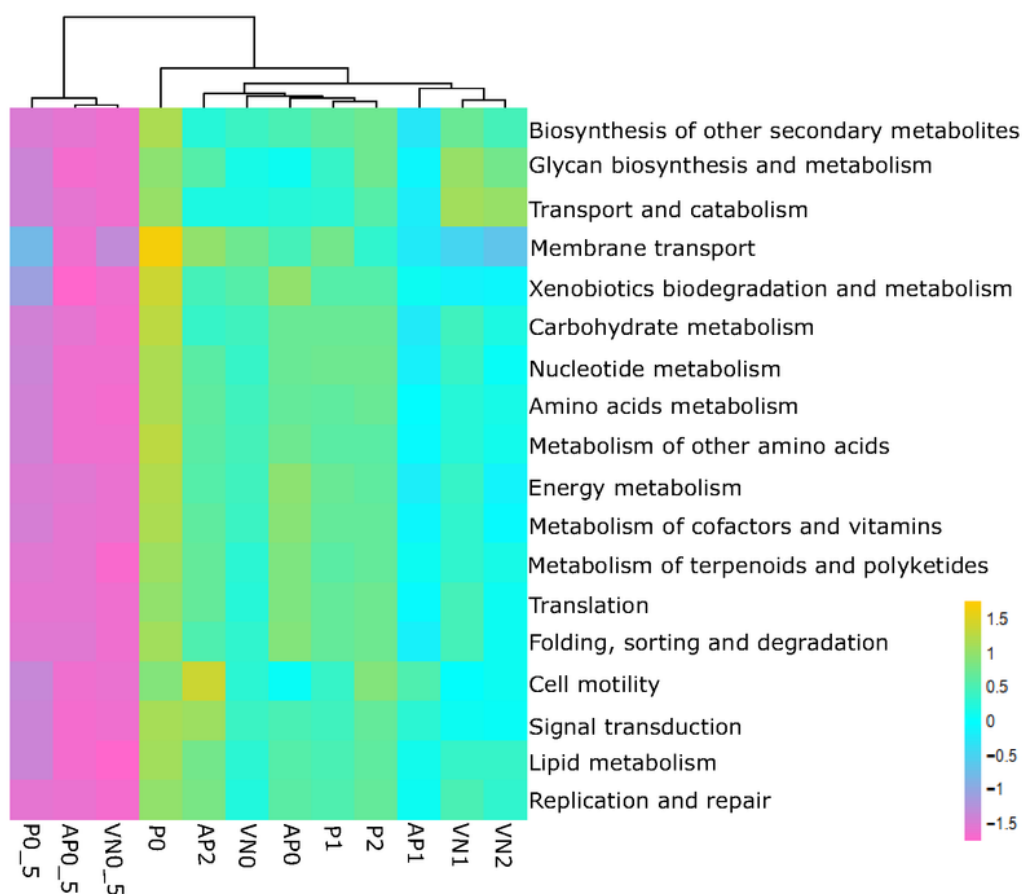
**Figure 4**

The diversity and relative abundance of bacterial OTUs (A), phylum (B), and Genera (C) on artificial plants, *V. natans*, and *P. maackianus*.



**Figure 5**

Co-occurrence networks of the bacterial communities on artificial plants (A), *V. natans* (B), and *P. maackianus* (C) under Cd exposure based on pairwise Spearman's correlations between genera. The nodes were colored according to different bacterial phyla. A connection stands for a strong (Spearman  $r > 0.7$  or  $r < -0.7$ ) and significant ( $P$ -value  $< 0.05$ ) correlation. The green and pink lines (edges) represent, respectively, significantly strong positive and negative relationships.



**Figure 6**

Heatmaps of percentage relative abundance of predicted bacterial metabolic pathways (Level 2) on biofilm attached to artificial plants, *V. natans*, and *P. maackianus* exposed to Cd using KEGG annotation.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryMaterials.docx](#)