Impact of Exogenous Indoleacetic Acid on Nitrogen Cycling-Associated Bacteria in the Rhizosphere and Eutrophic Water Surrounding Hydrocotyle vulgaris Lam

Min Zhang 1,2, Wenliang Xiang 1,2,*,†, Feifei Song 3, Haoyu Zhu 1, Ting Cai 1, Jie Tang 1 and Qing Zhang 1

1 School of Food and Bioengineering, Xihua University, Chengdu 610039, China; zhangmin10062023@163.com (M.Z.); zhuhaoyu6508@163.com (H.Z.); caiting1124@mail.xhu.edu.cn (T.C.); wendyjiejie@tom.com (J.T.); biozhangqf@163.com (Q.Z.)
2 Center for River and Lake Ecological Conservation and Restoration, Xihua University, Chengdu 610039, China
3 Sichuan Qinghe Technology Co., Ltd., Chengdu 610039, China; xsongfei@163.com
* Correspondence: biounicom@mail.xhu.edu.cn
† These authors contributed equally to this work.

Abstract: Phytohormones have the potential to enhance the nutrient removal efficiency of aquatic plants in wastewater treatment. Here, we investigated the impact of indoleacetic acid (IAA) on nitrogen removal by Hydrocotyle vulgaris Lam during the remediation process of eutrophic water. This investigation involved evaluating the biological indicators of H. vulgaris Lam, the nitrogen salt removal efficiency in eutrophic water, as well as analyzing the bacterial structure and function in both the rhizosphere and eutrophic water surrounding H. vulgaris Lam. The results indicated that surface-sprayed 50 mg/L IAA significantly stimulated the growth of H. vulgaris Lam, including parameters such as blade number, leaf area, petiole length, stem thickness, stem length, and root length of H. vulgaris Lam. Furthermore, exogenous application of IAA significantly accelerated the nitrogen removal of NH₄⁻N, NO₃⁻N and total nitrogen (TN) in eutrophic water by promoting the NH₄⁻N uptake of H. vulgaris Lam and NO₃⁻N denitrification. These findings suggest a potential application for exogenous IAA to enhance the nitrogen removal of H. vulgaris Lam in eutrophication control.

Keywords: Hydrocotyle vulgaris Lam; indoleacetic acid; nitrogen cycling-related bacteria; eutrophic water

1. Introduction

Eutrophication is a type of water contamination resulting from excessive levels of nutrients, such as nitrogen and phosphorus. This phenomenon can have severe consequences for aquatic life and ecosystems, including oxygen depletion, the production of algal toxins, and issues related to water turbidity and odor [1]. Bioremediation is an eco-friendly restoration method that utilizes the metabolic processes of organisms like aquatic plants, microorganisms, and zooplankton to absorb and convert nutrients in eutrophied water [2]. The synergistic action between aquatic plants and microorganisms can promote the degradation of nitrogen salts in eutrophic waters [2]. Among them, four aspects are particularly relevant to the nitrogen cycle: aquatic plant nitrogen uptake, rhizosphere microorganism-mediated nitrogen transformation [3], symbiotic relationships among rhizosphere microorganisms and aquatic plants that promote the cycling and utilization of nitrogen [4], and root-secreted substances of aquatic plants that enhance the microbial degradation of nitrogen salts [3].

Hydrocotyle vulgaris Lam is a hydrophyte commonly utilized for the restoration and control of eutrophied water [6]; it can enhance the dissolved oxygen levels through photosynthesis, thereby improving the overall aquatic environment. Furthermore, its rapid
growth enables the formation of extensive vegetation cover within a short period, effectively suppressing algae proliferation in water bodies. The leaf photosynthesis of *H. vulgaris* Lam improves the redox environment and facilitates organic substance degradation. Additionally, the complex root systems provide a favorable habitat for microorganisms involved in pollutant degradation, contributing to water purification [6]. Microorganisms play crucial roles in these processes, such as nitrification and denitrification, ammonification, and organic matter decomposition; they can reduce nitrogen concentration while promoting phosphorus uptake by aquatic plants [7]. Previous studies have demonstrated that phytohormones significantly stimulated microalgal growth, leading to a maximum increase in total nitrogen (TN) removal by 184.3% and facilitating nutrient removal from wastewater treatment under artificial experimental conditions [8]. However, limited research has been conducted on rhizosphere microorganisms associated with aquatic plants. Therefore, further investigation is needed to explore the effects of phytohormones on rhizosphere microorganisms of aquatic plants.

Plant growth phytohormones, a class of chemicals, possess the capacity to modulate the physiological and developmental processes of plants. Indole-3-acetic acid (IAA), an auxin known to stimulate growth and metabolism, belongs to the category of phytohormones [9]. IAA is implicated in diverse biological events such as cell proliferation, organogenesis, and stem elongation. Numerous studies have demonstrated that exogenous application of IAA could augment the growth and activity of specific beneficial microorganisms, including nitrogen-fixing and phosphorus-solubilizing bacteria in the rhizosphere, thereby exerting positive effects on land plant development [10,11]. However, most of these studies focused on terrestrial plants and rarely involved aquatic plants. Therefore, the present study aimed to investigate the optimal concentration of IAA for enhancing nitrogen removal efficiency of *H. vulgaris* Lam. Additionally, this study also analyzed the effects of IAA on the structure and function of bacteria involved in nitrogen removal within the rhizosphere of *H. vulgaris* Lam and eutrophic water.

2. Materials and Methods

2.1. Experimental Design and Processing

*H. vulgaris* Lam with similar growth conditions were selected for experiments. They were thoroughly cleaned and disinfected with 1% potassium permanganate solution for 1 min, followed by five rinses with sterile water. Subsequently, they were transplanted into sterile water for pre-culturing. The healthy *H. vulgaris* Lam with similar size were uniformly pruned to maintain five leaves per plant. Twenty plants were randomly divided into four groups, each consisting of five parallels.

The eutrophic water in the Gaopan River, Chengdu, China was collected to explore the purification ability of *H. vulgaris* Lam. The initial ammonia nitrogen (NH₄⁺−N), nitrate nitrogen (NO₃−−N), TN, total phosphate, and chemical organic demand (COD) concentration were 16.3 ± 0.01, 20.6 ± 0.06, 44.99 ± 0.02, 3.76 ± 0.01, and 115.00 ± 0.50 mg/L, respectively. The *H. vulgaris* Lam was implanted into eutrophic water, and then cultured. The culture room was maintained at a constant temperature of 25 ± 2 °C, with full wave light for 12 h per day. Throughout the experiments, the water lost by transpiration and volatilization was quantitatively replenished with sterile water. The experimental groups were sprayed with 25, 50, 100, and 150 mg/L IAA every 7 d, while the control groups (CK) were treated with distilled water. The group with the most favorable IAA concentration for growth of *H. vulgaris* Lam was used for subsequent experiments.
2.2. Biological Parameters of *H. vulgaris* Lam and Water Quality Testing

The biological parameters of *H. vulgaris* Lam were recorded after a 15-day period. These parameters included leaf count, net growth in leaf area, chlorophyll content, petiole length, stem thickness, stem length, root length, and fresh weight. The concentration of chlorophyll was determined using the method described by Duca et al. [11].

The eutrophic water was sampled every 5 d to monitor the physical and chemical indicators according to standard methods [12]. The NH$_4^+$ was measured spectrophotometrically at 420 nm using Nessler’s reagent, while the NO$_3^-$ was measured by ultraviolet spectrophotometry at 220 and 275 nm. The TN was detected by alkaline potassium persulfate oxidation UV spectrophotometry at 220 and 275 nm.

2.3. Bacterial Enrichment

The bacteria from the rhizosphere and eutrophic water surrounding *H. vulgaris* Lam were collected after 10, 20, and 30 d of cultivation. The roots were aseptically cut and placed in sterile tubes containing 50 mL of potassium phosphate buffer (0.1 mol/L, pH 8.0). They were then shaken at 4 °C for 5 min using a culture shaker. Subsequently, the washed roots were transferred to vials with 20 mL of PBS (pH 7.2–7.4) and subjected to elution using ultrasound at a frequency of 40 Hz (160 W, 30 s/30 s) for 10 min. The rhizosphere bacteria were subsequently collected from the elution solution using 0.22 μm microporous filter membrane. Bacteria present in the eutrophic water surrounding the roots were extracted through 0.22 μm microporous filter membrane from 500 mL eutrophic water. The bacteria-loaded microporous filter membranes were stored at −80 °C.

2.4. Bacterial Community Structure Analysis and Functional Predictions

The bacterial pellet in filter membranes was transferred into a 1.5 mL tube with 200 mg of zirconium beads (0.1 mm diameter). DNA extraction was conducted according to the soil DNA isolation kit protocol (Chengdu Foregene Biotechnology Co. Ltd, Chengdu, China). DNA concentration and quality were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). The bacterial structure was evaluated by Illumina Miseq sequencing of 16s rRNA gene [13]. The V3–V4 hyper-variable region was amplified with primers 338F (5′-ACTCCTAGGGAGAGCA-3′) and 806R (5′-GGACTCHVGGGITWTAT-3′) binding adapter sequences and barcode sequences [14]. The thermal cycling conditions were as follows: an initial denaturation at 95 °C for 3 min, and 30 cycles of 95 °C for 60 s, 55 °C for 90 s, and 72 °C for 60 s, followed by a final extension at 72 °C for 10 min. The PCR products were purified using 1.8% agarose gel electrophoresis, and the desired fragments were sequenced on the Illumina Hiseq2500 platform (Shanghai Majorbio Bio-Pharm Technology Co., Ltd., Shanghai, China).

The high-quality sequences, generated by Quantitative Insights Into Microbial Ecology (QIIME) software version 1.9.1, were clustered to operation taxonomy units (OTUs) using UPARSE software version 7.1 “http://drive5.com/uparse/ (09 October 2023)” at 97% similarity level. A representative OTU was then annotated in bacterial 16S Silva “http://www.arb-silva.de (23 October 2023)” and assigned to a taxonomic identity in RDP Classifier version 2.11 “http://sourceforge.net/projects/rdpclassifier/ (05 November 2023)”. Alpha diversity was calculated with Mothur version V1.30.2 “https://mothur.org/wiki/calculators/ (9 November 2023)” and described using observed species, Shannon indices, and Chao1 indexes. Then, the rarefaction curve was generated to compare the level of bacterial OTU diversity. The beta diversity was analyzed in R version 4.2.3 with the vegan package (v2.5-6) and visualized with principal coordinate analysis (PCoA) with ggplot based on Bray–Curtis distance. The samples were clustered according to species composition and abundance with R version 4.2.3 with vegan package and visualized on a heatmap. The LEfSe analysis “http://huttenhower.sph.harvard.edu/galaxy/ (12 November 2023)” was used to discriminate the significantly different bacteria in groups. The community distinction in groups was assessed using a Venn diagram,
which depicted the shared and unique OTUs at a 97% similarity level. The bacterial networks in the water and *H. vulgaris* Lam rhizosphere were analyzed. Initially, OTUs with a relative abundance greater than 0.1% were selected using the ggClusterNet package in R to calculate the correlation between different microorganism OTUs (using Spearman’s correlation coefficient; $R^2 > 0.6; p < 0.05$). Subsequently, co-occurrence networks of bacteria within different groups were constructed, where each node represented an OTU. Based on this analysis, various network properties including the number of edges, average degree, clustering coefficient, and average path length were calculated.

The functional prediction of normalized OTU (absolute abundance) was conducted using PICRUSt with the GreenGenes database. The functional annotation of prokaryotic taxa (FAPROTAX) method utilized representative literature from artificial cultures to map prokaryotic taxa (e.g., genus or species) and metabolic or other functions (e.g., nitrification, denitrification). The PICRUSt results were supplemented with the FAPROTAX database to enable better functional prediction.

All analyses were carried out in triplicate, and the results were expressed as mean values ± standard deviation. The significant difference was assessed by two-way analysis of variance (two-way ANOVA) using R software.

3. Results and Discussion

3.1. Impact of IAA Concentrations on the Biological Properties of *H. vulgaris* Lam

Exogenous IAA has been shown to enhance the cell proliferation of aquatic plants. However, the appropriate IAA concentration can vary significantly among different plant species [9]. To determine the appropriate IAA concentrations for *H. vulgaris* Lam growth, 25, 50, 100, and 150 mg/L of exogenous IAA were sprayed on the leaf surface of *H. vulgaris* Lam. The biological indicators of *H. vulgaris* Lam exhibited that 50 mg/L had the best promotional effect compared with 25, 100, and 150 mg/L IAA concentrations (Figure 1). After 50 mg/L IAA treatment, the number of blades, net growth leaf area, chlorophyll II, petiole length, stem thickness, stem length, root length, and fresh weight of *H. vulgaris* Lam were approximately 1.16, 1.36, 1.17, 1.39, 1.23, 1.59, 1.20, and 1.96 times over those of CK, respectively. Although 100 mg/L IAA significantly promoted the increase in leaf areas, the other biological indicators were less promoted than 50 mg/L. Moreover, 150 mg/L IAA dose had significant inhibitory effects on these indicators. The possible reason reported in the literature was that appropriate IAA concentration promoted plant growth and development by binding to the transport inhibitor response1 (TIR1) protein [15]. The presence of high concentrations of IAA, on the other hand, hindered the growth and development of *H. vulgaris* Lam by disrupting endogenous hormone balance [6]. Furthermore, it has been reported that the fresh weight of aquatic plants is positively correlated with their ability to absorb and accumulate nutrients [4]. Therefore, the 50 mg/L IAA dose could be an efficient way to purify the eutrophic water by promoting the growth of *H. vulgaris* Lam.
3.2. Impact of IAA on the Purification Performance of Eutrophic Waters

*H. vulgaris* Lam is well-known for its efficient sewage purification and is frequently employed as an alternative aquatic plant for remediating landscape water pollution [6,16]. However, the combined use of phytohormones and *H. vulgaris* Lam for eutrophic water remediation has not been extensively researched. Figure 2A–C depict the removal performance of NH$_4^+$–N, NO$_3^−$–N, and TN from eutrophic water during a 45-day incubation period. At day 20, the experimental group treated with a spray containing 50 mg/L IAA exhibited an average residual NH$_4^+$–N at 0.59 mg/L, which is below the Grade III limit value specified by Environmental Quality Standards for Surface Water (1.0 mg/L; GB3838-2002) [17]. In contrast, the CK had an average residual NH$_4^+$–N of 3.06 mg/L at this time point. The experimental group achieved a higher NH$_4^+$–N removal rate from eutrophic water at day 20. The NO$_3^−$–N removal in the experimental group reached 92.52% at day 35, exhibiting a significant increase of 10.44% compared to the control group. At day 40, the TN
removal in the experimental group was an impressive rate of 95.58%, surpassing that of CK by 11.64%. These findings demonstrate that exogenous IAA treatment effectively enhanced the removal of nitrogen salt and improved the nitrogen purification efficiency of *H. vulgaris* Lam. The removal of nitrogen salt in eutrophic water also involves a variety of microorganisms, and these microbial communities are significantly responsive to changes in habitat environment [5,18,19]. Therefore, the synergy between plants and microorganisms is the main reason for improving the nitrogen purification of *H. vulgaris* Lam, as depicted in Figure 2D,E.

**Figure 2.** The removal of nutrients in the eutrophic water by *H. vulgaris* Lam. (A) The NH₄⁺–N content. (B) The NO₃⁻–N content. (C) The TN content. (ns: *p* > 0.05; *: *p* < 0.05; **: *p* < 0.01; ***: *p* < 0.001). (D) The schematic representation of synergistic action of plants and microorganisms for water purification. (E) The nitrogen cycling by rhizosphere bacteria.
3.3. Impact of IAA on Bacterial Structure of *H. vulgaris* Lam Rhizosphere and Eutrophic Water

The transformation and removal of nitrogen in eutrophic water are closely associated with the composition, abundance, spatial distribution, and temporal dynamics of microbial communities. Different plant species have been found to host distinct microbial communities [19,20], and the development of a plant plays a crucial role in microbial–plant interactions [20]. The Venn analysis (Figure S1A,B) revealed that there were 178 bacterial operational taxonomic units (OTUs) exclusively present in eutrophic water of CK (WCK), while 210 OTUs were specifically found in eutrophic water of *H. vulgaris* Lam treated with 50 mg/L IAA (WIAA), and a total of 704 OTUs were shared between them. In the rhizosphere of *H. vulgaris* Lam, there was an overlap of 788 OTUs between them, but still 208 OTUs exclusively present in the rhizosphere of CK (RCK) and another 140 OTUs in the rhizosphere of treatment groups (RIA).

Alpha diversity indices (Ace, Shannon, and Coverage index) for all samples are depicted in Table S1. All samples had a coverage index of more than 0.9883, meaning that bacteria were mostly detected in the eutrophic water and rhizosphere of *H. vulgaris* Lam. From day 10 to 30, the Ace and Shannon indices of CK exhibited similar trends as those observed in the experimental group. In WCK and WIAA, the Ace index initially increased from 744.19 and 699.95 at day 10 to 949.39 and 909.69 at day 20, then decreased to 771.23 and 759.33 at day 30, respectively, while in RCK and RIAA, it first decreased from 874.68 and 863.53 at day 10 to 750.86 and 740.80 at day 20, and then increased to 888.39 and 757.18 at day 30, respectively (Table S1). In general, exogenous application of IAA did not exert a significant impact on the evolutionary pattern of bacteria in either eutrophic water or the rhizosphere of *H. vulgaris* Lam; however, there was a noticeable decline in the Ace index of experimental groups. The Shannon index was used to quantify the diversity and complexity within a microbial community. Here, it further exhibited this difference between experimental and control groups of eutrophic water and *H. vulgaris* Lam rhizosphere, indicating that IAA affected the community diversity in them (Table S1).

Network analysis was utilized to further understand the interactions within the microbial community and co-occurrences among OTUs in both the experimental and control groups [21]. The network in experimental groups exhibited a reduction in both node and edge density as compared to CK, as shown in Figure 3A,B. Hierarchical cluster analysis showed that bacterial communities were divided into four clusters, and PCoA 1 and PCoA 2 accounted for 67.51% of variance, as shown in Figure 3C. Interestingly, the bacterial composition of eutrophic water was significantly different from that of *H. vulgaris* Lam rhizosphere, regardless of IAA treatment. Several factors, such as microenvironmental differences, plant root secretions, symbiotic relationship between aquatic plants and rhizosphere microorganisms, and adhesion and adsorption of nutrients, might contribute to this difference [3,4]. However, the PCoA also revealed a statistically significant difference ($p = 0.045$) in bacterial community structure in eutrophic water and rhizosphere of *H. vulgaris* Lam after treatment with 50 mg/L IAA, compared to CK. The exogenous application of IAA promoted the growth and development of *H. vulgaris* Lam, and subsequently increased the chlorophyll content to enhance its photosynthetic capacity, thus leading to the microenvironmental differences of control and experiment groups. This may be the main reason why the IAA had a significant impact on bacterial structure in the rhizosphere and eutrophic water surrounding *H. vulgaris* Lam, and this impact became more obvious with the extension of IAA treatment time (Figure 3C–F).
Figure 3. The effect of IAA on bacterial structure of *H. vulgaris* Lam rhizosphere and eutrophic water. (A,B) The cooccurrence networks of bacteria in different groups. (C) The principal coordinate analysis (PCoA), based on the Bray–Curtis distance, showing the differences of bacterial structure in the water and *H. vulgaris* Lam rhizosphere. (D) The distance heatmap on OTU level. (E) The bar diagram of bacterial composition in water samples at the family level. (F) The bar diagram of bacterial composition in *H. vulgaris* Lam rhizosphere at the family level (F). WCK, water of CK; WIAA, water of IAA treatment with 50 mg/L; RCK, rhizosphere of CK; RIAA, rhizosphere of IAA treatment with 50 mg/L. WCK-10, WIAA-10, RCK-10, RIAA-10, etc., samples at day 10.

In eutrophic water and the rhizosphere of *H. vulgaris* Lam, the impact of IAA on bacterial structure was further elucidated through bacterial succession. In WCK, the
dominant bacteria (>5.00% at the family level), namely Comamonadaceae (66.88%) and Beijerinckiaceae (6.76%) at day 10, shifted to Comamonadaceae (9.92%), Sphingomonadaceae (8.08%), Spirosomaceae (8.38%), Reyrnellaceae (5.36%), Methylphilaceae (9.11%), and env.OPs_17 (11.48%) at day 20, followed by Flavobacteriaceae (23.19%), Sphingomonadaceae (11.15%), Spirosomaceae (6.42%), Micrococcaceae (14.01%), and Rhizobiales_Incertae_Sedis (5.72%) at day 30. After IAA treatment with 50 mg/L, in WIAA, they shifted from Comamonadaceae (67.09%) and Beijerinckiaceae (7.17%) at day 10 to Comamonadaceae (9.07%), Flavobacteriaceae (7.64%), Sphingomonadaceae (8.26%), Spirosomaceae (10.34%), Methylphilaceae (6.50%), and Microbacteriaceae (6.63%) at day 20, then to Comamonadaceae (10.18%), Flavobacteriaceae (22.63%), Micrococcaceae (5.95%), Microbacteriaceae (5.40%), Crocinitomicaceae (7.42%) Sphingobacteriaceae (5.71), and Neisseriaceae (5.09%) at day 30. In the rhizosphere of *H. vulgaris* Lam, the bacterial community composition also exhibited temporal shifts. In RCK, the dominant bacterial families were Comamonadaceae (39.96%), Nitrospiraceae (7.30%), and Sphingomonadaceae (5.08%) at day 10, which shifted to Comamonadaceae (5.08%), Nitrospiraceae (9.64%), Rhodobacteraceae (6.38%), Sphingomonadaceae (5.83%), Micrococcaceae (5.70%), Xanthomonadaceae (5.63%), and norank_p_WPS-2 (5.93%) at day 20, and further shifted to Comamonadaceae (5.65%), Rhodobacteraceae (6.75%), Sphingomonadaceae (6.70%), Micrococcaceae (7.34%), and Blastocatellaceae (7.52%) at day 30. After IAA treatment with 50 mg/L in RIAA at day 10, the dominant families were Comamonadaceae (25.80%) and Nitrospiraceae (11.17%). At day 20, the composition further changed to Comamonadaceae (15.92%), Nitrospiraceae (7.82%), Rhodobacteraceae (7.49%), and Sphingobacteriaceae (10.21%). Finally, at day 30, the dominant families were Comamonadaceae (15.91%) and Rhodobacteriaceae (10.47%). The microbial succession is a ubiquitous phenomenon in response to environmental changes [4,10]. In general, exogenous IAA had significant impacts on the occurrence, propagation, distribution, and succession of bacteria in the eutrophic water and rhizosphere of *H. vulgaris* Lam.

### 3.4. Regulation of Nitrogen Cycle-Related Bacterial community by IAA

Biogeochemical cycling of nitrogen is typically associated with six distinct nitrogen-transforming processes: nitrogen fixation, nitrification, denitrification, anaerobic ammonium oxidation, assimilation, and ammonification [7], as shown in Figure 4A. In the eutrophic water and rhizosphere of *H. vulgaris* Lam, the abundance of nitrogen removal bacteria at the family level is illustrated in Figure 4B,C. The results revealed that nitrogen fixation, denitrification, and photosynthesis contributed to the removal of nitrogen in eutrophic water, while nitrogen fixation, nitrification, denitrification, and photosynthesis contributed to the elimination of nitrogen in the water surrounding the rhizosphere of *H. vulgaris* Lam. In nitrogen cycling, the photosynthetic bacteria Spiraceae and Rhodobacter are associated with the nitrogen cycle, wherein nitrogen plays a crucial role in their protein and amino acid metabolism, which is intricately linked to photosynthesis under light conditions [22]. Beijerinckiaceae, Rhizobiales, and Ilimatobacteraceae were identified as nitrogen-fixing bacteria [23]. Nitrospiraceae, Brevibacillaceae, Bacillaceae, and Microcillaceae were classified as nitrifying bacteria [24,25]. The primary denitrifying bacteria included Comamonadaceae, Methylphilaceae, Rhizobiales_Incertae_Sedis, and Hyphomicrobiaceae [26−28]. When compared with WCK and RCK, respectively, the exogenous IAA did not result in any significant changes to the bacterial taxa involved in nitrogen cycling in WIAA and RIAA, but it did alter their relative abundance (Figure 4B,C). In RIAA, the relative abundance of bacteria associated with the nitrogen cycle exhibited an approximate increase of 1.13-fold compared to that of RCK. Interestingly, in WIAA, it was lower than that of WCK. This suggested that the exogenous IAA promoted the recruitment of bacteria involved in the nitrogen cycle to the rhizosphere of *H. vulgaris* Lam.
Figure 4. The effect of IAA on the nitrogen cycle-related bacteria in eutrophic water and rhizosphere of *H. vulgaris* Lam. (A) The bacterial transformation of nitrogen compounds. (B) The relative abundance of nitrogen removal bacteria at family level in eutrophic water. (C) The relative abundance of nitrogen removal bacteria at family level in *H. vulgaris* Lam rhizosphere. (D,E) The bacteria associated with nitrogen cycling.

Based on absolute abundance OTU, LEfSe analysis further revealed the co-occurrence of bacteria associated with nitrogen cycling (Figure 4D,E). In eutrophic water, the
Pseudolabrys, Rhizobacter, Ideonella, Brevundimonas genera, and Comamonadaceae and Rhizobiales_Incertae_Sedis were identified as co-biomarkers. Of them, the abundance of Pseudolabrys and Rhizobiales_Incertae_Sedis in WCK was significantly higher than that in WIAA. Conversely, the abundance of Rhizobacter, Ideonella, Brevundimonas, and Comamonadaceae in WIAA was significantly higher than that in WCK (Figure 4D). In the rhizosphere of H. vulgaris Lam, the co-biomarkers included the Paracoccus, Thermus, and Novosphingobium genera, Microscillaceae, Comamonadaceae, Nitrosomonadaceae, and Steroidobacteraceae families, and Rhizobiales order (Figure 4E). In RCK, the abundance of Steroidobacteraceae and Novosphingobium was significantly higher than that in RIAA. However, the abundance of Paracoccus, Thermus, Microscillaceae, Comamonadaceae, Nitrosomonadaceae, and Rhizobiales in RIAA was obviously higher compared to RCK. The findings revealed that the exogenous IAA had influenced the dynamics of bacterial community structures associated with nitrogen cycling in eutrophic water and the rhizosphere of H. vulgaris Lam.

The functional prediction revealed that exogenous IAA significantly enhanced the activities of nitrogen cycling in both eutrophic water and the rhizosphere of H. vulgaris Lam, particularly in the rhizosphere (Figure 5A,B). However, this enhancement was observed across various denitrification processes, including nitrate reduction, nitrate respiration, denitrification of nitrate and nitrite, as well as nitrous oxide denitrification during denitrification (Figure 5A). The nitrification was weak at days 10–30 in both eutrophic water and the rhizosphere. Although nitrogen fixation occurred, it was more obvious in experiments than the control [27,29]. It is well-known that aquatic plants prefer to uptake NH₄⁺–N rather than NO₃⁻–N. When NH₄⁺–N was preferentially absorbed by H. vulgaris Lam (Figure 2A), the residue of NO₃⁻–N provided the main nutrition and substrate for the growth and propagation of denitrifying bacteria. Therefore, the activities of denitrification were obviously observed (Figure 5A). Usually, denitrification occurs well in the absence of oxygen, but some facultative anaerobic bacteria and even aerobic bacteria can also participate in denitrification, such as Comamonadaceae, Hyphomicrobiaceae, Rhizobiales, Pseudomonas, Alcaligenes, Paracoccus, and Bacillus [7,26,27,30]. The exogenous IAA enhanced the photosynthetic activity of H. vulgaris Lam (Figure 1), thereby increasing oxygen transport to the eutrophic water and elevating the dissolved oxygen content. Simultaneously, IAA also stimulated the development and secretion of roots, thus promoting the colonization of facultative anaerobic denitrifying bacteria on roots, particularly those belonging to Comamonadaceae. In WIAA, the abundance of facultative anaerobic denitrifying bacteria ranged from 233 to 556 reads between days 10 and 30, slightly surpassing the range of 100–403 reads observed in WCK. Conversely, RIAA exhibited a significantly higher abundance, with a range of 1778–2350 reads compared to the range of 669–1087 reads found in RCK. Therefore, the denitrification effects caused by exogenous application of IAA to H. vulgaris Lam were more significant than those of CK, especially in RIAA.
Figure 5. The heatmap of bacteria associated with nitrogen cycling based on FAPROTAX. (A) The heatmap of bacteria related to nitrogen cycle. (B) The bacterial abundance related to nitrogen cycle. (C) The spatial and temporal schematic diagram of water purification by *H. vulgaris* Lam.

4. Conclusions

In this study, the application of 50 mg/L IAA on *H. vulgaris* Lam through surface spraying stimulated its growth and enhanced the NH$_4^+$–N uptake of *H. vulgaris* Lam, as well as the structure and function of facultative anaerobic bacteria associated with NO$_3^-$–N denitrification in both the rhizosphere and eutrophic water surrounding *H. vulgaris* Lam, especially for facultative anaerobic denitrifying bacteria in the rhizosphere. Therefore, the nitrogen salt removal was accelerated in eutrophic water. These findings highlight the potential application of IAA for synergistic remediation of eutrophic water by aquatic plants and microorganisms; however, further detailed studies are required.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w16070924/s1, Figure S1: Venn diagrams showing bacterial differences in common OTUs between water environment (A) and *H. vulgaris* Lam rhizosphere (B) under 50 mg/L IAA treatment; Table S1: Alpha diversity index.

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Conflicts of Interest: Author Feifei Song was employed by the company Sichuan Qinghe Technology Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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