Using *Ipomoea aquatic* as an environmental-friendly alternative to *Elodea nuttallii* for the aquaculture of Chinese mitten crab

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Elodea nuttallii (EN) is widely used in Chinese mitten crab (CMC) rearing practice, but it is not a native aquatic plant and cannot endure high temperature. Thus, large EN mortality and water deterioration events could occur during high temperature seasons. The aim of this study was to identify the use of local macrophytes in CMC rearing practice, including *Ipomoea aquatic* (IA) and *Oryza sativa* (OS). A completely randomized field experiment was conducted to investigate the crab yield, water quality, bacterioplankton community and functions in the three different systems (EN, IA, and OS). Average crab yields in the different macrophyte systems did not differ significantly. The IA and OS systems significantly decreased the TN and NO_3^--N quantities in the outflow waters during the rearing period compared to the EN system, and the IA and OS plants assimilated more nitrogen than the EN plant. Moreover, the significant changes of bacterioplankton abundances and biodiversity in the three systems implied cleanliness of rearing waters were concomitantly attributed to the differential microbial community and functions. In addition, principle component analysis successfully differentiated the bacterioplankton communities of the three macrophytes systems. Environmental factor fitting and the co-occurrence network analyses indicated that pH was the driver of bacterioplankton community structure. Functional predictions by PICRUSt considered a higher potential for microbial denitrification in the IA and OS systems. Notably, the OS plants stopped growing in the middle of the rearing period. Thus, the IA system rather than the OS system could be a feasible and environmental-friendly alternative to the EN system in CMC rearing practice.

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Abstract

Elodea nuttallii (EN) is widely used in Chinese mitten crab (CMC) rearing practice, but it is not a native aquatic plant and cannot endure high temperature. Thus, large EN mortality and water deterioration events could occur during high temperature seasons. The aim of this study was to identify the use of local macrophytes in CMC rearing practice, including *Ipomoea aquatic* (IA) and *Oryza sativa* (OS). A completely randomized field experiment was conducted to investigate the crab yield, water quality, bacterioplankton community and functions in the three different systems (EN, IA, and OS). Average crab yields in the different macrophyte systems did not

differ significantly. The IA and OS systems significantly decreased the TN and NO_3^--N quantities in the outflow waters during the rearing period compared to the EN system, and the IA and OS plants assimilated more nitrogen than the EN plant. Moreover, the significant changes of bacterioplankton abundances and biodiversity in the three systems implied cleanliness of rearing waters were concomitantly attributed to the differential microbial community and functions. In addition, principle component analysis successfully differentiated the bacterioplankton communities of the three macrophytes systems. Environmental factor fitting and the co-occurrence network analyses indicated that pH was the driver of bacterioplankton community structure. Functional predictions by PICRUSt considered a higher potential for microbial denitrification in the IA and OS systems. Notably, the OS plants stopped growing in the middle of the rearing period. Thus, the IA system rather than the OS system could be a feasible and environmental-friendly alternative to the EN system in CMC rearing practice.

Introduction

The Chinese mitten crab (CMC), *Eriocheir sinensis,* is considered an invasive species in Europe and North America (Brodin & Drotz 2014; Hanson & Sytsma 2008), but it is an expensive delicacy in Asia (Chen & Zhang 2007). In 2014, 796,621 tons of farmed Chinese mitten crabs were produced (Zeng et al. 2013); crabs were primarily bred in ponds and lakes (Zeng et al. 2013). The mitten crabs produced in Yangcheng Lake, Suzhou, China, are of high quality and have high economic value (Gu et al. 2013). Most of the crabs produced in Yangcheng Lake are exported to Shanghai, Hong Kong, and high-profit foreign markets.

Aquatic plants are required for mitten crabs farming. The plants provide shelter for the crabs

during exuviation, which is an important part of crab growth (Meng et al. 2013). In addition, aquatic plants assimilate excess nutrients, improve water cleanliness, and absorb solar radiation to maintain cool water temperatures. These properties increase crab growth, yield, and quality (Zhan & Yang 2015).

Elodea nuttallii (EN), a perennial aquatic plant native to North America, provides these benefits, and is thus widely used in CMC aquaculture (Wang et al. 2016). However, EN cannot withstand high temperatures (Zhan & Yang 2015). In Yangcheng Lake, summer air and water temperatures typically reach 35–40°C and 26–34ºC, respectively, which frequently leads to massive EN die off, resulting in serious water quality deterioration (Wu et al. 2016). Under such conditions, crab growth is negatively affected due to loss of shelter for exuviation hiding places and poor water quality (Zhan & Yang 2015). Consequently, alternative aquatic plants are required to facilitate mitten crab aquaculture in areas such as Yangcheng Lake.

Local plants that have adapted to local conditions are the best candidates for EN replacement. For example, *Ipomoea aquatica* (IA) is a semiaquatic, tropical/subtropical plant that might be applicable to mitten crab aquaculture (Zhang et al. 2014). IA grows well in shallow waters, withstands high temperatures, and efficiently removes nutrients (e.g., nitrogen and phosphorous) from water bodies (Liu et al. 2007; Wei et al. 2017). Furthermore, the tender shoots and leaves of IA are consumable, and providing additional economic value. Alternatively, *Oryza sativa* (OS) is a submerged grain crop that is common in Asia, and its assimilation ability of nitrogen and phosphorus similar to IA (Nawaz & Farooq 2017). OS is thus another candidate for EN replacement.

Therefore, in this study, we aimed to answer three questions. Which of the two locally adapted plants would most adequately replace EN, similarly improving crab yield and quality? How do the candidate plant systems affect water quality and the associated environmental characteristics? Considering microorganisms play important roles in nutrient conversion in wastewater (Daims et al. 2006), are the two plant systems associated with different bacterioplankton communities that differentially affect water quality? Answers to these questions are of critical importance to crab farmers, and those who are concerned with water quality. Despite the importance of these questions, they remain unanswered. We thus aimed to addresses the above questions by evaluating candidate aquatic plant systems, and exploring the associated bacterioplankton communities.

Material and methods

Pond construction, seedling preparation, and floating system construction

All of the experiments were performed in Lianzigang, Suzhou, China (31°27'40.18"N, 120°43.5'5.32"E). This region has a subtropical monsoon climate, with an annual average rainfall of 1076.2 mm (http://www.pmsc.cn/). Annual average temperature in Lianzigang is 15–17°C, with high temperatures of 35–40°C in July and August (http://www.pmsc.cn/). All of the experiments were conducted between May 1 and November 30, 2017.

The crab-rearing system used here consisted of a water inlet, a water outlet, a micro-porous aeration system, a pond, floating macrophytes, and vertical posts to anchor the macrophytes (Fig. 1). The pond, which was mechanically excavated, had a total area of 1,000 m^2 and was 1.5-m deep. The pond was separated into nine sub-ponds with cement walls that were 0.4 m wide and

1.5 m high. Each sub-pond had an area of ~100 m^2 and an independent staff gauge. Sub-pond bottoms were left in a natural state to allow crabs to burrow, and to facilitate EN root establishment and growth. IA, OS, and EN were grown in separate sub-ponds with three replicates per plant using a completely randomized design.

Sub-ponds were disinfected using sodium hypochlorite. After disinfection, water was pumped into each sub-pond to a depth of ~10 cm. Basal fertilizer (45 kg ha^{-1} of compound-fertilizer; $N:P_2O_5:K_2O$ = 15:15:15) was applied 2–3 days before seedling transplantation.

OS and IA seedlings were prepared by sowing seeds on a patented nutritional matrix (0.16 m^2) containing sufficient nutrients for seedling growth. The matrix had sufficient buoyancy to allow seedlings to float on the surface of the water. Seeds were germinated on the matrix, grown for 30 days in a greenhouse at 25°C, and then transferred to the bottom of the appropriate sub-ponds. Seedling matrices were arranged side by side until seedling coverage reached 60% of the total water surface area. Additional water was then pumped into each sub-ponds to increase the water depth to 20 cm, and seedlings were allowed to re-establish growth for 5–7 days. Upon growth re-establishment, water was again gradually and gently pumped into the sub-ponds to increase water depth to 1.0 m. At this depth, all of the seedlings were floating. Seedlings were fixed in place using ropes attached to buoys, which were fastened to posts in the sides of the cement dividers. EN seedlings were directly transplanted from other ponds. EN cluster spacing was 0.5 m × 0.5 m, with forty seedlings per cluster. The transplanted EN plants covered ~60% of the total water surface area. Upon growth re-establishment, water was pumped into the EN sub-ponds to a depth of 1.0 m.

105 **Crab pond management, nutrient measurements, and crab yield determination**

106 Crab ponds were managed using standard Chinese mitten crab rearing methods (Zhan & Yang
107 2015). Juvenile crabs, with an average weight of 15 g, were purchased from a local company
108 (Su'an Fishery Co., Ltd., Nantong, China) and added to the experimental sub-ponds at a density
109 of about 12,000 individuals per hectare. Crab feed (bait) was purchased from the Tongwei Group
110 (http://www.tongwei.com/). The nutrient composition of the feed was varied to meet the
111 different needs at each of the growth stages. Crabs were fed twice per day dependent on growth
112 stage, as recommended by the bait manufacturer. Crabs were not dosed with antibiotics or
113 chemical fishery drugs. A micro-porous aeration system was used nightly to ensure sufficient
114 aeration. Approximately ~5 cm of water was pumped out of all ponds each week, and replaced
115 with an equal volume of fresh water from Yangcheng Lake (depending on local precipitation).

116 Outflow water samples were taken every 7–10 days during the rearing period, and
117 immediately frozen at -20°C. At the end of the experiment, nutrients in the outflow samples were
118 measured using an auto analyzer (SKALAR SAN^{++}, Breda, the Netherlands); the nutrients
119 measured included total nitrogen (TN), total phosphorous (TP), ammonium-N (NH_4^+-N), nitrate-
120 N (NO_3^--N), and nitrite-N (NO_2^--N). Absolute cumulative nutrient quantity in discharged water
121 was measured by nutrient concentration in discharged water and outflow water quantity
122 estimated by staff gauge. Outflow pH was measured using a WTW portable pH meter (ProfLine
123 3310, Weilheim, Germany). Inflow water samples were taken and measured every month during
124 the rearing period, and average nutrient concentrations were finally calculated. Dissolved oxygen
125 (DO) concentrations were not measured, because preliminary studies identified large spatial and

126 temporal variations in DO concentrations (Fig. S1) due to uncontrollable factors (i.e., air
127 temperature, air pressure, water disturbances, aeration, activities of aquatic organisms, and
128 photosynthesis of plants and algae) (Dai et al. 2013).

129 The biomass yields (TBM) of EN, IA, and OS were estimated by dry matter productivity in
130 unit area (DM) and aquatic plant areas (APA) in each pond. The fresh plant tissues in 1 m^2 area
131 were collected and oven-dried with 3 replicates in each pond at the end of the experiment, and
132 average dry weight in 1 m^2 area was considered as DM. APA was equal to the 60% of total area
133 of each pond. Thus, the TBM could be calculated by the equation: TBM = DM×APA. Trimmed
134 plant tissues were included in the biomass production estimates. As IA and EN has a sprawling
135 growth pattern, it was periodically cut back outside of the rope-restricted area to maintain ~60%
136 coverage to keep constant aquatic plant coverage (Trimming details see Fig. S1-S2). Nutrients
137 assimilated by the plants were calculated based on plant biomass and nutrient concentrations.
138 The tissue-mixed plant samples that collected in different growth stages were used for nutrient
139 concentration measurement. Table S1 showed the average nutrient concentrations of plants.

140 To assess crab production, mature crabs were harvested using crab traps, and remaining crabs
141 were captured by hand at night, after the pond water was completely drained. Males and females
142 were manually separated and weighed.

143 **Characterization of bacterioplankton communities**

144 As crab rearing is sensitive to high air temperatures (Yuan et al. 2017), the bacterioplankton
145 communities during periods of high temperatures were more interesting. Thus, the
146 bacterioplankton community was assessed on July 2, 2017, when the maximum air temperature

147 reached 37°C. To obtain sufficient bacterioplankton biomass for community profiling via DNA
148 sequencing, we collected 10 L of water at 20 cm depth from each replicate pond. Water samples
149 were filtered through a 0.22-μm polycarbonate membrane (Millipore, Billerica, MA, USA).
150 DNA was extracted from the filtered biomass using a FastDNA Spin Kit for soil (MP bio, Solon,
151 OH, USA), following the manufacturer's protocols. Extracted DNA concentration was
152 determined with a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington,
153 NC, USA); DNA quality was assessed with gel electrophoresis on a 1% agarose gel. The V4
154 hypervariable region of the bacterial 16S rRNA gene was amplified using PCR, with the primers
155 563F (5'-AYTGGGYDTAAAGVG-3') and 802R (5'-TACNVGGGTATCTAATCC-3')
156 (Cardenas et al. 2010), following previously described protocols (Wang et al. 2017). The
157 amplified PCR products were purified using an AxyPrep DNA Gel Extraction Kit (Axygen
158 Biosciences, Union City, CA, USA), and quantified using a QuantiFluor-ST kit (Promega,
159 Madison, WI, USA), following the manufacturer's instructions. Purified amplicons were pooled
160 in equimolar concentrations and sequenced on an Illumina MiSeq platform (Illumina, San Diego,
161 CA, USA) at Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China), following standard
162 protocols.

163 Raw sequence reads were demutiplexed using QIIME (v.1.9.1) (Caporaso et al. 2010a).
164 Barcoding adapters and PCR primers were cleaved using cutadapt (v.1.16) (Martin 2011). Low-
165 quality reads were removed from the dataset with USEARCH10 (Edgar & Flyvbjerg 2015),
166 using the "fastq_filter" command with the parameters maxee=1 and truncqual=15. The
167 remaining paired-end reads were merged using the "fastq_mergepairs" command in

USEARCH10 (Edgar & Flyvbjerg 2015). Merged read abundances were normalized across samples by randomly subsampling 28,000 sequences from each sample. A zero-radius OTU (zOTU) table was produced from the sequence reads using the Unoise3 algorithm (Edgar 2018). The taxonomic classification of each zOTU was assigned using the UCLUST algorithm against the Silva (SSU123) 16S *r*RNA database with default parameters (Caporaso et al. 2010b; Edgar 2010).

An additional OTU table was generated to predict bacterioplankton functions. The abundance-normalized sequences were clustered at the 97% nucleotide similarity cutoff level into OTUs using the "pick_closed_reference_otus.py" function in QIIME (v.1.9.1) (Caporaso et al. 2010b). OTUs were taxonomically classified using the UCLUST algorithm (Edgar 2010) against the Greengenes (gg_13_5) reference database (DeSantis et al. 2006) with default parameters. The resulting OTU table was analyzed using PICRUSt (v.1.1.3) (scripts "normalize_by_funtion.py", "predict_metagenomes.py", "categorize_by_function.py", and "metagenome_contributions.py") (Langille et al. 2013). The PICRUSt algorithm produced a table of functional Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs (KOs). To obtain OTU-specific counts of genes (da Fonseca et al. 2019; Fan et al. 2018) associated with nitrification and denitrification, the PICRUSt script "metagenome_contributions.py" with -l option was applied to selected KOs (K00370, K00371, K00374, K02567, K02568, K00368, K15864, K04561, K02305, K00376, K10535, K10944, K10945, and K10946) (Script S1).

Statistical analysis

Statistical analyses were primarily performed in R (v.3.3.2) (Team 2013). To identify

189 significant differences among treatments, the Levene and Kolmogorov-Smirnov tests were used
190 to check the homogeneity of variances and data normality, respectively. One-way ANOVA was
191 used to determine the significance among the treatments, and Tukey's HSD test was then applied
192 for multiple comparisons. If the measurement variable did not meet the normality assumption, a
193 Kruskal-Wallis test was performed instead of one-way ANOVA. We considered $P < 0.05$ to be
194 statistically significant, unless otherwise noted.

195 The bacterioplankton communities were analyzed using the vegan package (Dixon 2009) in R.
196 The matrix of zOTU abundances was transformed prior to distance-based analyses using the
197 "decostand" function with the Hellinger method (Legendre & Gallagher 2001). Principal
198 component analyses (PCAs) were performed using the "rda" function in vegan to visualize
199 differences among the bacterioplankton communities from the different macrophyte systems.
200 Environmental variables were then fitted and projected onto an ordination using "envfit"
201 function in vegan based on 1,000 permutations. Bonferroni-adjusted *P* values were then
202 calculated using the "p.adjust" function in the stats package in R.

203 To investigate the correlation of microbial taxa and environmental variables, we constructed a
204 co-occurrence network using CoNet (Faust & Raes 2016), based on the zOTU table and the
205 environmental variables. In the co-occurrence analysis, the read count matrix was first filtered,
206 and only those zOTUs with at least seven minimum occurrence values across the nine samples
207 were retained. Pair-wise associations among zOTUs and environmental factors were calculated
208 using the Pearson, Spearman, Kendall, Bray-Curtis, and Kullback-Leibler correlation methods.
209 The initial top and bottom edge numbers were set at 1,000. For each edge and each measure of

association, 1,000 permutation scores and 1,000 bootstrap scores were computed. The resultant networks were visualized using Cytoscape (v.3.2.1) (Shannon et al. 2003).

Results

System performance, crab yield, and water quality

The macrophyte systems floated steadily throughout the whole experiment despite two moderate windstorms. Thus, the floating systems described here were suitable for plant growth on water surfaces. All of the plants grew well initially (between May and August). However, OS began to go to seed (indicating the start of the reproductive stage) in early August, about 25 days earlier than the normal (September, if OS is grown in a field). After early August, OS plants yellowed and the roots darkened. In contrast, IA and EN grew well until the end of the experiment.

Crab yields among the three macrophyte systems did not differ significantly. The average yields of male and female crabs were ~740 kg ha^{-1} and ~267 kg ha^{-1}, respectively (Fig. 2). Individual weight is one of the most important factors determining the value of commercial crabs. Here, the individual weight distributions did not differ significantly among plant systems ($P > 0.05$). The median weights of male and female crabs were 168 g and 109 g, respectively (Fig. 2).

The nitrogen assimilated by IA was estimated to be 118 kg ha^{-1}, based on macrophyte biomass weight and nutrient concentrations. This level of assimilation was 3.5 times that of EN, and 1.5 times that of OS (Fig. 3). In contrast, the phosphorous assimilated by IA, EN, and OS did not differ significantly.

The average TN, TP, NH_4^+-N and NO_3^--N concentrations of inflow waters in the rearing

period were 1.25 ± 0.44 (Mean ± SD) mg L^{-1}, 0.03 ± 0.02 mg L^{-1}, 0.25 ± 0.43 mg L^{-1} and 0.57 ± 0.47 mg L^{-1}, respectively (Fig. 4). The TN content in 31.3% of the samples from the EN system exceeded the Environmental Quality Standards for Surface Water (GB3838-2002) limit for type III water. In contrast, the TN content in 9.4% and 15.6% samples from the OS and IA systems, respectively, exceeded the type III water quality limit (GB3838-2002) (Fig. 4). The number of samples that exceeded the TP and NH_4^+-N type III water quality limits did not differ substantially among treatments. No samples exceeded the water quality limit for NO_3^--N (10 mg L^{-1}). Cumulative curve analysis indicated that amount of TP and NH_4^+-N accumulated over the course of the experiment (up to November 27) did not differ significantly different among plant systems. However, the levels of accumulated TN and NO_3^--N were significantly higher in the EN system than in the IA or OS systems (Fig. 5). Average pH was 7.48 in the IA system, 8.16 in the EN system, and 7.9 in the OS system (Fig. 6).

Bacterioplankton communities and predicted functions associated with nitrification/denitrification

The abundances of nearly all of the bacterioplankton phyla differed significantly among macrophyte systems (Fig. 7 A). The abundances of *Acidobacteria*, *Chloroflexi*, *Firmicutes*, *β-Proteobacteria,* and *δ-Proteobacteria* were significantly higher in the OS and IA systems as compared to the EN system, while the abundances of *Actinobacteria*, *Armatimonadetes,* and *γ-Proteobacteria* were significantly lower. OTU-based diversity (α-diversity) was higher in the OS and IA systems than in the EN system (Fig. 7 B), suggesting that bacterioplankton communities were more complex in the OS and IA systems than in the EN system.

252 PCA indicated that the bacterioplankton communities were discrete among the different plants,
253 with the first two axes explaining 83.67% of the total variation (Fig. 8 A). After fitting
254 environmental variables (Table S2) and bacterioplankton community ordination, we found that
255 pH was significantly associated with community differences (Bonferroni-adjusted $P < 0.05$) (Fig.
256 8 A). The co-occurrence network analysis also identified pH as the only environmental factor
257 that co-varied with certain taxa. These pH-correlated taxa included zOTUs belonging to the
258 phyla *Acidobacteria*, *Chloroflexi*, *Firmicutes*, *Proteobacteria,* and *Planctomycetes* (Fig. 8 B).
259 The total average abundance of co-occurring zOTUs was 7.7%, suggesting that pH influenced
260 the relative abundance of the most abundant members of each community.
261 Potential functions of the bacterioplankton communities were predicted using PICRUSt. We
262 focused specifically on functional genes associated with nitrification and denitrification, as these
263 processes are highly related to nitrogen cycling and are likely to affect nitrogen concentration in
264 pond water. Genes associated with all of the steps of denitrification (*napA*, *napB*, *nirK*, *norB*,
265 *norC,* and *nosZ*; (Kanehisa et al. 2017)) were generally more abundant in the IA and/or OS
266 systems than in the EN system. However, the abundances of *narI*, *narG,* and *narH,* which are
267 only involved in the reduction of nitrate to nitrite (Kanehisa et al. 2017), were lower in the IA
268 and/or OS systems (Fig. 9). The abundances of the nitrifying genes *pmoA-amoA*, *pmoB-amoB,*
269 and *pmoC-amoC,* which are involved in the oxidation of ammonia to hydroxylamine (Kanehisa
270 et al. 2017), were significantly higher in the OS system than in the IA and EN systems. The
271 abundance of *hao* was not significantly different among the three macrophyte systems, although
272 the abundances of *narG* and *narH* were lower in the IA and OS systems than in the EN system

(Fig. 9).

Discussion

The macrophyte system described here floated steadily throughout the whole experiment. In addition, mitten crab yield and quality did not differ significantly among plant systems (Fig. 2). Thus, our results demonstrated that local plants were a feasible alternative to EN. Indeed, our data indicated that IA was be the best local replacement for EN, as this plant grew well in ponds and provided sufficient shade. In contrast, OS is not a suitable alternative to EN, as it stopped growing in the middle of the experimental period. The mechanisms underlying the different performances of OS and IA were related to the different growth behaviors and nutrient requirements of the two species. OS has several distinctive growth stages, including tillering, heading, ripening and et al. (Zhang et al. 2018), which typically require different levels of nutrients (Fairhurst et al. 2007). For example, during the tillering stage, OS biomass increases much faster than during other stages of OS growth, and consequently requires a more intensive nutrient supply (Nawaz & Farooq 2017). If the nutrient supply needs are not met (i.e., due to low nutrient concentrations in the water), many tillers become non-productive, and the few remaining productive tillers enter the reproductive stage earlier than normal (Nawaz & Farooq 2017). In submerged paddy fields, the intensity of nutrient supply is manipulated by top-dressing fertilizers (Fairhurst et al. 2007). However, this technique cannot be used in crab-rearing ponds because it would pollute the water.

Unlike OS, IA does not exhibit obvious physiological differences among growth stages. IA has sprawling growth, with biomass increasing over rearing time (from May to November)

294 (Shaltout et al. 2010). Thus, low concentrations of nutrients in the water are sufficient. We
295 consequently concluded that IA is an attractive macrophytic alternative to EN in crab-rearing
296 ponds.

297 Based on the Chinese national water quality standards (GB3838-2002) (SEPA), 31.3% of the
298 outflow samples from the EN system contained TN levels in excess of limits for type III waters
299 (Fig. 4). Thus, the outflow from ponds using the EN system is likely to pollute the downstream
300 environment. Fewer outflow samples from the IA and OS systems, as compared to the EN
301 system, had TN levels above the type III water quality limits. These data suggest IA or OS
302 systems would generate less environmental pollution than EN systems. This suggestion was
303 reinforced by the absolute cumulative nutrient quantities in outflow samples (Fig. 5), which
304 showed that the TN and NO_3^--N concentrations were significantly higher in the EN system, as
305 compared to the IA and OS systems. Indeed, IA and OS assimilated more nitrogen from the
306 water than did EN (Fig. 3). Thus, it was possible that IA and OS more effectively improved
307 water quality than EN, which is a desirable property for macrophytes grown in crab-rearing
308 ponds. An alternative explanation was that the differences in water quality among plant systems
309 were a result of the activities of macrophyte-specific bacterioplankton communities.

310 Bacterioplankton communities differed significantly among the three macrophyte systems (Fig.
311 6), possibly because of the different root deposits produced by the three plants. Terrestrial and
312 macrophytic plants release unique root exudates that drastically alter bacterioplankton
313 community structure (Baudoin et al. 2003; Casamatta & Wickstrom 2000; Nelson et al. 2013;
314 Tanaka et al. 2012; Zhao et al. 2013). Bacterioplankton community composition might also be

315 regulated by pH, as pH was the only environmental factor that was significantly associated with
316 the relative abundances of specific bacterioplankton taxa (Fig. 8). These results were consistent
317 with previous reports, which indicated that pH affects the community structures of both
318 terrestrial bacteria (Fierer & Jackson 2006; Lauber et al. 2009; Rousk et al. 2010) and
319 bacterioplankton (Ren et al. 2015). Moreover, the significant increases in bacterioplankton
320 diversity with lower water nutrients in the IA and OS treatments (Figs. 5, 7 B) indicated that
321 biodiversity helped improve water quality, as observed in other aquatic systems (Cardinale 2011;
322 Gregoracci et al. 2012).

323 The predicted functions of the bacterioplankton community also differed among the
324 macrophyte systems, particularly those related to nitrification and denitrification. Most
325 denitrifying genes, especially those associated with the reduction of nitrite, nitric oxide, and
326 nitrous oxide, were more abundant in the IA and OS bacterioplankton communities, as compared
327 to the EN bacterioplankton community (Fig. 9). Moreover, denitrifier is heterotrophic bacteria,
328 and abiotic environmental factors usually control denitrification process, i.e., pH, temperature
329 and organic carbons (OC). The IA and OS systems that could provide labile OC derived from
330 root exudates (Wang et al. 2018; Xu et al. 2008; Zhu & Cheng 2011) favored denitrification.
331 Overall, the biotic and abiotic influences on denitrification in the IA and OS systems were
332 thought to be co-occurring, consistent with the conclusion in riparian wetlands (Xiong et al.
333 2017). In addition, concentrations of NO_3^--N (and TN) were lower in the IA and OS systems than
334 in the EN system. Thus, the IA and OS systems might have a higher denitrification potential than
335 the EN system. Meanwhile, there were no obvious differences in the abundance patterns of

nitrification-associated genes among macrophyte systems. The genes responsible for the oxidation of ammonium to hydroxylamine (*pmoA-amoA*, *pmoB-amoB,* and *pmoC-amoC*) were significantly more abundant in the OS system (but not the IA system) as compared to the EN system, but the abundances of these genes were relatively low across all of the plant systems, with only ~2–20 copies per sample. In addition, the gene abundances in nitrification process usually could not accurately predict nitrification potential, which can be affected by other abiotic factors (Francis et al. 2005; Rocca et al. 2015; Yao et al. 2018). Thus, nitrification potential may not vary significantly among these three macrophytes, and further studies are required to validate this coupled nitrification-denitrification processes in future.

Conclusions

EN is routinely cultivated in ponds used for mitten crab aquaculture. However, EN temperature sensitivity often leads to plant deterioration and decreased water quality at high ambient temperatures, negatively affecting crab production. Here, we successfully designed a floating system to support the growth of IA and OS on the surfaces of crab-rearing ponds. We then compared the crab yield, outflow water quality, and bacterioplankton communities among ponds with EN, IA, and OS macrophyte systems. Our results indicated that IA growth behavior was preferable to that of OS. Crab yields did not differ significantly among systems. Moreover, outflow water quality, as indicated by TN and NO_3^--N concentrations, was better in the IA and OS systems than in the EN system, due to the greater nitrogen assimilation of IA and OS as compared to EN. In addition, the microbial communities associated with IA and OS had a greater denitrification potential than the microbial community associated with EN. Thus, our results

357 indicated mitten crabs could be successfully reared using native aquatic plant. Specifically, IA
358 was a suitable and environmental-friendly replacement for EN, but OS was not.

359 Data Availability

360 Demultiplexed sequences and metadata are available from the NCBI Sequence Read Archives
361 (SRA) under accession number SRP136316.

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Figure 1

Experimental design and schematics for three macrophyte systems.

Elodea nuttallii (EN), *Oryza sativa* (OS), and *Ipomoea aquatica* (IA). Each macrophyte was planted in a separate sub-pond (three replicate ponds per species). The sub-ponds were connected by a series of PVC pipes. Pond water was replenished from the Yangcheng Lake water, and discharged water flowed into a constructed wetland. The growth of all three macrophytes was restricted to 60% of the pond areas. A micro-porous aeration system was used nightly to ensure sufficient aeration. Photo credit: Linlin Shi.

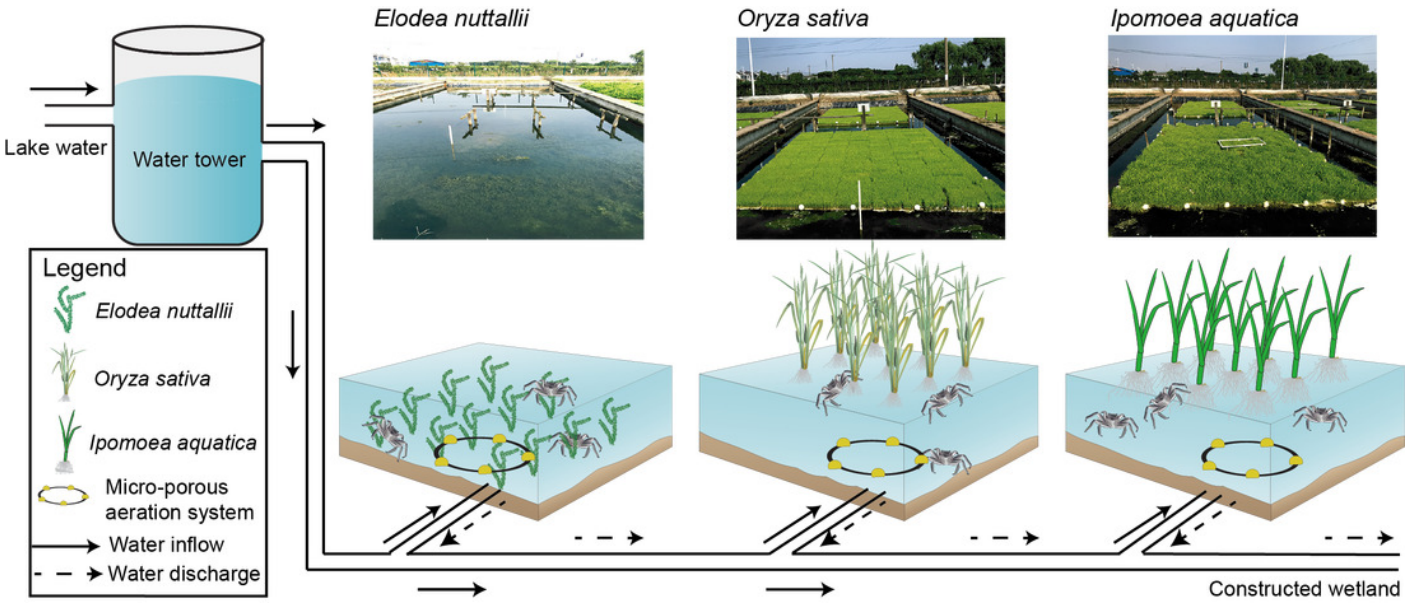


Figure 2(on next page)

The yield and individual weight distributions of the Chinese mitten crabs did not differ significantly among macrophyte systems.

Error bars denote the standard deviations of the mean (n=3). EN, *Elodea nuttallii*; OS, *Oryza sativa;* IA, *Ipomoea aquatica*.

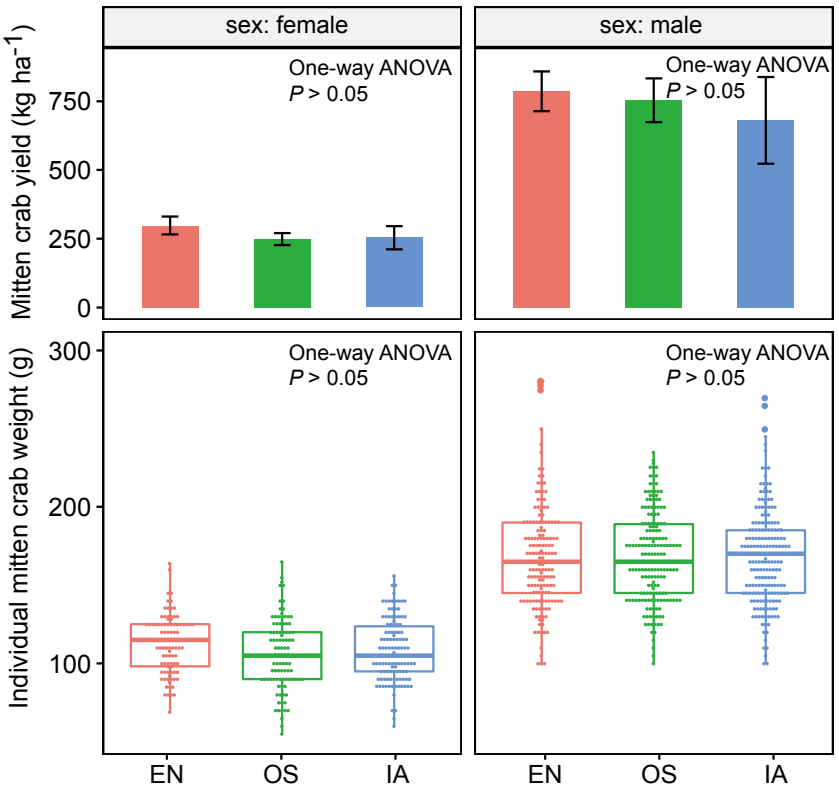

sex: female
sex: male
One-way ANOVA
P > 0.05
Mitten crab yield (kg ha-1)
0
250
500
750
Individual mitten crab weight (g)
100
200
300
EN
OS
IA

Figure 3(on next page)

Assimilation of nitrogen and phosphorous by *Elodea nuttallii* (EN), *Ipomoea aquatica* (IA), and *Oryza sativa* (OS).

Different letters above bars represent significant differences among plant systems ($P < 0.05$; Tukey's HSD test). Error bars denote the standard deviations of the means (n=3).

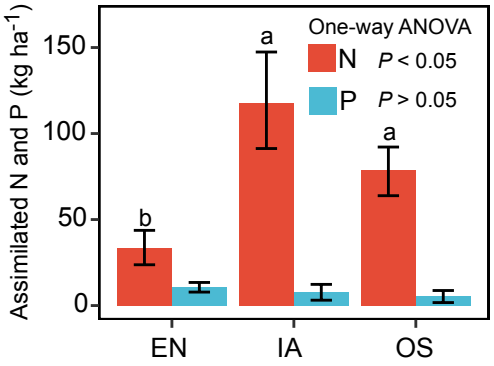

Assimilated N and P (kg ha-1)
150
100
50
0
a
b
a
One-way ANOVA
N P < 0.05
P P > 0.05
EN
IA
OS

Figure 4(on next page)

Figure 4 (A-D) separately shows the TN, TP, NH_4^+–N and NO_3^-–N concentrations in the rearing pond.

Error bars denote the standard deviations of the means (n=3). The brown dashed lines represent the average nutrient concentrations in the inlet waters during the rearing period. The grey dashed lines represent the type III water quality limits from the Chinese Environmental Quality Standard for Surface Water (GB3838-2002). NO_3^--N concentrations were all below the type III water quality limit (10 mg L^{-1}). EN, *Elodea nuttallii*; OS, *Oryza sativa;* IA, *Ipomoea aquatica*.

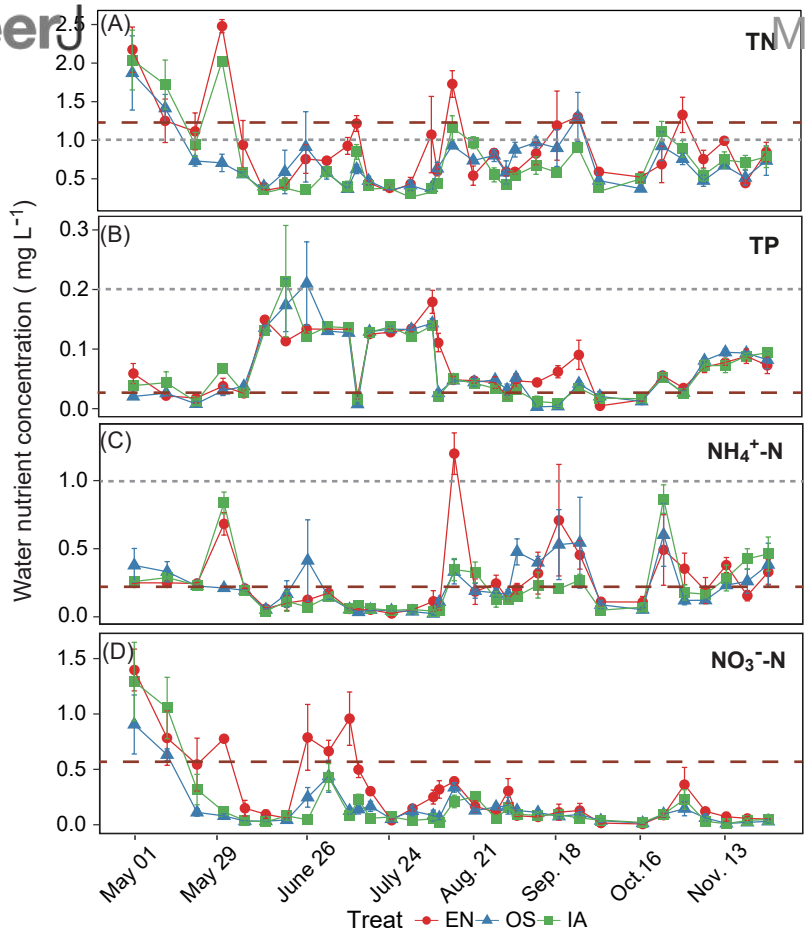

(A)
TN
(B)
TP
(C)
NH4+-N
(D)
NO3--N
Water nutrient concentration (mg L-1)
May 01
May 29
June 26
July 24
Aug. 21
Sep. 18
Oct.16
Nov. 13
Treat EN OS IA

Figure 5(on next page)

Figure 5 (A-D) separately shows the absolute cumulative TN, TP, NH_4^+–N and NO_3^-–N quantities in rearing period.

The statistical significance of differences in cumulative nutrient quantity among macrophyte systems was determined with a Kruskal-Wallis test (n=3) at the end of the rearing periods (November 27). Different lowercase letters represent significant differences ($P < 0.05$; Tukey's HSD test). EN, *Elodea nuttallii*; OS, *Oryza sativa;* IA, *Ipomoea aquatica*.

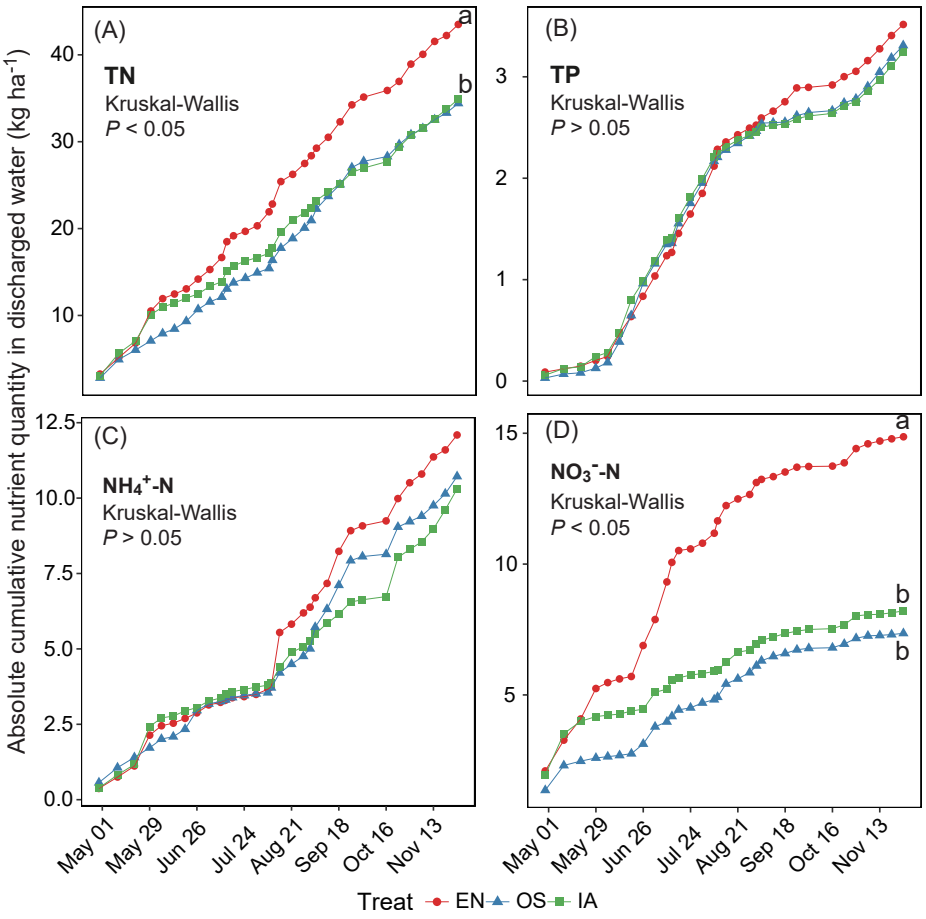

(A)
TN
Kruskal-Wallis
P < 0.05
a
b
(B)
TP
Kruskal-Wallis
P > 0.05
(C)
NH4+-N
Kruskal-Wallis
P > 0.05
(D)
NO3--N
Kruskal-Wallis
P < 0.05
a
b
b
Absolute cumulative nutrient quantity in discharged water (kg ha-1)
May 01
May 29
Jun 26
Jul 24
Aug 21
Sep 18
Oct 16
Nov 13
Treat EN OS IA

Figure 6(on next page)

pH of the outflow water samples during the rearing period.

EN, *Elodea nuttallii*; OS, *Oryza sativa;* IA, *Ipomoea aquatica*.

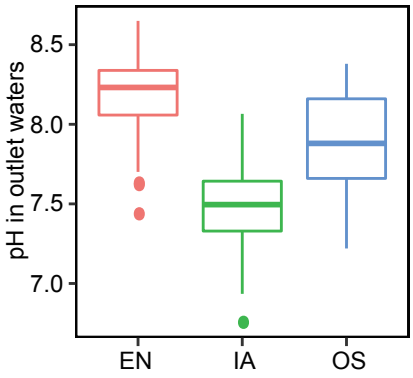

pH in outlet waters
8.5
8.0
7.5
7.0
EN
IA
OS

Figure 7 (on next page)

Bacterioplankton abundances and diversity indices of the three macrophytes systems.

(A) The relative abundances of the dominant bacterial phyla in the bacterioplankton communities, and (B) the associated alpha diversity. Asterisks indicate significant differences in abundances among the macrophyte systems ($P < 0.05$ level; one-way ANOVA; n=3). Error bars denote the standard errors of the means. Different letters above bars represent significant differences among treatments ($P < 0.05$; Tukey's HSD test; one-way ANOVA; n=3). EN, *Elodea nuttallii*; OS, *Oryza sativa;* IA, *Ipomoea aquatica*.

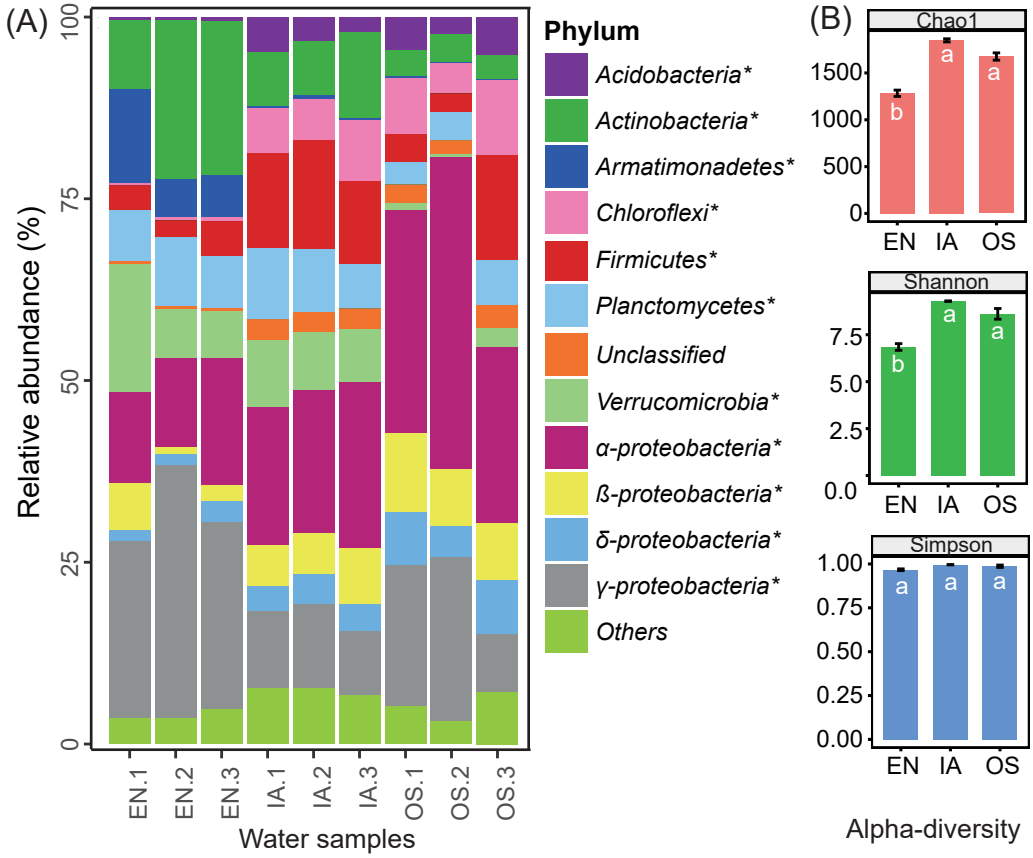

(A)
Relative abundance (%)
0
25
50
75
100
EN.1
EN.2
EN.3
IA.1
IA.2
IA.3
OS.1
OS.2
OS.3
Water samples
Phylum
Acidobacteria*
Actinobacteria*
Armatimonadetes*
Chloroflexi*
Firmicutes*
Planctomycetes*
Unclassified
Verrucomicrobia*
α-proteobacteria*
ß-proteobacteria*
δ-proteobacteria*
γ-proteobacteria*
Others
(B)
Chao1
1500
1000
500
0
b
a
a
EN
IA
OS
Shannon
7.5
5.0
2.5
0.0
b
a
a
EN
IA
OS
Simpson
1.00
0.75
0.50
0.25
0.00
a
a
a
EN
IA
OS
Alpha-diversity

Figure 8(on next page)

Bacterioplankton community structure and environmental factors.

(A) Principal component analysis (PCA) of bacterioplankton community composition and environmental variables. The significant environmental variable (Bonferroni-adjusted $P < 0.05$) is shown with a black arrow; other factors are shown in gray. EN, *Elodea nuttallii*; OS, *Oryza sativa;* IA, *Ipomoea aquatica*. (B) Subnetwork showing the correlation between bacterial taxa and pH. Squared nodes correspond to environmental parameters and circle nodes correspond to zOTUs. Circle nodes not assignable to genus are labeled with the names of higher taxonomic ranks, and node colors represent phyla. Red and blue colors of edge represent negative and positive correlations, respectively.

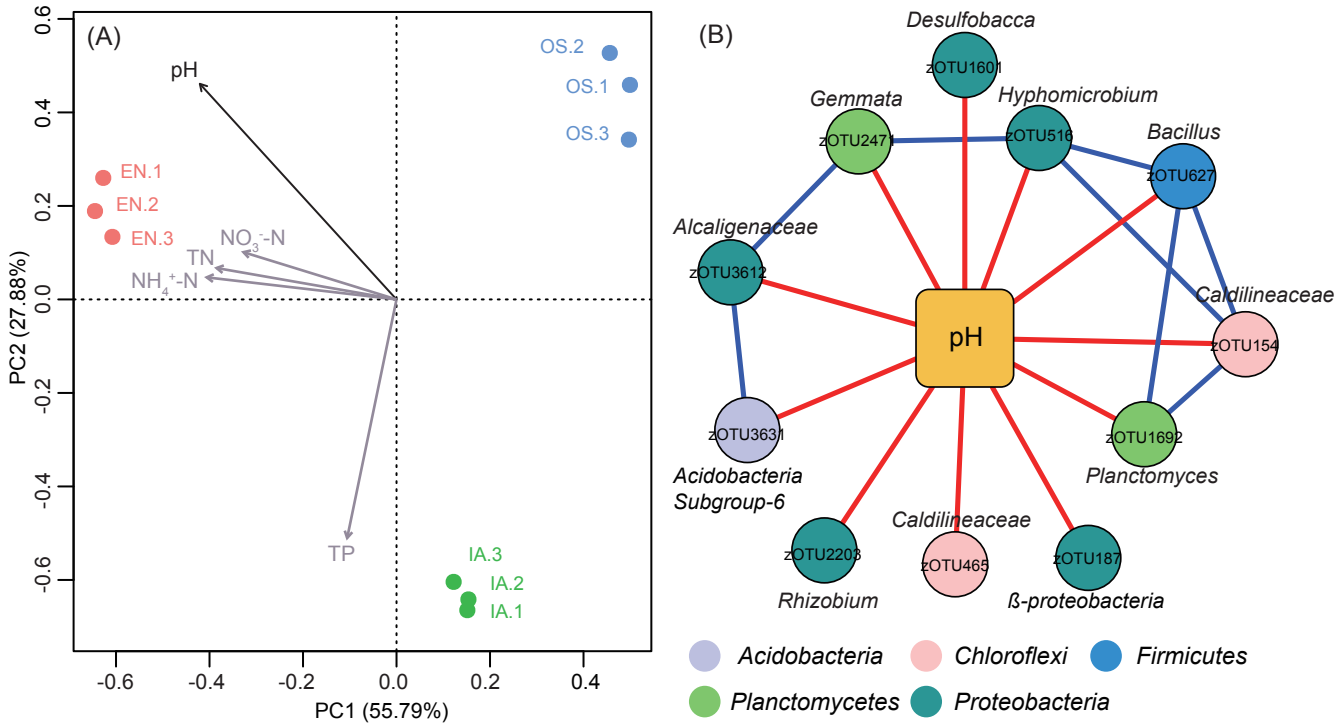
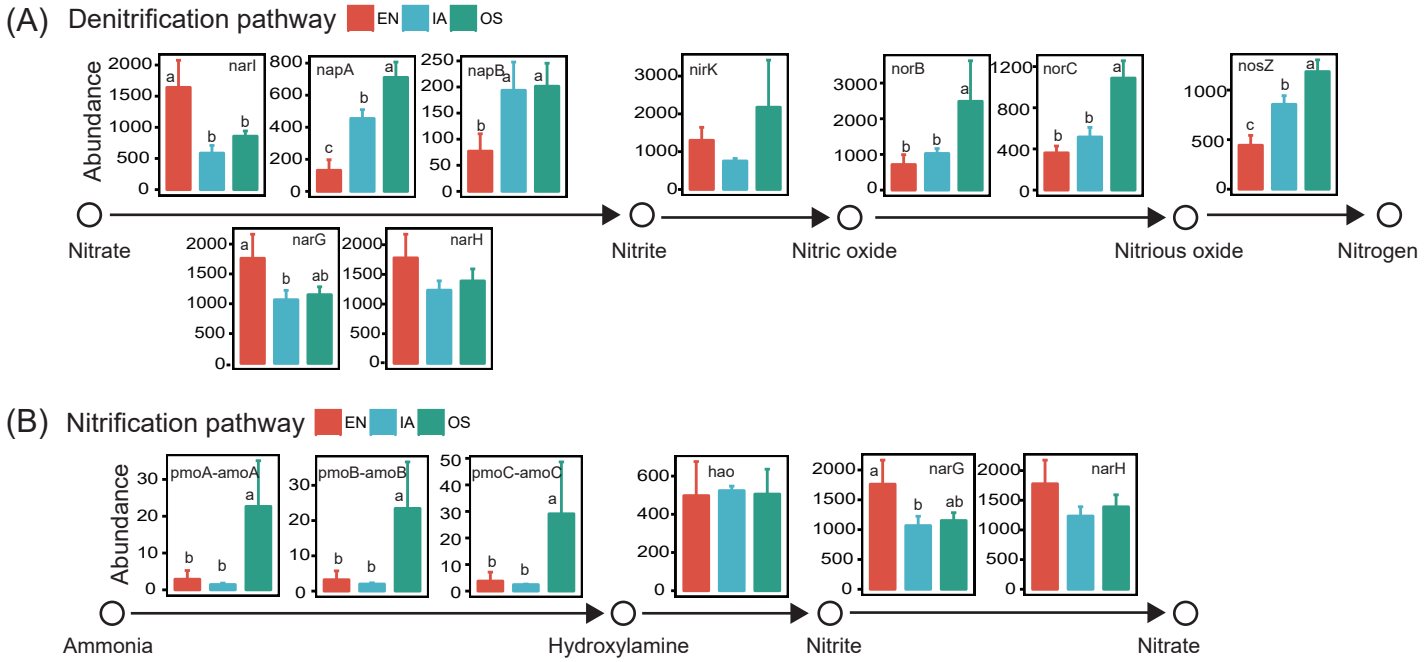

(A)
pH
OS.2
OS.1
OS.3
EN.1
EN.2
EN.3
NO3-N
TN
NH4+-N
TP
IA.3
IA.2
IA.1
PC2 (27.88%)
PC1 (55.79%)
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
(B)
Desulfobacca
zOTU1601
Gemmata
zOTU2471
Hyphomicrobium
zOTU516
Bacillus
zOTU627
Alcaligenaceae
zOTU3612
pH
Caldilineaceae
zOTU154
zOTU3631
Acidobacteria
Subgroup-6
zOTU1692
Planctomyces
Caldilineaceae
zOTU2203
Rhizobium
zOTU465
zOTU187
ß-proteobacteria
Acidobacteria
Chloroflexi
Firmicutes
Planctomycetes
Proteobacteria

Figure 9(on next page)

The abundances of the bacterioplankton genes associated with nitrification and denitrification among macrophyte systems, as predicted by PICRUSt.

(A) and (B) shows the denitrification and nitrification predicted gene abundances, respectively. Error bars denote the standard deviations of the means (n=3). Different lowercase letters above bars represent statistically significant differences ($P < 0.05$; one-way ANOVA followed by Tukey's HSD tests). EN, *Elodea nuttallii*; OS, *Oryza sativa;* IA, *Ipomoea aquatica*.


(A) Denitrification pathway
EN
IA
OS
Abundance
narI
napA
napB
nirK
norB
norC
nosZ
narG
narH
Nitrate
Nitrite
Nitric oxide
Nitrious oxide
Nitrogen
(B) Nitrification pathway
EN
IA
OS
Abundance
pmoA-amoA
pmoB-amoB
pmoC-amoC
hao
narG
narH
Ammonia
Hydroxylamine
Nitrite
Nitrate