Mechanisms of enhanced inorganic phosphorus accumulation by periphyton in paddy fields as affected by calcium and ferrous ions

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HIGHLIGHTS
• Calcium and ferrous ions promoted inorganic P (Pi) accumulation on the periphyton.
• P precipitation and coprecipitation with carbonates or Fe(OH)3 were main mechanisms.
• Efficiency of Fe(II) was higher than Ca(II) in enhancing Pi abiotic accumulation.
• Periphyton would help reduce P fixation in soil and P loss from the paddy fields.

GRAPHICAL ABSTRACT

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ABSTRACT
The effect of periphyton propagation in paddy fields on phosphorus biogeochemical cycling has received little attention. In this phytotron study, inorganic phosphorus (Pi) accumulation by periphyton was investigated for varying inputs of calcium [Ca(II)] or ferrous iron [Fe(II)], and lighting conditions. Results indicated that additions of Ca(II) or Fe(II) enhanced abiotic accumulation of Pi by up to 16 times, and decreased solution Pi concentration by up to 50%, especially under light condition. The enhanced Pi accumulation into periphyton intensified with increasing Pi concentration, and Pi accumulation showed a positive linear relationship with Ca or Fe accumulation. Abiotic accumulation of Pi induced by Ca(II) was mainly through Ca-phosphate precipitation, and co-precipitation of Pi with carbonates at pH > 8. Accumulation with added Fe(II) was mainly considered to be through Fe(III) phosphate precipitation coupled with adsorption of Pi by ferric hydroxides. Moreover, Fe(II) was more effective than Ca(II) in promoting abiotic accumulation of Pi by periphyton. Our results indicate the potential for controlling environmental factors to enhance the role of periphyton in biogeochemical cycling and P-use efficiency in paddy rice fields and to reduce P discharged to neighboring water bodies.

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1. Introduction
Paddy fields comprise the largest anthropogenic wetland ecosystem, and they subjected to intensive human activities (Kögel-Knabner et al., 2010). Rice is the staple food for more than half of the world’s population and phosphorus (P) is a limiting nutrient for rice production
Farmers commonly apply a large base dose of P fertilizer before planting rice (Ayaga et al., 2006; Lan et al., 2012). However, since P uptake by rice is minimal during the seedling stage, excess fertilizer P is either fixed by soil particles, such as iron (Fe) and aluminum (Al) oxides/hydroxides and calcium (Ca) bounded carbonates, or leached out of paddy fields (Arai and Sparks, 2007; Lgwe et al., 2010; Lan et al., 2012). Then, during the critical flowering and maturation stages of high P demand, plant-available P to rice is low. Consequently, P use efficiency is low in paddy fields, and these fields are an important nonpoint source of P runoff (MacDonald et al., 2011; Yang and Lu, 2014; Zhou and Zhu, 2003). Better management strategies based on a mechanistic understanding of P biogeochemical processes are needed to improve P use efficiency and reduce P discharged from paddy fields.

Periphyton is ubiquitous in paddy fields because light conditions, water, temperature, and nutrients are suitable for periphyton to proliferate in the early stage of rice growth (Hagerthey et al., 2012; Kirk, 2004; Yang et al., 2016). Periphyton is an under-water micro-floral community that attaches to substrates such as submerged plants or plant parts, rocks, and sediments (Larned, 2010). Its components include algae, bacteria, protozoa, metazoans, epiphytes associated with inorganic minerals such as Fe and Al hydroxides, and Ca/Mg carbonates (Wu et al., 2012, 2014). Periphyton can act as a potential P sink in wetlands and is also regarded as a buffer for P precipitation and release between sediments and water (Dodd, 2003; Drake et al., 2012; Lu et al., 2016a; Wu et al., 2011; Zamalloa et al., 2012). Phosphorus is normally in excess for rice during the early stages of growth (Ayaga et al., 2006; Lan et al., 2012). In this stage, the periphyton microorganisms thrive under sufficient light and could potentially accumulate P, which should reduce P fixation by soils and P discharged to the neighboring water bodies. Periphyton would decompose and potentially release P as the canopy cover fills during later stages of rice growth (Drake et al., 2012). Hence, the timing of biogeochemical P cycling through periphyton could be in synchrony with the timing of crop needs, thereby improving P use efficiency and reducing P loss from the paddy fields. However, this potentially beneficial effect of periphyton on P cycling in paddy fields has received little attention.

Periphyton can modify the local chemical environment and retain P by biotic as well as abiotic accumulation (Scinto and Reddy, 2003). The localized pH in the zone of actively photosynthesizing periphyton increases by up to 1 unit, which favors Ca-phosphate precipitation, and concurrent deposition of carbonate-phosphate complexes (Woodruff et al., 1999). Moreover, periphyton can cause super-saturated O₂ concentrations near the soil surface through photosynthesis, which encourages deposition of metal phosphates (Dodd, 2003). Furthermore, periphyton can act as a sink for P by reducing P fixation and releasing P to the paddy fields. However, this potentially beneficial effect of periphyton on P cycling in paddy fields has received little attention.

2. Materials and methods

2.1. Periphyton culture

Periphyton was cultured from a water sample collected at Pullen Park Lake, a eutrophic lake in Raleigh of North Carolina, USA. The water was incubated in two glass fish tanks of 60 × 25 × 30 cm (l × w × h) under controlled temperature and light conditions in a phytotron. Polypropylene fiber carriers (FCs, Jineng Environmental Protection Company of Yixing, China) were used as a substrate for periphyton growth and enrichment. The FCs were disinfected using 95% alcohol solution and rinsed with deionized water. The FCs were submerged into the 45 L fish tanks filled with 30 L of lake water, and modified BG-II medium composed of the following minerals was added: 16 mg L⁻¹ Na₂CO₃, 85.1 mg L⁻¹ NaNO₃, 8.71 mg L⁻¹ K₂HPO₄, 37 mg L⁻¹ MgSO₄·7H₂O, 36 mg L⁻¹ CaCl₂·2H₂O, 2.86 mg L⁻¹ H₂BO₃, 0.18 mg L⁻¹ MnCl₂·4H₂O, 4.36 mg L⁻¹ Na₂EDTA·2H₂O, 3.15 mg L⁻¹ FeCl₃·6H₂O; trace elements of 0.22 mg L⁻¹ ZnSO₄·7H₂O, 0.39 mg L⁻¹ Na₂MoO₄·7H₂O, 0.079 mg L⁻¹ CuSO₄·5H₂O, 0.012 mg L⁻¹ Co(NO₃)₂·6H₂O; and vitamins of 0.13 mg L⁻¹ Vitamin B12, 0.33 mg L⁻¹ Thiamin, 0.02 mg L⁻¹ Biotin. The fish tanks with FCs were incubated for 2 months in a manually controlled climatic (phytotron) box and exposed to a light/dark cycle of 14 h (28 °C)/10 h (20 °C), a light intensity of 12,000 lx, and a humidity 80%. At the end of the incubation, the periphyton colonized on the FCs were removed from the fish tanks and used for the experiments described below.

2.2. Periphyton characterization

The morphology of the phototrophic periphyton was characterized by optical microscopy (OM, NICON-80i, Japan) at 400 × magnification, scanning electron microscopy (SEM, Quanta200, FEI, Netherlands), confocal laser scanning microscopy (CLSM, Zeiss-LSM710, German), and XRD across an angle range of 0 to 70° 2θ (D/max-2C, Rigaku Corporation, Japan). Periphyton biomass used throughout this study were on an oven-dry basis, for which hydrated periphyton were dried at 105 °C for 24 h.

2.3. Inorganic P accumulation with batch experiments

Parameters that were evaluated in the overall set of experiments described below are summarized in Table 1.
Table 1
Summary of parameters used in different experiments reported here.

<table>
<thead>
<tr>
<th>Experiment type</th>
<th>Variables</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Part 1: Effect of periphyton on the physiochemical properties of suspension</td>
<td>With or without periphyton; light or dark conditions</td>
<td>Added P (0.2 mM), time (24 h), other nutrients same as BG-Ilmedium solutions</td>
</tr>
<tr>
<td>Batch</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Part 2: Effect of Ca(II) on pH, P, and Ca accumulation by periphyton</td>
<td>Added P (0.0–0.4 mM), light or dark conditions</td>
<td>Added Ca(II) (0.6 mM), time (24 h)</td>
</tr>
<tr>
<td>Batch Kinetics</td>
<td>Added Ca(II) (0.1–1.2 mM), light or dark conditions</td>
<td>Added P (0.2 mM), time (24 h)</td>
</tr>
<tr>
<td>pH control</td>
<td>pH (6–9), under light, dark, or light conditions coupled with 0.1% NaN3</td>
<td>Added P (0.2 mM), light conditions</td>
</tr>
<tr>
<td>Part 3: Effect of Fe(II) on pH, Eh, P, and Fe accumulation by periphyton</td>
<td>Both added P and Fe(II) (0.2 mM), light condition, first add P and then Fe</td>
<td></td>
</tr>
<tr>
<td>Kinetics</td>
<td>Added Fe(II) (0.0–0.2 mM), time (0–5 h)</td>
<td>Added P (0.2 mM), light conditions, first add P and then Fe</td>
</tr>
<tr>
<td>Batch</td>
<td>Added P (0.0–0.4 mM), light or dark conditions</td>
<td>Added Ca(II) (0.6 mM)</td>
</tr>
<tr>
<td>Added Fe(II) (0.0–0.2 mM), light or dark conditions</td>
<td>Added P (0.2 mM), time (24 h), first add P and then Fe</td>
<td></td>
</tr>
<tr>
<td>Fe/Pi addition sequence</td>
<td>Added Fe (0.05, 0.1, 0.2 mM), Fe and P, addition sequence</td>
<td>Added P (0.2 mM), light conditions, time (24 h)</td>
</tr>
</tbody>
</table>

Additional control: periphyton (0.4 g dry weight/L) and initial pH 6.0 in all experiments; except when otherwise indicated; other nutrients were at one-tenth strength of BG-IImedium solutions in the Ca and Fe experiments.

2.3.3. Ferrous ion effect
To determine the effect of Fe(II) on P accumulation by the periphyton, incubation experiments were conducted with various additions of Fe(II). These incubations were done in the glove-box under an inert (N2) atmosphere to determine the ability of periphyton to oxidize Fe(II) to Fe(III). The oxygen was removed from deionized water used to prepare experimental solutions by boiling for 5-min, then aspirating the water with N2 for 20 min. as it cooled. Except for Fe(II) and P, only one-tenth-strength BG-II nutrient medium solution was used, as noted above. The incubation procedure was similar to that mentioned above. Nutrient solutions containing 0.2 mmol L−1 FeCl2 and K2HPO4 with or without periphyton were used and FeCl2 was the last solution added to the flasks before incubation. After 0.2, 1.0, 2.0, and 5.0 h of incubation, pH and Eh were measured in the solutions and simultaneously, supernatants were filtered through a 0.45 μm cellulose-acetate filter membrane and then acidified to pH 1 with 6 mol L−1 HCl to inhibit Fe(II) oxidation and hydrolysis. Then Fe(III) and Fe(II) ions were measured according to (Lu, 2000), and P was determined using the ascorbic acid molybdenum-blue method (APHA, 1998). An additional experiment with treatments of different mixtures of Fe(II) (0–0.2 mmol L−1) and P concentrations (0–0.4 mmol L−1) was conducted to study the effect of periphyton on pH, Eh, and P accumulation under light and dark conditions, respectively.

Since Fe(II) oxidation by periphyton and hydrolysis was found to be quick in this study, we also evaluated the effects of the sequences of different Fe and P addition on P accumulation in periphyton. Nutrient solutions containing periphyton (without added including Fe and P) were initially adjusted to pH 6.0, and FeCl2 and K2HPO4 solutions were separately adjusted to pH 6.0 under an N2 atmosphere. Three different treatment sequences were evaluated (Table 1): (i) first adding K2HPO4 to nutrient solutions and then adding FeCl2 1 h later (P-Fe); (ii) first adding FeCl2 to nutrient solutions and then adding K2HPO4 1 h later (Fe-P); and (iii) first adding K2HPO4 to nutrient solutions and then adding freshly precipitated ferrihydrite (ferric hydroxide) 1 h later (P-Ferricydrate). The added concentration of Pi was fixed at 0.2 mmol K2HPO4 L−1, and FeCl2 concentrations were varied at 0.05, 0.1, 0.2 mmol L−1. Accumulated Pi and Fe were measured after 24 h of incubation under light conditions. Since Fe is a micronutrient to microorganisms and its assimilation is limited during the 24 h incubation time, Fe accumulation in this experiment was considered to be largely abiotic. The net Pi accumulation induced by added Fe was obtained by subtracting the amount of Pi accumulation in the control treatment (without Fe) from that in Fe treatment.

2.4. Kinetic P accumulation experiments
Kinetic studies determined the effect of periphyton on pH, P accumulation, Ca accumulation, or Fe accumulation for varying concentrations of added Ca(II) (0, 0.6, 0.9, 1.8 mmol L–1), or Fe(II) (0, 0.1 0.2 mmol L–1). The incubation procedure was similar to those of experiments described above. Solutions of CaCl2 or FeCl2 were the last solution added to the flasks before incubation. The pH, P, Ca(II), Fe(III), and Fe(II) ions were measured after 0.5, 2, 4, 8, 24, 48, 72, 96 h of incubation in the phytotron under light conditions. The net Pi, Ca or Fe accumulation by abiotic reactions were obtained by subtracting the amount of Pi, Ca or Fe accumulation in the control treatment (without Ca or Fe while diminishing interference other nutrient ions with Ca and P treatments. The incubation procedure was the same as described above, and incubation was done under both light and dark conditions. In order to separate biotic and abiotic processes affecting P accumulation by the periphyton, 0.1% NaN3 was added into one set of the treatment nutrient solutions to inhibit microbial activity (Lu et al., 2014). They found the microbial activity was absolutely inhibited with this method. pH was controlled at 6.0, 7.0, 8.0, and 9.0 by manually adjusting with 0.1 M HCl or NaOH.

2.3.2. Calcium ion effect
To investigate the effect of Ca(II) on Pi accumulation by the periphyton, different Ca(II)concentrations (CaCl2, 0–1.2 mmol L−1) and P concentrations (K2HPO4, 0–0.4 mmol L−1) were varied independently. Other than Ca and P added at treatment levels(Table 1), a one-tenth-strength BG-Ilnutrient solution was used to maintain microbial activity.
addition) from those in Ca or Fe treatment. Visual Minteq 3.1 software (Gustafsson J.P., 2014. Visual MINTEQ ver. 3.1) was used to estimate possible chemical reactions in periphyton suspensions.

2.5. Statistical methods

SPSS 15.0 (SPSS Inc., Chicago, IL, USA) was used to perform the statistical analysis of the data. Correlation analyses were conducted between Pi accumulation and Ca or Fe accumulation. Independent-sample t-tests were used to compare the pH, Eh, nutrient concentration change with and without periphyton, or Pi accumulation in different Ca(II), Fe(II), or Pi treatments, with $p < 0.05$ indicating statistical significance. All results are the average of three replicates with standard deviation ($\pm$SD) reported.

3. Results and discussion

3.1. Characteristics of periphyton

From the morphological characteristics of the original periphyton shown in Fig. 1A–C, green algae and filamentous cyanobacteria were the dominant community of the periphyton, likely along with associations of other bacteria, diatoms, and protozoa. Microalgae and filamentous cyanobacteria formed the base matrix, and their complex structures contained voids, channels, cavities, and pores that allow diffusion of nutrients and oxygen and facilitated P transport and adsorption inside the periphyton (Wu et al. 2012, 2014). The morphological characteristics of periphyton observed in this study were generally consistent with those in paddy fields (Wu et al., 2016). Moreover, microsurfaces of periphytic algae and filamentous cyanobacteria were covered with mineral-like materials (Fig. 1C), and calcite was the main crystalline mineral detected by the XRD (Fig. 1D). Previous studies also indicated that some periphytic algae were able to form carbonate minerals, such as calcite, on the micro-surfaces of periphyton, which benefit the P adsorption capability of periphyton (Scinto and Reddy, 2003).

3.2. Pi accumulation by the periphyton

3.2.1. Effect of periphyton on the physiochemical properties in the nutrient solutions

Results in Table 2 indicated that except for Fe, the pH, Eh, Ca, Mg, and Pi concentrations of nutrient solutions for the control treatments had minimal differences between light and dark conditions. Since the initial Pi concentration in the added nutrient solution was 6.2 mg L$^{-1}$, results for the light and dark control treatments suggested that P loss from the nutrient solutions in the absence of periphyton was <3%. However, the presence of periphyton under dark conditions increased the pH, Ca and Mg concentrations, but did not significantly ($p > 0.05$) decrease the P concentration. Under light conditions, the presence of periphyton increased the pH and Eh, and decreased the Ca, Mg, Fe, and P concentrations significantly ($p < 0.05$). Iron(III) ions that are initially in the nutrient solution as Fe-EDTA complexes likely precipitated as poorly crystalline Fe hydroxide under light conditions, thereby lowering dissolved Pi (and possibly Ca, and Mg) via adsorption or co-precipitation (Arai and Sparks, 2007). However, periphyton respiration under dark conditions promoted the release of Ca and Mg, and had little effect on P. Dodds (2003) found that anoxia associated with periphyton respiration at night may offset the effect of abiotic P accumulation during daytime. It is possible that Ca(II) and Fe(II) ions cycle through precipitation and dissolution of solid phases in paddy fields, with
important consequences for biogeochemical cycling of P (Arai and Sparks, 2007; Kögel-Knabner et al., 2010).

3.2.2. Effect of Ca(II) on Pi accumulation

Inorganic P accumulation by the periphyton was markedly affected by Ca(II) inputs. Results in Fig. 2A showed nearly a four-fold increase in Pi accumulation with increasing Ca(II) concentration and correspondingly, dissolved Pi concentrations decreased gradually (data not shown). When the added Ca(II) concentration was increased from 0 to 1.2 mmol L\(^{-1}\), Pi accumulation by the periphyton increased by 3.2 and 2.4 times under light and dark conditions, respectively. Moreover, Pi accumulation by the periphyton under light conditions was 5–10 times higher than that under dark conditions for the same Ca(II) concentration. Moreover, Pi accumulation by the periphyton increased significantly with increasing Pi concentration at constant Ca(II) concentration, especially under light conditions (Fig. 2B). These results demonstrated that Ca(II) promoted Pi accumulation by the periphyton and consequently decreased the Pi concentrations in the nutrient solutions, which was mainly affected by photosynthesis in the periphyton.

Microorganisms must assimilate P to maintain normal physiological activities. A previous study had shown that P reserves occurred as polyphosphate inside the cells of phototrophs, such as inside vacuoles of green algae (Guzzon et al., 2008). Moreover, the special morphological characteristics of periphyton contain numerous voids, channels, cavities, and pores (Fig. 1), which provide micro-spaces or adsorption sites for the periphyton to capture or intercept nutrients, including P (Dodds, 2003). Extracellular polymeric substances (EPS) may account for 50 to 90% of the total organic carbon of periphyton, which is considered as the primary matrix material and sorption sites (Flemming et al., 2007). Lu et al. (2014) found that adsorption due to EPS was a major process during Pi removal from wastewater. Therefore, periphyton could accumulate Pi through bio-sorption, including assimilation, entrapment, and adsorption as mentioned above, even when no Ca(II) was added (Fig. 2A) (Liu et al., 2016a, 2016b; Dodds, 2003; Lu et al. 2014, 2016b).

Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH</th>
<th>Eh(_7)</th>
<th>Ca (mg L(^{-1}))</th>
<th>Mg (mg L(^{-1}))</th>
<th>Fe (mg L(^{-1}))</th>
<th>Pi (mg L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (dark)</td>
<td>6.27</td>
<td>129 ± 2</td>
<td>10.60 ± 0.10</td>
<td>4.08 ± 0.12</td>
<td>0.60 ± 0.00</td>
<td>6.22 ± 0.14</td>
</tr>
<tr>
<td>Control (light)</td>
<td>6.23</td>
<td>117 ± 3</td>
<td>10.30 ± 0.49</td>
<td>3.98 ± 0.32</td>
<td>0.49 ± 0.05</td>
<td>6.09 ± 0.02</td>
</tr>
<tr>
<td>Periphyton (dark)</td>
<td>7.40</td>
<td>140 ± 4</td>
<td>13.16 ± 0.2</td>
<td>4.72 ± 0.14</td>
<td>0.59 ± 0.01</td>
<td>5.93 ± 0.09</td>
</tr>
<tr>
<td>Periphyton (light)</td>
<td>8.72</td>
<td>204 ± 2</td>
<td>9.43 ± 0.80</td>
<td>3.51 ± 0.29</td>
<td>0.21 ± 0.02</td>
<td>4.50 ± 0.04</td>
</tr>
</tbody>
</table>

Fig. 2. Inorganic P (Pi) accumulation by periphyton as affected by (A) added Ca(II) concentration (initial Pi concentration = 0.2 mmol L\(^{-1}\)), and (B) added Pi concentration (initial Ca(II) concentration = 1.2 mmol L\(^{-1}\)). (C) pH variation with Ca(II) concentration; (D) Pi accumulation as affected by different pH in light or dark conditions, or by NaN\(_3\) treatments (initial added Ca(II) concentration = 1.2 mmol L\(^{-1}\) and added Pi = 0.2 mmol L\(^{-1}\)).
Photosynthetic-driven changes in pH are likely the driver for Pi uptake under light conditions. Although the initial pH of each periphyton suspension was set to 6.0 at the start of incubation, the suspension pH increased to nearly 9.0 under light (photosynthetic) conditions. The pH increased to only 7.5 under dark conditions (Fig. 2C), probably due to a combination of acid-producing respiration and pH buffering by carbonates. Geochemical modeling using Visual Minteq 3.1 indicated that at pH < 8.0, the aqueous solutions in our suspensions were oversaturated with respect to Ca-phosphates, and also became oversaturated with respect to calcite as pH approached 9.0. Phosphate may co-precipitate with particulate carbonates, which promotes Pi accumulation (Otsuki and Wetzel, 1972). Calcite was detected by XRD in our original periphyton stock suspensions (Fig. 1D) which gives credence to a mechanisms of phosphate co-precipitation with calcite, in addition to possible Ca-phosphate precipitation.

Data reported in Fig. 2D show the overriding effect of pH on Pi uptake into periphyton. In one case, NaN₃ was used as a biocide to impede microbial respiration and assimilation (Lu et al., 2014), while the pH of periphyton suspensions in each treatment was manually adjusted with 0.1 mol L⁻¹ HCl or NaOH to a range between 6 and 9. Results showed that Pi accumulation by the periphyton increased with increasing pH, particularly at pH > 8. At pH < 8, Pi accumulation followed the order light treatment > dark treatment > light + NaN₃ treatment. Between pH 8 and 9, Pi accumulation increased 4–6 times, with no significant differences between treatments. These results not only demonstrated that assimilation was a minor mechanism for Pi accumulation by periphyton in our 24-h experiments, but it also inferred that the addition of Ca(II) and increasing pH induced by photosynthesis promoted Pi removal by Ca-phosphate precipitation or co-precipitation with carbonates at pH > 8 (Dodds, 2003; Lu et al., 2016b; Scinto and Reddy, 2003). On the contrary, <0.1 mg/g of Pi release was observed when periphyton was incubated without Pi addition in the dark condition (Fig. 2B). The difference in pH between light and dark conditions (Fig. 2C) explained the corresponding differences in Pi accumulation. It is noteworthy that Dodds (2003) found that periphyton respiration at night may partially offset abiotic accumulation of Pi during the daytime.

Results of kinetic experiments showed that the rate of Pi accumulation in periphyton suspensions was most rapid within the first 8–40 h, and increased with increasing Ca(II) concentration (Fig. 3A). Concurrently, the pH of periphyton suspensions increased from 6 to 9 within the first 8 h and then rose gradually to nearly pH 10 to our final measurement at 96 h (Fig. 3B). For Ca(II) additions of 0.9 and 1.8 mmol L⁻¹, the rate of Pi accumulation was slower at pH < 8 during the first 4 h, became rapid as pH increased above 8, and again slowed after 24 h (Fig. 3A, B). Results in Fig. 3C showed that Ca accumulation increased only when the suspension pH rose from 8 to 10, which was in agreement with the results obtained in pH control experiment (Fig. 2D). Collectively, our results indicated that photosynthesis induced an increase in suspension pH to > 8, which markedly promoted abiotic accumulation of Pi and Ca. Moreover, Pi accumulation was linearly correlated with Ca accumulation for each added Ca(II) concentration (with R² > 0.89, p < 0.01), and the slopes of linear regressions decreased with increasing Ca(II) concentration (Fig. 3D).

Inorganic P accumulation by the periphyton included abiotic and biotic processes. In the control (no Ca(II) addition) samples, we measured <0.1 mM Ca was in the aqueous phase of the periphyton suspensions, which was released from periphyton itself since no Ca was added to the diluted nutrient solution. Pi accumulation in the control samples...
was mainly by bio-sorption, resulting in a molar ratio of accumulated P\textsubscript{i}/Ca of 1.31. Increasing added Ca(II) concentration promoted abiotic Ca accumulation, likely as calcite or Ca-phosphate precipitation, which consequently reduced the molar ratio of accumulated P\textsubscript{i}/Ca to 0.70–0.81 (Fig. 3C, D). Molar ratios of net P\textsubscript{i}/Ca accumulated by abiotic reactions in this study were in the range of 0.66–0.71, which were close to molar ratios of P\textsubscript{i}/Ca accumulated in the different Ca(II) concentration treatments. When adsorption characteristics of calcite are not significantly changed during co-precipitation, the phosphate co-precipitation rate would be linearly related to the calcite precipitation rate (House, 1990). Thus the mechanism responsible for P\textsubscript{i} and Ca accumulation in each treatment should be similar. Our results suggested Ca-phosphate precipitation and co-precipitation of P with carbonates as dominant mechanisms for P\textsubscript{i} accumulation by the periphyton at different Ca(II) concentrations under light conditions (Fig. 2D). Based on our measured solution properties and Visual Minteq equilibrium calculations, our systems were oversaturated with respect to hydroxyapatite (Ca\textsubscript{5}(PO\textsubscript{4})\textsubscript{3}(OH)), tricalcium phosphate (Ca\textsubscript{3}(PO\textsubscript{4})\textsubscript{2}), and octacalcium phosphate (Ca\textsubscript{10}(OH)\textsubscript{2})(PO\textsubscript{4})\textsubscript{6}. The P\textsubscript{i}/Ca molar ratios in these precipitates were in the range of 0.6–0.75, which was in agreement with our measured ratios. Hydroxyapatite (Ca\textsubscript{5}(PO\textsubscript{4})\textsubscript{3}(OH)) as a main Ca-phosphate precipitation was widely observed in aerobic sludge granules (Huang et al., 2015; Manas et al., 2011). In dark conditions, P\textsubscript{i} accumulation by periphyton occurred mainly at high Ca or P concentrations (Fig. 2), which may be the result of Ca-phosphate precipitation. Some microbial species in periphyton could excrete mucus to capture Ca-phosphate and carbonate-phosphate precipitates on micro-surfaces of periphyton, and thus enhance its P adsorption capacity (Karageorgiou et al., 2007).

3.2.3. Effect of Fe(II) on P\textsubscript{i} accumulation
Dissolved Fe(II) can occur in paddy fields at concentrations > 10 mM after flooding (Kögel-Knabner et al., 2010; Kirk, 2004), which can also impact biogeochemical cycling of P. Incubations with added Fe(II) and P\textsubscript{i} in the presence and absence of periphyton for different periods of time under light conditions and an N\textsubscript{2} atmosphere showed changes in pH, Eh7, and Fe concentrations (Fig. 4). For the control treatment without periphyton, pH decreased by <0.5 units and Eh7 increased from 0 to 25 mV, while dissolved Fe(II) and total Fe [Fe(II) + Fe(III)] changed by ≤15% with increasing incubation time (Fig. 4). Because added FeCl\textsubscript{2} was the only Fe source in the nutrient solution, almost 90% of total dissolved Fe occurred as Fe(II). This result suggested that oxidation of Fe(II) in the nutrient solution without periphyton was limited. In contrast to the control, the presence of periphyton significantly increased the pH and Eh7, and decreased Fe(II) and total soluble Fe concentration in the suspensions, even after 0.2 h of incubation (Fig. 4). Differences between the systems with vs. without periphyton grew larger with incubation time. Although Fe is a micronutrient for microorganisms, its assimilation should be limited in such a short time period. After 0.2 h, pH and Eh7 in the periphyton systems were 1.1 units and 76 mV greater than those in the respective controls, and dissolved Fe(II) and total Fe concentrations were just 13% and 35% of their concentrations in the control. >65% of total dissolved Fe existed as Fe(III) after 0.2 h of incubation, indicating that the loss of dissolved Fe was due to rapid oxidation of Fe(II) to Fe(III) and precipitation of Fe(III) hydroxide. Both Fe(III) and Fe(II) could additionally be removed by complexation with negatively charged organic functional groups in periphyton (Hong et al., 2015).

The effect of Fe(II) concentration on P\textsubscript{i} accumulation is presented in Fig. 5. Results indicated that P\textsubscript{i} accumulation by the periphyton increased linearly with increasing Fe(II) concentration, and also with increasing P\textsubscript{i} concentration in the presence of Fe(II). The accumulation of P\textsubscript{i} under light conditions was modestly greater than in the dark condition, although pH and Eh7 under light were generally 1.7 units and 80 mV higher than those in the dark. When added Fe(II) was increased from 0 to 0.2 mmol L\textsuperscript{-1}, P\textsubscript{i} accumulation increased by >16 times. Nearly all added Fe(II) was accumulated by the periphyton under both lighting conditions. These results indicated that Fe(II) can significantly promote P\textsubscript{i} accumulation in the periphyton either under daylight or at night, and Fe(II) was much more effective that Ca(II) in promoting P\textsubscript{i} accumulation.

More detailed processes responsible for P\textsubscript{i} and Fe accumulation were investigated using kinetic experiments. Results showed not only that increasing Fe(II) additions significantly increased P\textsubscript{i} accumulation, but also that the Fe accumulation rate was in synchrony with P\textsubscript{i} accumulation rate (Fig. 6). Both P\textsubscript{i} and Fe accumulation rates were very rapidly during the first hour and then slowed. For example, when 0.1 mmol L\textsuperscript{-1} Fe(II) was added in the periphyton suspension, >52% of P\textsubscript{i} and 85% of Fe were accumulated on the periphyton within 30 min. When added Fe(II) was doubled to 0.2 mmol L\textsuperscript{-1}, >67% P\textsubscript{i} and 80% Fe were accumulated on the periphyton in 2 h. Thus, the Fe induced P\textsubscript{i} accumulation was very rapidly, which was quite different from that of the control without Fe as well as the Ca effect (Figs. 3 and 6). Mao et al. (2016) found that FePO\textsubscript{4} (s) and vivianite would be formed simultaneously when Fe(III) and Fe(II) normally co-exist in real water or wastewater. Moreover, they observed that when millimolar concentrations of Fe(II) salts were added to wastewater, strengite formation would

Fig. 4. Changes in (A) pH and Eh7, and (B) Fe concentration over time as affected by periphyton under light conditions and an N\textsubscript{2} atmosphere (initial Fe(II) and P\textsubscript{i} concentration = 0.2 mmol L\textsuperscript{-1}).
be much more likely in the pH range of 6.5–7.5 where Fe(III) would be generated more slowly by oxidation of Fe(II). However, Hauduc et al. (2015) suggested that formation of amorphous ferric hydroxides would take precedence over that of FePO₄(s) since the former was more insoluble than the latter over most of the pH range of interest. In our study, Fe accumulation in synchrony with Pᵢ accumulation also mainly occurred in the first few hours for periphyton suspensions was in the pH range of 6–8. Both Fe(III) and Fe(II) co-existed in the periphyton suspension, and most Fe(II) oxidation occurred in <1 h (Fig. 4). Thus, we hypothesized that Fe(III)-phosphate precipitation or adsorption of phosphate by ferric hydroxides were key mechanisms responsible for Pᵢ accumulation induced by added Fe(II). Huang et al. (2015) also observed Fe(III)-phosphate (Fe₇(PO₄)₆) formation in aerobic sludges. In addition, periphyton

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Fig. 5. Inorganic P (Pᵢ) accumulation by periphyton as affected by (A) added Fe(II) concentration (at added Pᵢ concentration = 0.2 mmol L⁻¹), (B) added Pᵢ concentration (at added Fe(II) concentration = 0.2 mmol L⁻¹); and (C) pH and Eh₇ variation with added Fe(II) concentration under light conditions (LC) and dark conditions (DC).
also adsorbed Fe(II) and Fe(III), which could promote P\textsubscript{i} adsorption by forming ternary complexes on periphyton surfaces (Arai and Sparks, 2007).

To gain more insights on possible mechanisms of P\textsubscript{i} accumulation induced by Fe(II) addition, we measured P\textsubscript{i} accumulation and molar ratios of net P\textsubscript{i}/Fe for different sequences of dissolved Fe and P\textsubscript{i} addition and for Fe added as ferrihydrite (ferric hydroxide). Results in Fig. 7A showed that net P\textsubscript{i} accumulation increased with increasing Fe concentration, and generally followed the order P-Fe treatment > Fe-P treatment > P-Ferrihydrite treatment. The molar ratios of net P\textsubscript{i}/Fe accumulation showed the same trend (Fig. 7B). For Fe(II) added at 0.05 mM, net P\textsubscript{i} accumulation and molar ratios of net P\textsubscript{i}/Fe accumulated were almost equal for the Fe-P and P-Ferrihydrite treatments. It should be noted that the Fe(II) added at 0.05 mM should mostly be accumulation in the periphyton within the 1 h before P\textsubscript{i} was added in this Fe-P treatment (Fig. 6). The similarity between the Fe-P and P-Ferrihydrite treatments suggested that Fe(II) added before P\textsubscript{i} likely oxidized and formed a ferrihydrite-like precipitate, which then adsorbed P\textsubscript{i}. Given that only surfaces of the ferrihydrite were accessible to P\textsubscript{i}, the molar P/Fe ratios were less in both the Fe-P and P-Ferrihydrite treatments than in the P-Fe treatment. As added Fe(II) was increased to 0.1 or 0.2 mM, some Fe(II) accumulation by periphyton continued when P\textsubscript{i} was added 1 h later in the Fe-P treatment (Fig. 6). This result suggested that Fe(III)-phosphate precipitation coupled with adsorption of P by ferric hydroxides both occurred at these higher Fe(II) additions. Consequently, the net P\textsubscript{i} accumulation and the P/Fe molar ratios in the Fe-P treatment at the higher Fe(II) inputs were greater than those in the P-Ferrihydrite treatment. Precipitation of Fe(III) phosphate could also occur in the P-Fe treatment because sufficient P\textsubscript{i} was present during oxidation of Fe(II) to Fe(III). Results in Fig. 6B indicated that the net molar ratios accumulated P\textsubscript{i}/Fe increased from 0.38 to 0.5 within the first 6 h, then progressively decreased as P\textsubscript{i} and Fe continued to accumulate over time. This change in P\textsubscript{i}/Fe trend suggested that the dominant mechanism of P\textsubscript{i} accumulation via Fe(II) addition varied over time and chemical ambient environment. The P/Fe trend was consistent in both Fe concentrations with Fe(III)-phosphate precipitation occurring mainly within the first 8 h, and P\textsubscript{i} adsorption on newly formed ferric hydroxide becoming dominant at longer incubation times.

### 3.3. Agronomic and environmental implications

Results from this study indicated that additions of Ca(II) or Fe(II) enhanced abiotic removal of P\textsubscript{i} from aqueous solution and accumulation into periphyton, especially under light conditions. Moreover, Fe(II) was more effective than Ca(II) in promoting accumulation of P\textsubscript{i}. For example, when Ca(II) concentration increased from 0 to 1.2 mM, P\textsubscript{i} concentration in the suspension decreased by 15% to 48%; and when Fe(II) concentration increased from 0 to 0.2 mM, P\textsubscript{i} concentration in the suspension decreased by 50%. The Ca(II), Fe(II) and P\textsubscript{i} concentrations chosen in this study are representative of paddy fields (Kögel-Knabner et al., 2010; Wu et al., 2016). Thus, periphyton propagated in paddy fields during the early stage of rice growth would serve as a sink for P\textsubscript{i}, thereby decreasing loss to surrounding water bodies. Consequently, propagation of periphyton should favor P retention in paddy soils and protect nearby surface waters from eutrophication (Wu et al., 2016).

Periphyton with accumulated P\textsubscript{i} can also act as a bio-fertilizer, an emerging area of fertilizer technology (Wu et al., 2016). Bio-fertilizer is formed by mixing nutrients with microbial aggregates that are capable of entrapping the nutrients or promoting beneficial chemical transformations (Wu, 2016). Photosynthesis can dramatically increase the pH and dissolved O\textsubscript{2} in the periphyton micro-environments. Abiotic P\textsubscript{i} accumulation by periphyton can be enhanced by Ca(II) addition, apparently via Ca-phosphate precipitation and co-precipitation with carbonate at pH > 8. These Ca-phosphates are thermodynamically stable (Liu et al., 2016b) and non-bioavailable until the pH of the periphyton micro-environments decreases, for example during respiration or oxidation of Fe(II) (Rzepecki, 2010). Moreover, periphyton can induce oxidation of Fe(II) generated in paddy soils under low redox conditions to Fe(III), thereby inducing abiotic accumulation of P\textsubscript{i} as Fe(III) phosphate or phosphate adsorbed on newly formed ferric hydroxides. These forms of P\textsubscript{i} are generally regarded as bioavailable through desorption, ligand exchange, or reductive dissolution (Liu et al., 2016b). Fortunately, P\textsubscript{i} accumulation induced by 1.2 mM Ca(II) and 0.2 mM Fe(II) were comparable, and thus the capacity of enhanced abiotic P\textsubscript{i} accumulation induced by Fe(II) was much more efficient than that of Ca(II). However, different Ca-phosphate and iron phosphate precipitates also have different solubility, which should be characterized in developing paddy-soil management systems that involve control of P cycling through periphyton. Management systems must also rely on controlling solution chemical properties to promote more P\textsubscript{i} accumulation into bioavailable forms.

### 4. Conclusions

Periphyton are ubiquitous in paddy fields, and our incubation results showed that the accumulation of inorganic P by periphyton can be enhanced by increasing Ca(II) or Fe(II) in periphyton. Our results suggested that abiotic accumulation of P\textsubscript{i} induced by Ca(II) was mainly through Ca-phosphate precipitation and co-precipitation of P\textsubscript{i} with calcite at pH > 8. Results also implied that accumulation of P\textsubscript{i} in periphyton induced by added Fe(II) was mainly through Fe(III) phosphate precipitation and co-precipitation.

Fig. 7. (A) Net P\textsubscript{i} accumulation and (B) the molar ratio of net P\textsubscript{i} and Fe accumulated by periphyton as affected by different Fe concentrations (unit: mmol L\textsuperscript{-1}, P\textsubscript{i} concentration = 0.2 mmol L\textsuperscript{-1}), sequence of Fe and P\textsubscript{i} addition under light conditions (P-Fe: first adding K\textsubscript{2}HPO\textsubscript{4} to nutrient solutions and then adding FeCl\textsubscript{2} 1 h later; Fe-P: first adding FeCl\textsubscript{2} to nutrient solutions and then adding K\textsubscript{2}HPO\textsubscript{4} 1 h later; and P-Ferrihydrite: first adding K\textsubscript{2}HPO\textsubscript{4} to nutrient solutions and then adding ferrihydrite 1 h later).
precipitation coupled with adsorption of P by ferric hydroxides. Moreover, Fe(II) was more effective than Ca(II) in promoting abiotic Pi accumulation for comparable added concentrations, a benefit for P retention and release to rice in paddy soils that undergo reduction and oxidation. In essence, our results indicate that the propagation of periphyton in paddy fields would favor retention of P within the field, thereby and protecting the surrounding surface waters from eutrophication.

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