Dynamics of phosphorus speciation and the *phoD* phosphatase gene community in the rhizosphere and bulk soil along an estuarine freshwater-oligohaline gradient

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Abstract:

Estuarine tidal marshes play a key role in phosphorus (P) retention and cycling; however, they are suffering

from small but significant increases in tidal saltwater intrusion. The likely impacts of these low-level saltwater intrusions on P availability and microbial activity are unclear. Here, we investigated soil P speciation, alkaline phosphatase (ALP) activity, and the *phoD* phosphatase gene community along a freshwater-oligohaline gradient in the Min River estuary, southeast China. The results indicated that with the transition from freshwater to oligohaline water, the levels of soil-water salinity, pH and sulfate (SO_4^{2-}) content were greater, and ALP activity was lower, which were associated with higher concentrations of organic P, available P, aluminum-bound P, calcium-bound P, and occluded P and lower levels of iron-bound P. There was a strong shift in the *phoD* phosphatase community composition in response to the freshwater-oligohaline gradient. Our findings showed that with the transition from freshwater to an oligohaline environment, in addition to the associated increases in salinity and soil pH and decreases in general microbial and biological activity and soil organic carbon, there is a shift in soil P toward more recalcitrant and immediately available fractions with less labile forms.

Keywords: Phosphorous; *phoD* phosphatase gene; Saltwater intrusion; Rhizosphere; Estuarine tidal marsh

1. Introduction

Tidal estuarine marshes, of which freshwater (salinity <0.5 ppt) and oligohaline (salinity = 0.5–5.0 ppt) marshes may represent a significant area (Odum 1988; Weston *et al.* 2014), are globally distributed ecosystems that play vital roles in ecological processes and nutrient cycling (Kirwan and Megonigal 2013; Tong *et al.* 2017). The complex biogeochemical cycles characteristic of these systems reflect various external stressors, such as human activity, hydrodynamics, and varying salinity, leading to spatial heterogeneity and uncertainty in the distribution of elements (Hu *et al.* 2018a). For example, estuarine studies of primary production have shown shifts in phosphorus (P) limitation to nitrogen limitation due to the transition between freshwater and seawater environments (Gireeshkumar *et al.* 2013; Hartzell *et al.* 2017). While nutrient cycling in coastal salt marsh systems has been well studied, little is known about nutrient dynamics in a low-salinity gradient from freshwater to oligohaline marshes, in which there are various microbial biogeochemical processes and plant communities (Weston *et al.* 2014). This knowledge gap currently limits the understanding of the wetland geochemical processes that drive nutrient cycling and the associated environmental responses.

Phosphorus is an essential element for living organisms and plays an important role in the regulation of primary productivity and ecosystem function in wetlands (Lin and Guo 2016). The immobilization and release processes of P in estuarine and coastal sediments are used in the quantification of the global P cycle due to the considerable levels of P sequestration and the potential associated contributions to water eutrophication (Gireeshkumar *et al.* 2013; Hartzell *et al.* 2010). Environmental conditions may drive the chemical speciation of P that subsequently determines its environmental fate, cycling, and bioavailability in estuarine sediments (Lin and Guo 2016). However, responses among P species to changes along a freshwater-saltwater gradient are known to differ. For example, in the transition from freshwater to saltwater, Gireeshkumar *et al.* (2013) reported decreased proportions of Fe-bound P (Fe-P) but increased proportions of calcium-bound P (Ca-P) and

total sulfur (TS) due to changes in sediment texture and redox conditions. In contrast, Paludan and Morris (1999) showed that aluminum-bound P (Al-P) was an important inorganic P (IP) pool, regardless of salinity, likely as a consequence of changes in ionic strength and aluminum availability. Previous studies of responses of P speciation to freshwater-saltwater gradients have tended to focus on the effects of wide ranges in salinity (Bai *et al.* 2017; Caraco *et al.* 1990; Gireeshkumar *et al.* 2013), while P responses to relatively narrow ranges in salinity, such as from freshwater to oligohaline, are poorly understood.

Soil microbes are key drivers of P transformation and dominate the composition of P forms (Fraser *et al.* 2015; Stout *et al.* 2014). Alkaline phosphatase (ALP) describes a large group of enzymes that generally originate from soil microbes and recycles organic P (OP) to orthophosphate via enzymatic hydrolysis (Ragot *et al.* 2015). Three ALP-encoding gene families, comprising *phoD*, *phoA*, and *phoX*, have been identified (Acuña *et al.* 2016). Among these genes, the distribution of the *phoD* phosphatase gene is widespread, and this gene is considered the key ALP gene in marine sediment. The abundance of the *phoD* phosphatase gene has been used as a measure of ALP bacterial diversity and distribution in a range of ecosystems (Fraser *et al.* 2015; Lagos *et al.* 2016). The soil ALP activity and *phoD* phosphatase genes are affected by biotic and abiotic factors. For example, Huang and Morris (2003) showed that ALP activity in tidal freshwater wetlands was positively correlated with aboveground plant biomass and negatively associated with soluble reactive P concentration. Acuña *et al.* (2016) found that ALP gene abundance in rhizosphere soils was positively correlated with ALP activity but negatively correlated with P availability. Increased levels of salinity have been associated with shifts in ALP activity, albeit with variable responses. Morrissey *et al.* (2014) reported a positive association between increased salinity and ALP activity as a consequence of increases in the bioavailability of organic substrates and changes in microbial community structure. However, Jackson and Vallaire (2009) observed that an increase in salinity to 3.5 ppt decreased the phosphatase activity by almost 20%. Therefore, systematic studies of P speciation, ALP activity, and *phoD* phosphatase genes would allow the evaluation of P dynamics

in tidal estuarine wetlands and the prediction of eutrophication risk due to P mobilization.

The Min River estuarine tidal marsh is the largest in southeastern China and is characterized by a transition from freshwater to oligohaline water (Tong *et al.* 2017), providing an ideal model environment to study responses of soil P availability and *phoD* gene community to variation along a freshwater-oligohaline gradient. Previous studies in this estuary have found greater levels of porewater sulfate (SO_4^{2-}), chloride concentrations (Hu *et al.* 2019), and plant biomass, along with a larger pool of iron oxides and lower levels of sulfide (Luo *et al.* 2019) in oligohaline marshes than in freshwater. However, variation in soil P dynamics and *phoD* phosphatase genes between freshwater and oligohaline marshes remains unclear. Thus, the objectives of this study were to (1) evaluate the P dynamics and differences in P speciation and the associated drivers in estuarine marshes and (2) quantify the responses of ALP activity and *phoD* community composition to freshwater and oligohaline transition and the associated interactions with P availability. We hypothesized that soil P availability is greater at oligohaline sites due to shifts in P-related physicochemical properties (first hypothesis) and that the transition from freshwater to oligohaline reshapes the bacterial *phoD* gene community and modulates ALP activity due to the associated changes in nutrient levels (second hypothesis).

2. Materials and methods

2.1. Study sites

The study area at the Min River estuary (Fig. 1) is in a region with a humid, subtropical monsoon climate, where the average annual temperature and precipitation are 19.85 °C and 1905 mm, respectively (Tong *et al.* 2017). The tides are semidiurnal over a 24-h cycle, and the soil surface is completely exposed at low tide (Tong *et al.* 2014). Further details of the Min River estuary are described in our previous studies (Luo *et al.* 2019; Tong *et al.* 2017). We selected three tidal marsh study sites (Fig. 1) that included a freshwater site in the Tajiaozhou wetland (A; 25°56′59.9″N, 119°21′07.8″E) with an average salinity of 0.08 ±0.02 ppt and two

oligohaline sites at the mouth of the Min River estuary in the Bianfuzhou (B; 26°03′12.0″N, 119°33′25.1″E) and Shanyutan (C; 26°0′50.7″N, 119°40′28.4″E) wetlands that are affected by tidal saltwater intrusion and have average salinities of 1.27 ±0.09 and 3.31 ±0.14 ppt, respectively (Luo *et al.* 2019). At each marsh, we selected an area dominated by the native sedge grass *Cyperus malaccensis*.

2.2. Soil and water sampling

In each marsh, two plots (each with 1 m × 1 m dimension) were established in 2018, one vegetated and the other unvegetated. Three soil samples were randomly collected from each plot. For rhizospheres, entire plants with roots were sampled from the vegetated plots, and rhizosphere soil was collected by scraping the soil that was attached to the roots. For bulk soils, surface soil (0-20-cm deep) was collected from the unvegetated plots and immediately placed in sterile, vacuum-sealed polyethylene bags that were transported to the laboratory in a portable refrigerator containing ice within 12 h. The overlying water was simultaneously collected with the soil samples from each plot during low tide, when the soil surface was exposed, and filtered using a 0.45-μm cellulose membrane filter (Millipore Sigma, MA, USA).

At the laboratory, soil samples were sieved through a 2-mm mesh within an anaerobic glove box and then divided into three subsamples. One subsample was immediately stored at -80 °C prior to DNA extraction and bacterial *phoD* gene analysis, one subsample was stored at 4 °C for the measurement of ALP activity, and the remaining subsample was immediately freeze-dried and stored in a glass vacuum desiccator for the determination of soil physicochemical properties.

2.3. Analysis of soil and water physicochemical properties

Soil pH was measured *in situ* using an IQ150 meter (IQ Scientific Instruments, Carlsbad, USA), and electrical conductivity (EC) was monitored using an EC meter (FieldScout 2265FS, Spectrum Technologies, Aurora, USA). Soil total organic carbon (TOC) and TS concentrations were determined using a Vario MAX element

analyzer (Elementar, Frankfurt, Germany), and carbonate was removed using 10% HCl before TOC determination. Soil particle size distribution was determined using a Malvern Mastersizer-2000 laser particle size analyzer (Malvern Instruments, Malvern, UK). The overlying water SO_4^{2-} concentration was measured using ion chromatography (Dionex, Sunnyvale, USA), salinity was measured directly using a Salt 6+ salinity meter (Oakton Instruments, IL, USA), and total P concentration (TP_w) was determined using a continuous flow analyzer (Auto Analyzer 3, Bran+Luebbe, Germany) following digestion with K_2SO_4.

2.4. Soil P speciation

Phosphorus speciation was characterized as total P (TP), inorganic P (IP), or organic P (OP) and was analyzed according to the protocol method presented in Ruban *et al.* (1999) and Ruban *et al.* (2001). Further, the IP fraction was classified as aluminum-bound P (Al-P), Fe-bound P (Fe-P), calcium-bound P (Ca-P), and occluded P (O-P) and was determined following a sequential extraction procedure based on differential solubility in different chemical extractants (Table S1), as described by Chang and Jackson (1957) and modified by Hartikainen (1979). This method has previously been used for analysis of freshwater and saltwater sediments (Laakso *et al.* 2016; Rahutomo *et al.* 2019; Ray *et al.* 2018; Zhang *et al.* 2015). Available P (AP) was measured following a 0.5 M $NaHCO_3$ extraction (Olsen and Sommers 1982). The P concentrations of extracts were analyzed using the molybdenum blue spectrophotometry method (Paludan and Morris 1999).

2.5. Soil ALP activity assay

We estimated the ALP activity from the production of p-nitrophenol (pNP) from *p*-nitrophenyl phosphate (p-NPP), as described by Tabatabai and Bremner (1969), where 1 g of soil (dry weight equivalent) was incubated using *para*-nitrophenyl phosphate (Macklin Biotechnology, Shanghai, China) as the substrate in a modified universal buffer at pH 11 (Tabatabai and Bremner 1969). Following incubation at 37 ºC for 1 h, the reactions were terminated using 1 M NaOH and centrifuged at 4000 rpm for 15 min. The colorimetric determination of

144 pNP formation in the supernatant was analyzed using a spectrophotometer at 410 nm (Thermo Fisher Scientific,
145 MA, USA), and the ALP activity was expressed as micrograms of pNP released by 1 g of soil (dry weight
146 equivalent) per hour (μmol g^{-1} h^{-1}).

147 ***2.6. Bacterial* phoD *gene analysis***

148 Total genomic DNA was extracted from 0.5 g of fresh soil using Fast DNA SPIN extraction kits (MP
149 Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions and was stored at -20 ℃ prior
150 to analysis. The quantity and quality of extracted DNAs were measured using a NanoDrop ND-1000
151 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis,
152 respectively.

153 The primer set ALPS-F730 (5'-CAGTGGGACGACCACGAGGT-3') and ALPS-1101 (5'-
154 GAGGCCGATCGGCATGTCG-3′) was used to amplify the bacterial *phoD* gene (Sakurai *et al.* 2008),
155 where PCRs (30 μL) comprised 15 μL of Phusion High-Fidelity PCR Master Mix (New England Biolabs), 0.2
156 μM of forward and reverse primers, and approximately 10 ng of template DNA. Amplification was performed
157 in a thermal cycler with an initial denaturation at 98 ℃ for 2 min, followed by 25 cycles of denaturation at
158 98 °C for 15 s, annealing at 50 ℃ for 30 s, and elongation at 72 ℃ for 30 s, with a final extension for 5 min at
159 72 ℃ (Huang *et al.* 2017). Purified amplicons were quantified on a microplate reader (BioTek, Vermont, USA)
160 using a Quant-iT PicoGreen dsDNA assay kit (Invitrogen, P7589) and were subsequently pooled in equal
161 amounts. Paired-end (2×300 bp) sequencing was performed using an Illumina MiSeq platform with a MiSeq
162 rReagent kit (v3) at Shanghai Personal Biotechnology (Personal Biotechnology Co., Ltd., Shanghai, China).
163 Although the ALPS primer have an amplification bias toward *Alphaproteobacteria* (Ragot *et al.* 2015; Tan *et*
164 *al.* 2013), this set of primers has been conducted by most of the previous studies (Acuña *et al.* 2016; Chen *et*
165 *al.* 2019a; b; Fraser *et al.* 2015; Fraser *et al.* 2017; Hu *et al.* 2018b; Huang *et al.* 2019; Luo *et al.* 2017;
166 Matsuoka *et al.* 2019; Sun *et al.* 2019; Tan *et al.* 2013; Valdespino-Castillo *et al.* 2014; Wan *et al.* 2019; Wei

et al. 2019), and making it possible to compare *phoD*-harboring bacterial community among studies.

2.7. Pyrosequence data processing

Raw sequence reads with exact matches to barcodes were assigned to respective samples and identified as valid sequences. After chimera detection, the remaining high-quality sequences were classed into operational taxonomic units (OTUs) using a sequence similarity threshold of 97%. To minimize differences in sequencing depth across samples, an averaged, rounded rarefied OTU table was generated by averaging 100 evenly resampled OTU subsets at <90% of the minimum sequencing depth for further analysis (QIIME, v1.8.0). Abundance at the phylum, class, order, family, genus, and species levels was compared among samples or groups using Metastats (White *et al.* 2009). Indices of OTU-level alpha diversity, such as the Chao1 richness estimator, abundance-based coverage estimator (ACE), Shannon's diversity index and Simpson's evenness index, were calculated using the OTU table in QIIME (Caporaso *et al.* 2010). We analyzed beta diversity to investigate the structural variation in microbial communities using UniFrac distance metrics based on the OTUs and visualized these results using principal coordinates analysis (PCoA) and pair-group method with arithmetic mean (UPGMA) hierarchical clustering (Ramette 2007).

2.8. Statistical analysis

When necessary, data (i.e., P concentrations and environment variables) were log-transformed to meet the ANOVA assumption of normality and homoscedasticity. Pearson's correlation coefficient was used to test the potential correlation between soil P concentration, *phoD* gene diversity, ALP activity, and environmental parameters, and the correlation matrix was visualized using the 'corrplot' package in R. Regression analysis was used to explore the relationship between AP concentration, ALP activity, and *phoD* gene diversity. Redundancy analysis (RDA) was performed to identify the main influencing factors of soil P dynamics using Canoco 4.5 (Microcomputer Power, Ithaca, USA). Overall distributions and variations in soil P fraction, environmental parameter, and *phoD* gene community among the study sites were summarized using a principal

components analysis (PCA) in Statistica 6.0 (StatSoft, Tulsa, USA).

3. Results

3.1. Soil and overlying water physicochemical properties

Physicochemical properties varied between the freshwater and oligohaline marshes (Table 1), where the levels of soil pH, EC, and TS were greater, but that of TOC was lower, at oligohaline sites. Overall, surface soil mainly comprised silt (55%), followed by sand (34%) and clay (11%). The proportions of silt and clay were greater, while that of sand was lower, at oligohaline sites. The salinity and SO_4^{2-} concentration of overlying water at oligohaline site C were greater than at freshwater site A, and there was no difference in TP_w concentration among the study sites. In general, there were no within-study site differences between the rhizosphere and bulk soil parameters; exceptions were for lower levels of pH, TS, and sand and higher levels of TOC and clay in the rhizosphere at oligohaline site B and respectively higher and lower levels of silt and sand in the rhizosphere at oligohaline site C (Table 1).

3.2. Soil P dynamics

There were some differences in soil P concentrations in the rhizosphere and bulk soils, where the rhizosphere TP concentration was lower at freshwater site A than at oligohaline site B. The bulk soil IP concentration was lower at the freshwater site than at oligohaline site B. The OP concentration was lower in rhizosphere and bulk soils at the freshwater site than at the two oligohaline sites (B and C). The AP concentration in rhizosphere soils was greater at oligohaline site C than at the freshwater site and oligohaline site B. The AP concentration in bulk soils was greatest at oligohaline site B and lowest at the freshwater site ($P < 0.05$; Figs. 2a-d). Overall, IP accounted for 66-89% of TP (Figs. 2a, b). Among the IP fractions, the concentrations of Fe-P in rhizosphere and bulk soils were greater at the freshwater site than at the two oligohaline sites, whereas the concentration

of Ca-P in the two soil profiles was lower (Figs. 2e, g). The Al-P concentration in the rhizosphere was lower at the freshwater site than at the two oligohaline sites and lower in the bulk soil at the freshwater site than at oligohaline site C (Fig. 2f). The O-P concentrations in the rhizosphere and bulk soils were lower at the freshwater site and oligohaline site B than at oligohaline site C (Fig. 2h). Al-P and O-P were the dominant forms of IP (38 and 30%, respectively), followed by Fe-P (21%) and Ca-P (11%) at all sites (Fig. S1). There were few within-site differences in the soil P concentrations between rhizosphere and bulk soils (Fig. 2). TP and Fe-P at the freshwater site were respectively lower and greater in the rhizosphere than in the bulk soils, while at oligohaline site B, the concentration of AP was lower in the rhizosphere than in bulk soils, but that of Fe-P was greater.

3.3. Soil ALP activities

Soil ALP activity was 7-fold greater in the freshwater (0.7 μmol $g^{-1} \cdot h^{-1}$) than in the oligohaline marshes (0.1 $g^{-1} \cdot h^{-1}$), but there were no within-study site differences in ALP activity between the rhizosphere and bulk soils (Fig. 3).

3.4. Richness and alpha diversity of the **phoD** *phosphatase gene*

A total of 73,350 qualified sequences of the *phoD* phosphatase gene were recorded from the soil samples. The richness and alpha diversity of the *phoD* phosphatase gene were greater in the rhizosphere than in the bulk soils at site B, and overall, these values were greatest at site B and lowest at site A (Table 2). There was no clear pattern in *phoD* gene diversity with the transition from freshwater to oligohaline environments. The PCoA showed that the bacterial *phoD* gene communities at freshwater site A were more loosely clustered and distinct from those at oligohaline sites B and C (Fig. 4).

3.5. **phoD** *phosphatase gene community structure*

Overall, the most abundant bacteria classes containing the *phoD* gene were the *Alphaproteobacteria, Betaproteobacteria* and *Gammaproteobacteria*, accounting for 30-80% of total sequences (Fig. 5a). The

234 dominant genera in all samples were *Pleomorphomonas*, *Streptomyces*, *Cupriavidus*, *Bradyrhizobium*, and
235 *Pseudomonas* (relative abundance >1%) (Fig. 5b). Specifically, *Streptomyces*, *Cupriavidus*, and
236 *Bradyrhizobium* were the dominant genera at the freshwater marsh (relative abundance: 43, 19, and 9%,
237 respectively), and *Pleomorphomonas*, *Streptomyces*, and *Bradyrhizobium* were dominant at the oligohaline
238 sites (relative abundance: 52, 22, and 5%, respectively).

239 The relative abundance of the *phoD-harboring* bacterial shifted significantly along a freshwater-
240 oligohaline gradient, where the relative abundance of the *Alphaproteobacteria* class was greater at oligohaline
241 sites, but that of the *Betaproteobacteria* was greater at freshwater sites. The relative abundance of the
242 *Pleomorphomonas* genus was greater, whereas those of *Streptomyces* and *Cupriavidus* genus were lower, at
243 oligohaline sites. Hierarchical cluster analysis showed that bacterial communities formed three groups that
244 represented the three study sites (Fig. S2). However, there were no within-site differences between rhizosphere
245 and bulk soil communities.

246 ***3.6. Relationships between soil P and* phoD *gene communities with soil and overlying water variables***

247 The concentrations of soil Al-P, Ca-P, O-P, and AP were positively correlated with soil pH, EC, TS and silt
248 content and negatively correlated with soil TOC and sand content. The soil Fe-P concentrations were
249 negatively correlated with pH, EC, and TS and positively correlated with soil TOC. The soil OP concentrations
250 were positively correlated with soil pH, EC and overlying water salinity (Fig. 6). The concentrations of soil
251 Ca-P and AP were positively associated with soil clay content. Soil Al-P, Ca-P, O-P, and AP were also found
252 to be positively related to overlying water salinity and SO_4^{2-} concentration.

253 We found that the relative abundance of *Pleomorphomonas* was negatively associated with the contents
254 of TOC and sand and positively associated with other environmental variables; that of *Streptomyces* showed
255 the opposite trend (Fig. 6). The soil ALP activity was positively correlated with soil TOC and sand content and
256 negatively correlated with soil pH and EC, contents of TS, clay, and silt, and overlying water salinity and SO_4^{2-}.

257 We examined the relationships between the AP concentration, *phoD* gene community, and ALP activity
258 (Fig. S3) and found that soil AP was positively correlated with the *phoD* gene diversity but negatively

correlated with the ALP activity. The soil AP was positively related to the relative abundance of *Pleomorphomonas* and negatively related to the abundance of *Streptomyces*. The soil ALP activity was negatively related to the bacterial *phoD* gene diversity and the relative abundance of *Pleomorphomonas*.

The first two axes of the RDA of the influence of biochemical variables (environmental variables, *phoD* gene community, and ALP activity) accounted for 78.6% of the variation in P dynamics ($P < 0.01$) that clustered into three groups (groups I, II, and III) representing the three study sites (A, B, and C, respectively) (Fig. 7). The soil P dynamics in Group I (freshwater) were primarily influenced by the soil contents of TOC, ALP activity, and the relative abundance of *Streptomyces*, while in Groups II and II (oligohaline), they were primarily driven by overlying water salinity and SO_4^{2-}, soil TS and pH, *phoD* gene diversity, and the relative abundance of *Pleomorphomonas*.

4. Discussion

4.1. Soil P speciation responses to a freshwater-oligohaline gradient

Previous studies have indicated that P dynamics and associated speciation varied from freshwater to saltwater due to shifts in soil physicochemical and microbial processes (Gireeshkumar *et al.* 2013; Paludan and Morris 1999). As expected, we observed higher of soil-water salinity, pH and SO_4^{2-} content, lower soil TOC, and greater associated soil AP concentration in the transition from freshwater to oligohaline sites (Table 1; Fig. 2), supporting our first hypothesis that soil P availability is greater at oligohaline sites due to shifts in the P-related physicochemical properties. It is possible that the salinity brought by seawater influences P dynamics through changes to adsorption and desorption reactions triggered by an increase in ions in the sediment that compete with phosphate ions (PO_4^{3-}) for the sorption sites (Qu *et al.* 2018). This response was demonstrated by the positive correlation between the AP concentration and overlying water salinity (Fig. 6). The abundance of electron-accepting SO_4^{2-} in the oligohaline marshes (Table 1) may enhance the release of P as a result of sulfate

281 reduction (SR), but it may also compete with PO_4^{3-} for anion adsorption sites (Caraco *et al.* 1990), thereby
282 regulating P availability. However, we did not find a difference in soil IP concentration among the sites, even
283 though the OP levels were greater in the oligohaline sites than in freshwater (Figs. 2b, c), possibly as a result
284 of a slower capacity for P-mineralization in oligohaline soils than in freshwater, as indicated by the lower ALP
285 activity in the oligohaline soils (Fig. 3).

286 The concentrations of Al-P, Ca-P, and O-P in the IP fraction increased from freshwater to oligohaline sites
287 (Figs. 2f-h), while Fe-P markedly decreased (Fig. 2e), representing a shift from Fe-P to Ca-P and Al-P due to
288 the reduction of Fe along a freshwater-oligohaline gradient. These findings are consistent with the greater
289 availability of SO_4^{2-} in oligohaline sites (Table 1). This greater availability promotes the formation of sulfide
290 and subsequent Fe(II) sulfides due to the high rates of SR, which increase the release of PO_4^{3-} due to the
291 reduction of Fe(III)-bound-P, thus reducing Fe-P storage in the soil (Dierberg *et al.* 2011; Luo *et al.* 2019). It
292 is also likely that greater levels of alkalinity generated by SR in anoxic soils may inhibit P sorption onto iron
293 oxides in soils (Caraco *et al.* 1989). The shifts in soil physicochemical properties from freshwater to
294 oligohaline sites also affected the P fraction. We found that soil concentrations of Al-P, Ca-P, and O-P were
295 positively associated with silt content in the soil and negatively associated with sand content (Fig. 6), indicating
296 that soil texture is a driver of P dynamics in estuarine marshes. This finding may be explained by differences
297 in the substrate surface area and reactivity, as the greater surface area of silt particles provides more binding
298 sites for PO_4^{3-} adsorption in the soil (Gireeshkumar *et al.* 2013). Our findings also indicate that the transition
299 from freshwater to an oligohaline environment produces more strongly occluded P in soil so that the long-term
300 P storage capacity in soil is enhanced with moderate increases in salinity.

301 We found that the IP fraction of soil P was primarily controlled by Al-P (38%), followed by O-P (30%),
302 Fe-P (21%), and Ca-P (11%) (Fig. S1). The Min River estuary is located in the subtropics, where the weathering
303 of parent rock is relatively strong under the warm, humid climate, resulting in high levels of organic matter

304 rich in Fe/Al oxide (Luo *et al.* 2014; Luo *et al.* 2019). The release of Fe and Al facilitates the enrichment and
305 migration of P as the adsorption carriers, thereby controlling the IP fraction. In this study, the average soil TP
306 concentration across study sites in the estuary (698 $mg \cdot kg^{-1}$) did not vary with the transition from freshwater
307 to oligohaline sites (Fig. 2a). In addition, the TP concentrations were similar to those recorded in estuaries in
308 Portugal (Coelho *et al.* (2004) and elsewhere in China (Jin *et al.* (2013) but lower than those recorded from
309 estuaries in the US (Jordan *et al.* 2008) and Sri Lanka (Gireeshkumar *et al.* 2013) (Table S2). Within-estuary
310 consistency in soil TP content may be the result of a combination of changing dominance of the actions of
311 various forms of P and their trade-offs because we found that while some P-fractions were more abundant at
312 oligohaline sites (AP and O-P), other, mostly moderately labile forms, were less abundant. Our finding that
313 soil TP, and its associated speciation, did not differ between rhizosphere and bulk soils was inconsistent with
314 previous studies (Hinsinger 2001; Luo *et al.* 2017). This inconsistency may be due to the periodic flooding
315 and associated variations in salinity that drive complex hydrological and nutrient cycling in estuarine
316 environments (Hu *et al.* 2018a), which partially offset possible underlying rhizosphere effects on soil P
317 dynamics. Moreover, the resuspension of sediment driven by tide and river runoff may have stimulated P
318 immobilization to change the transformation of soil P fractions (Labry *et al.* 2016). Thus, shifts in soil P
319 fractions from freshwater to oligohaline sites may be a consequence of the direct effects of salinity and the
320 indirect effects of altered soil/water physicochemical properties due to saltwater intrusion.

321 ***4.2. Effects of salinity on ALP activity and the* phoD *gene community***

322 Greater rates of ALP activity were observed in the freshwater than the oligohaline sites, regardless of soil type
323 (rhizosphere/bulk) (Fig. 3). These findings corroborate a study by Jackson and Vallaire (2009) but are in
324 contrast to those by Morrissey *et al.* (2014) and Labry *et al.* (2016). It is known that pH plays a crucial role in
325 the regulation of phosphatase activity, and ALP activity is predominant in alkaline environments (Stout *et al.*
326 2014). However, and somewhat surprisingly, we did not observe this phenomenon in our study because the

soil ALP activity was negatively correlated with pH (Fig. 6). This absence of a positive effect of pH on ALP activity may be due to the limited variation in pH value (6.4-7.4; Table 1) or the low concentrations of PO_4^{3-} that drive ALP activity in P-deficient environments (Fraser *et al.* 2015; Labry *et al.* 2016). In this study, soil AP concentrations were greater in the oligohaline sites than the freshwater site, decreasing the requirement for ALP synthesis to facilitate P uptake in microbes and plants, as indicated by the negative association between ALP activity and soil AP (Fig. S3d). It should be noted that the ALP activity of this study refers to potential phosphatase activity rather than actual soil phosphatase activity because the pH has been modified, which allows it to be compared to other studies (Chen *et al.* 2019a; b; Fraser *et al.* 2015; Fraser *et al.* 2017).

Although *phoD* genes have been investigated extensively under various environmental conditions (Fraser *et al.* 2015; Ragot *et al.* 2015), the influence of variations with the transition from freshwater to oligohaline environments remains unclear. Here, the alpha-diversity of bacterial *phoD* genes did not vary with the transition (Table 2), possibly due to the relatively narrow range of salinity (0.03–2.92 ppt) at the study sites, which may not have been sufficient to result in differences in diversity. In contrast, the soil bacterial *phoD* gene community compositions differed between the freshwater and oligohaline sites (Fig. 5). Overall, *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* were the most abundant bacterial classes in the study sites (>59% of total bacteria; Fig. 5), supporting other studies of various types of soil (Lagos *et al.* 2016; Luo *et al.* 2017; Tan *et al.* 2013) and indicating that the *phoD*-harboring bacteria community composition is stable across different environments. The greater relative abundance of *Pleomorphomonas* and the lower abundance of *Streptomyces* at oligohaline sites relative to freshwater (Fig. 5) may reflect their responses and adaptations to nutrient availability and salinity fluctuations (Spohn *et al.* 2015).

Our analysis of the community composition of *phoD*-harboring bacterial genera showed that the relative abundance levels of some genera, such as *Pleomorphomonas*, were much greater at oligohaline sites, while that of *Streptomyces* was lower (Fig. 5). In addition to the direct influences of soil osmotic potential and water

stress caused by salinity (Chambers *et al.* 2013), changes in soil pH, TOC, and texture with the transition from freshwater to oligohaline environments may have affected *phoD* gene community composition. For example, we found that soil TOC was positively associated with the relative abundance of *Acidobacteria* (Fig. 6), likely because substrates rich in C favor growth of some *phoD*-harboring species, leading to increases in the abundance of the *phoD* genes and ALP activity (Luo *et al.* 2017). It is important to note that soil texture, which is correlated with variations in bacterial community composition, may affect the adsorption of chemicals by distinct microbial communities due to differences in surface properties and microenvironments (Hemkemeyer *et al.* 2015). Overall, these results support our second hypothesis that the transition from freshwater to oligohaline environments shapes bacterial *phoD* gene communities and influences ALP activity.

4.3. Linking ALP activity and **phoD** *gene community structure with P availability*

Soil ALP activity has been regarded as an indicator of changes in organic P mineralization and bacterial *phoD* gene abundance in soils (Acuña *et al.* 2016; Huang and Morris 2003). The negative relation between the soil ALP activity and AP concentration (Fig. S3d) provides additional evidence that ALP activity is only induced at low P levels due to the inhibited synthesis of phosphatases at high AP concentrations (Acuña *et al.* 2016; Fraser *et al.* 2015). The differences in relative abundance of the dominant *phoD* gene community may play a key role in P availability because soil AP concentration was positively associated with the relative abundance of *Pleomorphomonas* (Fig. S3b) but negatively associated with that of *Streptomyces* (Fig. S3c). Both of these dominant genera are important for P solubilization and mineralization through the production of P-hydrolyzing enzymes (Acuña *et al.* 2016; Ragot *et al.* 2015).

In general, potential ALP activity may have been directly regulated by the *phoD* phosphatase gene community due to the greater production efficiency of extracellular alkaline phosphatases (Luo *et al.* 2017). The high taxonomic diversity of the *phoD* gene community renders it better able to tolerate changes in salinity and potentially affects ALP production (Fraser *et al.* 2015). Moreover, the responses of the *phoD* gene

community to the freshwater-oligohaline transition might be partially depend on P availability (Fraser *et al.* 2015), and high levels of P might inhibit *phoD* gene expression (Vershinina and Znamenskaya 2002). However, this pattern only was observed in the *phoD* gene diversity, which showed a negative correlation with the ALP activity. Measuring the gene and transcript levels with more universal *phoD* primers is thus warranted (Ragot *et al.* 2015) and will provide a full understanding of ALP production and *phoD* gene diversity along an estuarine freshwater-oligohaline gradient.

4.4. Implications and uncertainties

In summary, our data clearly showed the variations of soil P fractions, environmental parameters, and *phoD* gene communities among the study sites, where the levels of salinity and pH were greater, the soil texture was finer, and the contents of O-P/Al-P were greater at the oligohaline sites, which were associated with greater levels of soluble and available P. In contrast, freshwater sites were characterized by coarser textured soils, higher levels of TOC and Fe-P, lower levels of O-P and AP, and higher levels of ALP activity, despite the low pH levels. These results indicate higher microbial and general biological activity, lower P retention capacity and greater biological effort required for P uptake in freshwater conditions. Furthermore, the overall PCA analysis (Fig. 8) indicated that the most diverse bacterial community and the higher, more evenly distributed stocks of P among the rhizospheres and bulk soils occurred at the moderately saline site (site B). Thus, we conclude that with the transition from freshwater to oligohaline sites, the associated increases in soil-water salinity and SO_4^{2-} and decreases in general microbial activity are key drivers of P fractions and availability, which indicates that greater potential P losses may reduce nutrient availability for estuarine plants and microbes under longer-term, climate change-mediated rises in sea levels.

Although these patterns have important implications for our understanding of wetland geochemical processes that drive P cycling, some uncertainties and future works must be carefully considered. First, hydrological conditions, such as periodic tidal processes and seawater intrusion across the estuarine tidal

marshes, might have significant effects on P speciation and the *phoD* gene community. Therefore, increasing the consideration of tidal effects can contribute to more accurate estimates of soil P dynamics and microbial community along a freshwater-oligohaline gradient. Second, our results support the conclusion of Tan *et al.* (2013) and Ragot *et al.* (2015) that the ALPS primers seems to have an amplification bias caused by the primers specificity, for example, numerous sequences were assigned to *Alphaproteobacteria,* which suggested this primer set could be biased toward *Alphaproteobacteria* rather than the real distribution of *phoD*. Therefore, although the present study provided some insights into the *phoD* bacterial communities along an estuarine freshwater-oligohaline gradient, the interpretation and comparison of our results must be conducted with some caution. Future research should consider newly designed primers based on metagenome databases (Ragot *et al.* 2015), which probably provide better coverage of the *phoD* diversity.

5. Conclusions

Our results suggest that the transition from freshwater to an oligohaline environment drives the increases in soil-water salinity, pH and SO_4^{2-} content, a decrease in soil TOC, the associated increases in concentrations of AP, Al-P, Ca-P, and O-P, and a decrease in Fe-P concentrations. These findings support our first hypothesis that soil P availability is greater at oligohaline sites due to shifts in P-related physicochemical properties. These findings also highlight the role of salinity as a substantial factor in P availability, where increases in salinity and the associated changes in soil-water physicochemical properties (SO_4^{2-}, pH, and TOC) as a consequence of saltwater intrusion may exacerbate losses of P to water, eventually leading to losses in P-fertility for plants and microbes. The lower levels of soil ALP activity and the altered composition of *phoD* gene communities at oligohaline sites indicate that even small increases in the salinity levels and the associated shifts in environmental factors can be caused by saltwater intrusion and may regulate microbial activity and reshape the *phoD* gene community composition in estuarine tidal marshes, supporting our second hypothesis. Our

results showed that the moderately saline study site (site B) was characterized by the most diverse bacterial community and higher and more evenly distributed stocks of P among the rhizosphere and bulk soils. Our results increase our understanding of the main processes and mechanisms involved in the estuarine P dynamics and *phoD* phosphatase gene communities. However, detailed studies and analyses of spatiotemporal coupling and tidal action that control the available soil P pools are required.

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