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Short Communication

Orthophosphate uptake onto woodchips in denitrifying bioreactors is enhanced by anoxic-(anaerobic-)oxic cycling

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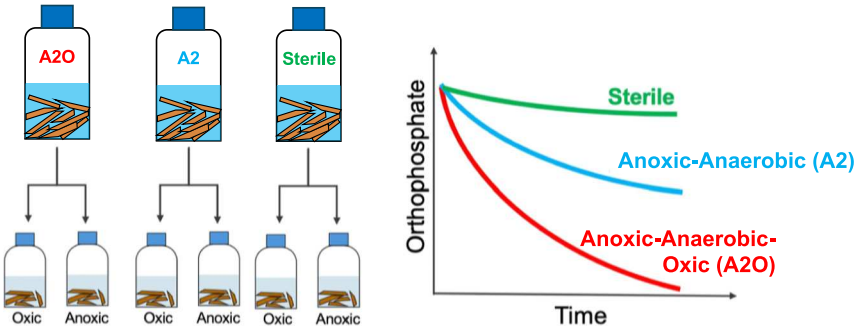
HIGHLIGHTS

- Anoxic-oxic cycling enhances biological orthophosphate uptake onto woodchips.
- Polyphosphate accumulating organisms may contribute to P removal by woodchips.
- Greatest orthophosphate uptake corresponded to peaks in *ppk* expression.
- Drying and reflooding of woodchip media improves treatment of both N and P.

GRAPHICAL ABSTRACT



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ABSTRACT

Woodchips are widely used as a low-cost and renewable organic carbon source for denitrifying biofilms in passive nutrient removal systems. One limitation of wood-based biofiltration systems is their relatively poor removal of phosphorus (P) from subsurface drainage and stormwaters, necessitating the use of additional filter media when co-treatment of nitrogen (N) and P is required. Here, we show that anoxic-oxic cycling of woodchip media, which enhances nitrate (NO_3^-) removal by increasing the mobilization of organic carbon from wood, also improves orthophosphate (P_i) uptake onto woodchips. Orthophosphate removal rates in flow-through woodchip columns ranged from 0 to 34.9 μg PO_4^{3-} L^{-1} h^{-1} under continuously-saturated (anoxic) conditions, and increased to 17.5 to 71.9 μg PO_4^{3-} L^{-1} h^{-1} in columns undergoing drying-rewetting (oxic-anoxic) cycles. The highest P_i removal efficiencies were observed in the first 20 h after reactors were re-flooded, and were concurrent with maxima in polyphosphate kinase (*ppk*) gene expression by the polyphosphate accumulating organisms (PAOs) *Accumulibacter* spp. and *Pseudomonas* spp. Batch experiments confirmed that anoxic-anaerobic-oxic pre-incubation conditions led to orthophosphate uptake onto woodchips as high as 74.9 ± 0.8 mg PO_4^{3-}/kg woodchip, and batch tests with autoclaved woodchips demonstrated that P_i uptake was due to biological processes and not adsorption. NO_3^- removal in batch tests was also greatest under oxic incubation conditions, attributed to greater carbon availability in hypoxic to anoxic zones in woodchip biofilms. While further research is needed to elucidate the mechanisms controlling enhanced P_i uptake by woodchip biofilms under anoxic-(anaerobic-)oxic cycling,

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these results suggest a role for enhanced P_i uptake by PAOs in a nature-based system for treatment of nonpoint source nutrients.

1. Introduction

Nonpoint source nutrient pollution is a leading cause of eutrophication and associated water quality problems around the world. There is growing attention to dual nutrient reduction targets that focus on managing both nitrogen (N) and phosphorus (P) in watersheds (Christianson et al., 2017; Paerl et al., 2016), and prominent water quality management plans including the Chesapeake Bay total maximum daily load (TMDL) include targets for both N and P. Woodchips are widely used as a low-cost and renewable organic carbon source and biofilm carrier for denitrifying microorganisms in passive nutrient removal systems including stormwater bioretention systems (Davis et al., 2009; Peterson et al., 2015), biofilters for septic system leachates (Chen et al., 2022; Lopez-Ponnada et al., 2017) or aquaculture effluents (Aalto et al., 2020; Sharrer et al., 2016), and woodchip bioreactors treating agricultural tile drainage (Warneke et al., 2011; Christianson and Schipper, 2016; Schipper et al., 2010). While woodchip amendment to saturated zones of treatment systems enhances removal of nitrate (NO_3^-) via heterotrophic denitrification from waters with low C/N ratios (Peterson et al., 2015), woodchip media typically have poor effectiveness for P removal (Sharrer et al., 2016; Sanchez Bustamante-Bailon et al., 2022; Perera et al., 2024). Fresh woodchips can even be a source of orthophosphate (P_i) release, though such P_i release is usually short-lived (Sharrer et al., 2016; Husk et al., 2018). The lack of effective treatment of P by woodchip media has necessitated additional filter media for P, usually incorporating materials rich in calcium or iron (Fe) and aluminum (Al) oxides (Husk et al., 2018) including steel byproducts or mining residuals (Christianson et al., 2017; Hua et al., 2016), when dual nutrient reduction goals require co-treatment of N and P.

Research from our group (McGuire et al., 2021; McGuire et al., 2023) and elsewhere (Maxwell et al., 2019) has shown that exposing woodchip media to alternating oxic and anoxic conditions through drying-rewetting cycles accelerates NO_3^- removal by enhancing the release of labile organic carbon, including low molecular weight organic acids (LMWOA), for denitrifying biofilms. A metatranscriptomic analysis revealed that greater organic carbon availability was driven by oxygen (O_2)-dependent stimulation of fungal lignin-degrading enzymes during air exposure (McGuire et al., 2023), enhancing the mobilization of labile carbon from lignocellulosic woody residues when woodchip beds were reflooded. Anoxic-anaerobic-oxic transitions coupled with changes in LMWOA availability can also promote the microbial uptake of P_i via synthesis of intracellular polyphosphate (polyP) (Onnis-Hayden et al., 2011). This process is well-known in the context of enhanced biological phosphorus removal (EBPR) in activated sludge wastewater treatment plants (He and McMahon, 2011a; McMahon et al., 2002a), where there is typically a net release of P_i under anaerobic conditions (in the absence of O_2 or NO_3^- as terminal electron acceptors) replete with available carbon, while there is net uptake of P_i in the presence of O_2 or NO_3^- to support polyP synthesis. There is growing interest in the roles of polyP accumulating organisms (PAOs) in the biogeochemical P cycle in natural ecosystems (Akbari et al., 2021; Saia et al., 2021; Saia et al., 2017; Hupfer et al., 2007; Watson et al., 2019; Duggan DiDominic et al., 2024), but the potential role of EBPR-like processes in nature-based treatment systems like denitrifying woodchip media is not well understood.

In this communication we examine how anoxic-(anaerobic-)oxic cycling affects P_i uptake into woodchip biofilms in denitrifying woodchip bioreactors treating synthetic agricultural tile drainage. We use the term "anoxic" to refer to conditions in which O_2 is depleted but NO_3^- is present, while "anaerobic" refers to depletion of both O_2 and NO_3^-. We combine chemical observations in column and batch experiments with expression analysis of polyphosphate kinase (*ppk*) genes involved in polyP synthesis (McMahon et al., 2002a; McMahon et al., 2002b). The working hypothesis for this study was that anoxic-(anerobic)-oxic cycling will enhance P_i removal from water through a biological uptake process, providing a practical approach to improve the co-treatment of N and P from nonpoint sources.

2. Materials and methods

2.1. Column experiments

Hardwood woodchips were excavated from a 7-year old woodchip bioreactor in Freeville, NY, USA, and packed into two horizontal flow-through columns in the laboratory. This bioreactor treats agricultural tile drainage at the Homer C. Thompson Vegetable Farm, and additional details on the bioreactor are available in previous research reports on this site (Israel et al., 2023; Hassanpour et al., 2017). The laboratory reactor columns were 1.5 m long with an inner diameter of 0.1 m. A series of pore water sampling ports were installed at distances 23, 36, 73, 86, 125, and 135 cm from the column inlet (see Fig. S1 for a schematic of laboratory reactors). Dissolved O_2 (DO) in each reactor was measured using two O_2 microsensors (PreSens) located 55 and 105 cm from the column inlet. A synthetic agricultural drainage water containing 40 mg/L NO_3^- (as $NaNO_3$), 2.5 mg/L NH_4^+ (as NH_4Cl), and 1.8 mg/L PO_4^{3-} (as Na_2HPO_4) in dechlorinated tap water, at an average pH of 7.7, was pumped through the reactors with a design hydraulic retention time (HRT) of 12 h. Actual HRTs determined using bromide tracer tests, as described in McGuire et al. (2021), are reported in Table S1.

Two experiments (Replicates 1 and 2) were performed to evaluate the effects of drying-rewetting (DRW) cycles on bioreactor performance, and were originally designed to test the effects of O_2 exposure on mobilization of labile carbon from wood and resulting impacts on denitrification and nitrous oxide (N_2O) production (McGuire et al., 2021). Each experiment included one DRW reactor and one continuously saturated (CS) reactor. The DRW reactor was drained for 48 h once per week before being re-flooded. This cycling lasted for five weeks in Replicate 1 and eight weeks in Replicate 2. After Replicate 1, woodchips were removed from both reactors, mixed in a bucket to homogenize, and then returned to the columns for Replicate 2. Experiments were performed at room temperature (21 ± 2 °C).

Pore water samples were collected at approximately 6 h and 100 h after reactors were re-flooded, and 0.22 μm-filtered samples were analyzed for a suite of chemical analytes including N species, dissolved organic carbon, and P_i. During weeks 4 and 6 of Replicate 2, high-frequency pore water sampling was performed in the two days after reactors were re-flooded to capture the reactor dynamics across oxic-anoxic transitions. P_i removal rates were determined using:

$$P_i\ Removal\ Rate = \frac{P_{i,influent} - P_{i,effluent}}{HRT} \quad (1)$$

$P_{i,influent}$ and $P_{i,effluent}$ are influent and effluent P_i concentrations (μg PO_4^{3-} L^{-1}), and HRT is the actual HRT (hr). P_i removal efficiencies were determined using:

$$P_i\ Removal\ Efficiency = \frac{P_{i,influent} - P_{i,effluent}}{P_{i,influent}} \times 100\% \quad (2)$$

Three woodchip specimens were collected at $t = 0$, 8, 16, 28, and 52 h after reactor re-flooding and were flash-frozen in liquid nitrogen prior to storage at −80 °C for later nucleic acid isolation within six months of sample preservation and multi-omics analysis (McGuire et al., 2023). Data on N and C chemistry, as well as -omics data on N and C transformations in column experiments, have been published previously

(McGuire et al., 2021; McGuire et al., 2023). Here, we report P_i concentrations and removal rates using samples that were collected as part of the earlier study, but are published here for the first time. In McGuire et al. (2021) we showed that while DRW cycling significantly enhanced NO_3^- removal relative to CS conditions, denitrification was usually incomplete in both cases so NO_3^- was present throughout bioreactor columns. The bulk fluid in bioreactor columns was therefore characterized by anoxic rather than anaerobic conditions so we refer to the bioreactor column experiments as experiencing "anoxic-oxic" cycling conditions.

2.2. Analysis of ppk gene expression across oxic-anoxic transitions

Total RNA was extracted from frozen woodchips using an RNeasy PowerSoil Total RNA Kit (QIAGEN). Three sub-samples were isolated for each time point to collect a more representative sample, and RNA from sub-samples was then pooled and purified with an RNeasy MinElute Cleanup Kit (QIAGEN). RNA was shipped on dry ice to the Department of Energy (DOE) Joint Genome Institute (JGI) in Berkeley, CA, USA, for metatranscriptomic sequencing. rRNA depletion was performed at JGI using the QIAseq FastSelect 5S/16S/23S kit. Additional details on nucleic acid isolation and sequencing are available in McGuire et al. (2023). Translated nucleic acid-protein similarity analysis was performed using DIAMOND (Buchfink et al., 2015) in blastx mode to classify *ppk1* and *ppk2* transcripts. The reference data sets consisted of representative protein sequences of *Accumulibacter* and *Pseudomonas* spp. gathered from the NCBI database. A maximum e-value of 10^{-3} was set during DIAMOND, and a sequence identity of $\geq$60 % and a contiguous match length of $\geq$25 amino acids were used as filters to identify valid hits for protein classification.

2.3. Woodchip batch experiments

Batch experiments were performed to further investigate the processes leading to greater P_i removal under anoxic-anaerobic-oxic cycling. 32 g of woodchips (dry weight) were collected from the flow-through bioreactor columns, lightly rinsed with tap water, and added to 150 mL of synthetic media containing 170 mg/L NO_3^- as $NaNO_3$, 30 mg/L PO_4^{3-} as Na_2HPO_4, 250 mg/L KCl, and 84 mg/L $NaHCO_3$ in glass media bottles, adjusted to pH 7.1. Woodchips were pre-incubated with continuous shaking on an orbital shaker at 180 rpm under either continuously anoxic-anaerobic (A2) conditions or alternating anoxic-anaerobic-oxic (A2O) conditions for 20 days at room temperature (22 °C). We use the term "anoxic-anaerobic" because over the course of the pre-incubation the reactors shifted from anoxic to anaerobic conditions as NO_3^- was depleted. This did not involve sequential cycling between anoxic and anaerobic conditions. Anoxic-anaerobic incubations were performed in media bottles sealed with gas-tight bromobutyl stoppers, and were degassed for 10 min with N_2 after sealing. A2O experiments were performed by sealing bottles under anoxic conditions for 48 h and then opening them to the atmosphere for 48 h. This cycle was repeated five times. Dissolved oxygen concentrations during oxic conditions were confirmed to be in the range of 7–8 mg/L, and during anoxic/anaerobic conditions were confirmed to be <0.1 mg/L, with periodic measurements using a hand-held dissolved oxygen probe (YSI Pro20). After the fifth cycle, the media was replaced by a fresh synthetic media. Woodchips that had been pre-incubated under anoxic-anaerobic conditions were then incubated for 50 h under either oxic or anoxic conditions, and woodchips from the A2O pre-incubation were also incubated for 50 h under either oxic or anoxic conditions. Filtered water samples were periodically collected for analysis with ion chromatography (PO_4^{3-}, NO_3^-). Dissolved organic carbon (DOC); specific ultraviolet absorbance at 254 nm ($SUVA_{254}$), a measure of organic carbon aromaticity; and total P were measured at 50 h. All experimental conditions were performed in triplicate. A separate set of adsorption experiments were performed with the same woodchip/liquid ratios, using double-autoclaved woodchips, to assess abiotic P_i uptake by woodchips under both oxic and anoxic conditions.

2.4. Woodchip surface characterization

Protein concentrations on woodchip surfaces from batch experiments were determined as a proxy for microbial biomass. 1.4 g of woodchips (dry weight) were vortexed with 1.0 g of glass beads and 5 mL of a cell lysis buffer (1 % SDS, 40 mM Tris-HCl, 5 mM EDTA, pH 8) in a 50 mL centrifuge tube. The samples were then centrifuged for 20 min and the supernatant was collected for protein analysis. Protein concentrations were determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific) and normalized by the woodchip concentration to determine the surficial protein concentrations in mg protein/kg woodchip (dry weight).

Metal (iron, aluminum, manganese) concentrations on woodchip surfaces were determined to assess potential P_i adsorption to surface metal oxides, which is thought to contribute to P_i adsorption in woodchip media (Sanchez Bustamante-Bailon et al., 2022). 7 g of woodchips (dry weight) from the batch experiments were extracted with 20 mL of 6 M HCl for in 50 mL centrifuge tubes in a tube rotator. The supernatant was filtered and diluted in 2 % nitric acid for ICP-MS analysis.

2.5. Analytical methods

Anion concentrations (PO_4^{3-}, NO_3^-) in filtered water samples were determined using ion chromatography (Dionex ICS-2100). Dissolved organic carbon (DOC) was measured in filtered water samples using a Shimadzu TOC analyzer (NPOC method). $SUVA_{254}$, a measure of DOC aromaticity (Weishaar et al., 2003), was measured using a Shimadzu UV2600 spectrophotometer. Total elemental concentrations (P, Fe, Al, Mg, and Mn) were measured with an ICP-MS (Agilent 7800).

3. Results and discussion

3.1. Effects of drying-rewetting cycles on P_i removal in bioreactor columns

Analysis of P_i in column influent and effluent showed that P_i removal was systematically improved by drying-rewetting cycles (Fig. 1). Influent P_i concentrations were similar in both reactors, with the DRW influent tending at times to be approximately 0.1 mg/L PO_4^{3-} lower than the CS reactor. In the first sampling of Replicate 1 the woodchips in both reactors acted as a source of P_i (Fig. 1A), but after this isolated observation both the CS and DRW reactor acted as sinks for P_i in Replicate 1. Apart from the first measurement, P_i removal rates ranged from 0 to 21.9 μg PO_4^{3-} L^{-1} h^{-1} (median 12.6 μg PO_4^{3-} L^{-1} h^{-1}) in the CS reactor, and from 26.4 to 62.7 μg PO_4^{3-} L^{-1} h^{-1} (median 37.3 μg PO_4^{3-} L^{-1} h^{-1}) in the DRW reactor (Fig. 1A). P_i removal rates in the DRW reactor were always greater than the CS reactor, and P_i removal rates tended to be greater in the first sample after re-flooding compared to four days after re-flooding. P_i removal expressed in terms of removal efficiencies is shown in Fig. S2. Note that removal rates and efficiencies in the DRW reactor reported here overestimate P_i removal when averaged over multiple weeks, since for the 48 h each week when the DRW reactors are drained there is no treatment.

Overall in Replicate 2, P_i removal rates ranged from −1.3–34.9 μg PO_4^{3-} L^{-1} h^{-1} (median of 20.4 μg PO_4^{3-} L^{-1} h^{-1}) in the CS reactor and 16.9–71.9 μg PO_4^{3-} L^{-1} h^{-1} (median of 29.4 μg PO_4^{3-} L^{-1} h^{-1}) in the DRW reactor (Fig. 1B). There was no difference in P_i removal rates between the columns during the first 20 d of the experiment, with both columns characterized by removal rates between 13.8 and 38.1 μg PO_4^{3-} L^{-1} h^{-1}. However, after the fourth drying-reflooding cycle the P_i removal rates markedly improved at $t = 6$ h after re-flooding, and this pattern was sustained for the following five drying-reflooding cycles. After the fourth drying-reflooding cycle, P_i removal rates ranged from 50.0 to 71.9 μg PO_4^{3-} L^{-1} h^{-1} at t = 6 h, while removal rates were just

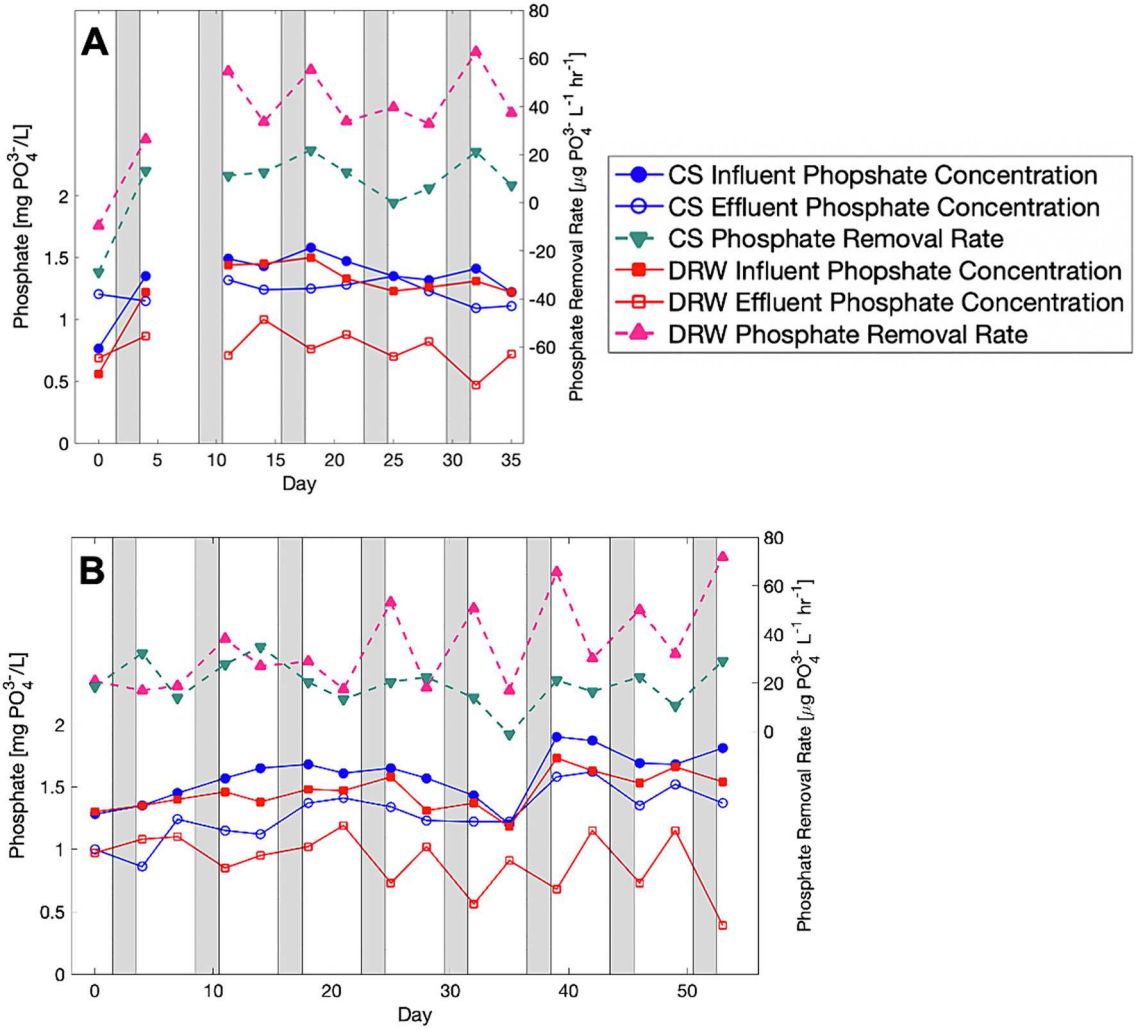

Fig. 1. Influent and effluent orthophosphate concentrations, and orthophosphate removal rates, in continuously-saturated (CS) and drying-rewetting (DRW) woodchip bioreactor columns in (A) Replicate 1 and (B) Replicate 2. Shaded grey areas indicate the 48 h period when the DRW reactor was drained each week; the CS reactor was not drained. Negative removal rates indicate that the woodchips were a source of orthophosphate at that sampling point.

16.9–31.9 μg PO_4^{3-} L^{-1} h^{-1} at $t = 100$ h after re-flooding. The difference between the t = 6 h and t = 100 h time points in the DRW reactor was more pronounced in Replicate 2 than Replicate 1; the reasons for this are not clear. Rates in the CS reactor led to P_i removal efficiencies of 4–23 % (excluding isolated observations of woodchips as a P_i source) (Fig. S2), which is consistent with prior studies of P_i removal in woodchip bioreactors operated under continuously-saturated anoxic conditions (Christianson et al., 2017; Sharrer et al., 2016; Povilaitis et al., 2020).

More frequent sampling of P_i removal and dissolved O_2 was performed in the first 50 h after reactor re-flooding in Weeks 4 and 6 of Replicate 2 to focus on the oxic-anoxic transition that led to enhanced P_i removal. Dissolved O_2 concentrations decreased rapidly after re-flooding (Fig. 2A-B), but in week 6, there was a transient increase in dissolved O_2 at the downstream sampling port that may have been caused by manual sampling of woodchips for nucleic acid extraction. In both weeks, dissolved O_2 in the upstream port decreased more slowly than the downstream port, and the slow decrease during week 6 indicates that low levels of O_2 persisted in the bulk liquid for >50 h after reactors were re-flooded. P_i removal was greatest in the first 15 h after reactors were re-flooded. In both weeks, the maximum P_i removal efficiency was observed 10–12 h after reactors were re-flooded. This was followed by a continuous decrease in P_i removal efficiency over the next 40 h.

3.2. Polyphosphate kinase (ppk) gene expression across the oxic-anoxic transition

Accumulibacter spp. and *Pseudomonas* spp. are well-documented PAOs in environments including activated sludge and soils (Nikel et al., 2013; Renninger et al., 2004; Camejo et al., 2016; Flowers et al., 2013; He and McMahon, 2011b), and expression of *ppk* genes has been linked to polyP accumulation (Renninger et al., 2004). Transcripts of *ppk1 and ppk2* from *Accumulibacter* spp. and *Pseudomonas* spp. both increased from $t = 0$ h after re-flooding to a maximum at $t = 16$ h, followed by a decrease until $t = 52$ h (Fig. 2C). The maximum *ppk* expression was concurrent with the maximum P_i uptake efficiency, and both *ppk* expression and P_i uptake decreased after these maxima were achieved. In week 6, when *ppk* expression was measured, O_2 levels in the central portion of the column were between 0.05 and 0.5 mg/L when *ppk* expression and P_i removal efficiencies were at their maxima.

3.3. Batch reactor experiments

Autoclaved woodchips did not take up P_i from solution, indicating that for our woodchips P_i uptake was driven by microbial processes and not adsorption (Fig. 3A). P_i adsorption onto woodchip surfaces of up to 13 ± 2.5 mg P_i/kg woodchip has been reported previously (Sanchez Bustamante-Bailon et al., 2022), likely due to high concentrations of Fe-

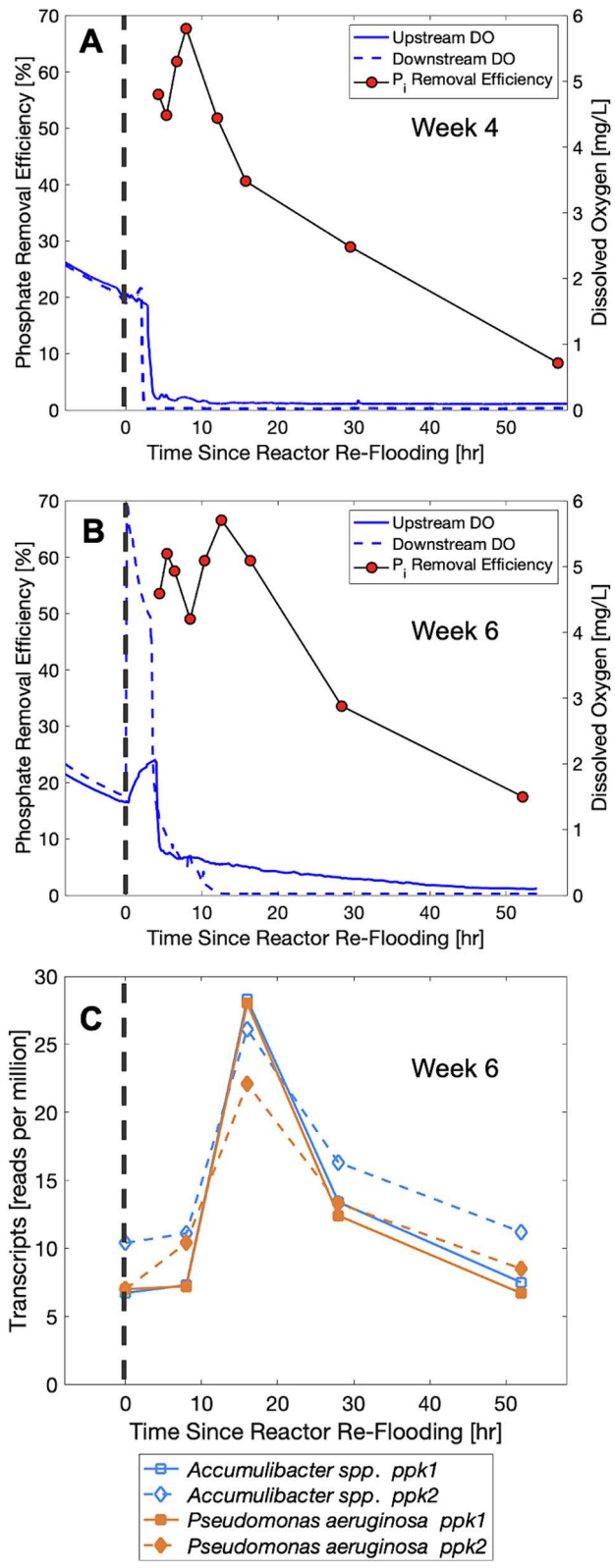

Fig. 2. P_i removal efficiencies, dissolved oxygen, and *ppk* gene expression across the oxic-anoxic transition when the DRW reactor was re-flooded after a 48 h dry period. The vertical grey dashed line at $t = 0$ h indicated when columns were re-flooded. P_i removal efficiency in the DRW reactor after drying-re-wetting cycles in (A) week 4 and (B) week 6 of Replicate 2. The blue lines show dissolved O_2 concentrations at locations 55 cm (upstream) and 105 cm (downstream) from the reactor inlet. (C) *ppk* transcripts from known PAOs across the oxic-anoxic transition during Week 6.

and Al-oxides on woodchip surfaces which serve as good adsorbents for P_i (Sanchez Bustamante-Bailon et al., 2022; Perera et al., 2024). The lack of abiotic P_i uptake in our study may be due to relatively low surface concentrations of Fe (80.8 ± 30.8 mg/kg dry weight) and Al (30.1 ± 5.6 mg/kg dry weight) (Fig. S3), which were more than an order of magnitude lower than concentrations measured in an earlier study of P_i adsorption onto woodchips (Sanchez Bustamante-Bailon et al., 2022).

During the 20 d pre-incubation, woodchips from the A2 pre-incubation transitioned from being a net sink of roughly 13 mg/L P_i over the first six days while NO_3^- was present before becoming a source of P_i after NO_3^- was fully depleted and the system became anaerobic (Fig. S4). Woodchips in the A2O pre-incubation served as a net sink of P_i during oxic and anoxic phases, but were a source of P_i in anaerobic phases after NO_3^- was depleted. After the media was replaced, woodchips in both oxic and anoxic incubation conditions took up P_i from solution over the 50 h incubation, with greater uptake in oxic conditions compared to anoxic conditions (Fig. 3A). NO_3^- was present in all conditions throughout the 50 h incubation, so the batch experiments in this stage did not become anaerobic (Fig. 3B). Woodchips that had been pre-incubated for 20 d in A2 conditions took up 23.0 ± 2.6 and 48.3 ± 7.9 mg PO_4^{3-}/g woodchip (dry weight) from fresh media in anoxic and oxic conditions, respectively. For woodchips from the A2O pre-incubation, P_i uptake was 30.8 ± 3.6 and 74.9 ± 0.8 mg PO_4^{3-}/g woodchip (dry weight) in anoxic and oxic conditions, respectively. The A2O pre-incubation increased P_i uptake relative to woodchips from the A2 pre-incubation by 55 % in oxic conditions and 34 % in anoxic conditions. ICP-MS analysis of total P confirmed that aqueous concentrations of P species other than orthophosphate were negligible (Fig. S5).

There was no difference in woodchip surface protein concentrations, a measure of microbial biomass, between the A2 vs. A2O pre-incubation conditions (Fig. 4A). This indicates that the greater P_i uptake by woodchips from the A2O pre-incubation was not driven by greater biomass that may have grown due to oxic and more carbon-rich conditions, but rather more efficient P_i uptake by the same biomass concentration. Our data do not provide any information on shifts in the microbial community composition that may have occurred, but prior work from our group showed that six weeks of oxic-anoxic cycling in the flow-through bioreactor columns did not significantly impact the taxonomic diversity of the woodchip biofilm community (McGuire et al., 2023). Biomass-normalized P_i uptake ranged from 0.0067 ± 0.0008 g P/g biomass (assuming cells were 50 % protein by mass) in woodchips from the A2 pre-incubation under anoxic conditions, to 0.0204 ± 0.0026 g P/g biomass in woodchips from the A2O pre-incubation under oxic conditions.

DOC release from woodchips was greater under oxic conditions than anoxic conditions (Fig. 4B), consistent with prior research from our group highlighting the importance of oxygen in stimulating degradation of lignocellulosic woodchip biomass (McGuire et al., 2021; McGuire et al., 2023). There was not a significant difference between the A2 and A2O pre-incubation on DOC release in oxic incubation conditions. However, there was a significant difference in carbon quality released from woodchips related to pre-incubation conditions, with the A2O pre-incubation leading to a DOC pool with greater aromaticity, likely due to a larger contribution of water-soluble phenolic lignin residues (Fig. 4C).

Notably, the fastest NO_3^- removal was observed under oxic incubation with woodchips from the A2O pre-incubation, followed by anoxic incubation with woodchips from the A2O pre-incubation (Fig. 3B). The slowest NO_3^- removal in biotic experiments was observed with woodchips from the A2 pre-incubation, and removal was similar in oxic and anoxic conditions. Active NO_3^- removal in oxic incubation conditions while the bulk liquid contained 7–8 mg/L DO can be explained by the presence of anoxic to hypoxic zones within woodchip biofilms where denitrification or potentially dissimilatory NO_3^- reduction to ammonium (DNRA) could occur. Faster NO_3^- removal in woodchips from the A2O pre-incubation, including under oxic conditions, is likely due to greater organic carbon availability for denitrification or DNRA in anoxic to

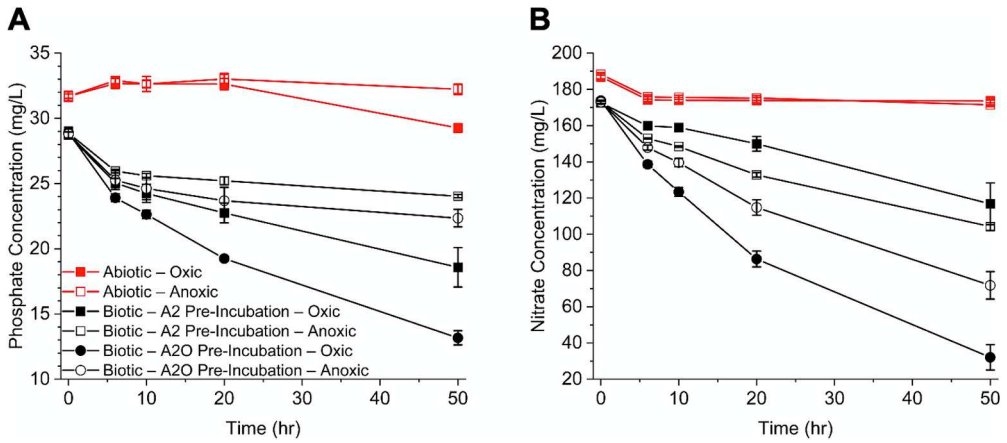

Fig. 3. Removal of (A) P_i and (B) NO_3^- in batch experiments with woodchips that had undergone a 20 day pre-incubation under either continuous anoxic-anaerobic (AA) or alternating anoxic-(anaerobic-)oxic (A2O) conditions. Open symbols represent incubation under anoxic conditions, and filled symbols represent incubation under oxic conditions. Circles are from woodchips from the A2O pre-incubation, and squares are from woodchips from the A2 pre-incubation. Red symbols are from autoclaved controls (note that autoclaved controls had not undergone the 20 days of antecedent pre-incubation).

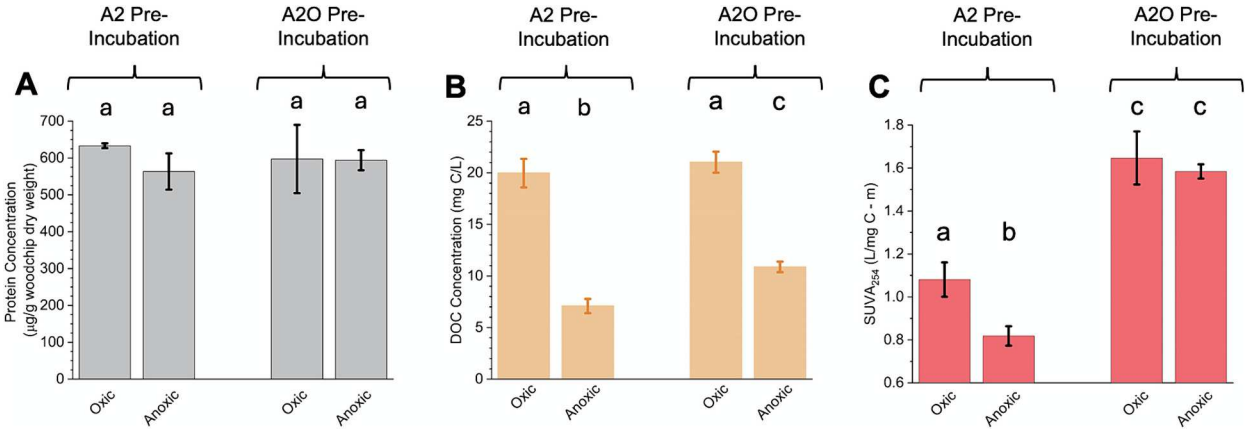

Fig. 4. (A) Protein concentrations on woodchip surfaces as a measure of biomass concentrations; (B) Dissolved organic carbon (DOC) concentrations; and (C) $SUVA_{254}$ measured after 50 h batch woodchip incubations with woodchips from either anoxic or oxic-anoxic pre-incubations. Lowercase letters above bars represent statistically significant differences using ANOVA with Tukey's honestly significant difference post-hoc test ($p < 0.05$).

hypoxic zones of woodchip biofilms.

3.4. Biological mechanisms for orthophosphate uptake

There has been one prior research report which documented improved removal of P_i by woodchip media after two reactor drying-rewetting cycles (Hua et al., 2016), but this study did not investigate the underlying mechanisms for this observation or determine whether it was driven by biotic or abiotic processes. Our batch reactor data with autoclaved woodchips demonstrate that uptake of P_i onto woodchips in our experimental setting was a biological process, with no evidence for adsorption of P_i onto woodchip surfaces (Fig. 3A). Microbial cells assimilate P_i from the environment to support growth of biomass, which is typically 1–3 % P by mass (Rittmann and McCarty, 2012). One plausible explanation for enhanced P_i uptake into woodchip biofilms from the A2O pre-incubation is therefore that greater organic carbon availability led to greater biomass on woodchip surfaces, which has been seen in woodchip bioreactors dosed with exogenous labile carbon (Zhang et al., 2024). However, there were no significant differences in surface protein concentrations between woodchips from the A2O and A2 pre-incubations (Fig. 4A), indicating that P_i uptake was not due to greater overall biomass synthesis, but rather was likely related to luxury P_i uptake by PAOs in the biofilms from the A2O pre-incubations. Phosphorus uptake in the oxic incubation following A2O pre-incubation represented 2.0 ± 0.3 % of the woodchip surface biomass, while in the anoxic incubation following A2 pre-incubation P uptake represented just 0.67 ± 0.08 % of the woodchip surface biomass. It is also possible that P_i uptake could be driven by interactions with biofilm extracellular polymeric substances (EPS) rather than intracellular uptake (Li et al., 2015).

Polyphosphate accumulating organisms are capable of higher levels of Pi uptake, accumulating up to 15 % P by mass in intracellular polyP granules (Rittmann and McCarty, 2012). There are several lines of evidence from our study which are consistent with a role for PAOs in the enhanced P_i uptake observed in woodchips from the A2O pre-incubation. First, alternating oxic-anaerobic conditions are known to select for PAOs (Rittmann and McCarty, 2012). Second, P_i uptake in batch experiments was greatest in oxic incubation conditions, consistent with classical wastewater EBPR systems where there is a net uptake of P_i under oxic conditions in aeration basins. In column experiments, P_i uptake was greatest in the first 15 h after reactors were re-flooded, when there were still low levels of dissolved O_2 (Fig. 2B). It is also possible that trapping of air in the woodchip pore structure after re-flooding (McGuire and Reid, 2019) could sustain aerobic microbial processes in biofilms after dissolved O_2 was depleted from the bulk liquid. Third, we observed a maxima in *Accumilibacter* and *Pseudomonas* spp. *ppk* gene expression in

column experiments that was concurrent with the greatest P_i removal efficiency (Fig. 2C), suggesting a potential link between *ppk* expression by these well-characterized PAOs (He and McMahon, 2011b; Racki et al., 2017; Wang et al., 2021) and P_i uptake in the woodchip biofilms. Transcriptional profiles of *ppk* in *Accumulibacter* spp. or other PAOs (e.g., *Microlunatus phosphovorus*) have been shown to respond to environmental triggers of polyP synthesis including changes in acetate concentrations (He and McMahon, 2011a) or oxic vs. anoxic or anaerobic conditions (Zhong et al., 2018). However, we caution that because PPK enzymes are involved in a range of physiological processes besides polyP accumulation, the presence of *ppk* in metatranscriptomic datasets does not necessarily provide a specific biomarker for PAO activity (McMahon et al., 2002a). So while the temporal association between *ppk* expression and P_i uptake is an interesting observation, it does not conclusively demonstrate an EBPR-like process in our system.

Release of P_i from woodchips was only rarely observed in the column or batch experiments. This may be due to the fact that NO_3^- was usually present throughout the columns (McGuire et al., 2021) and was not fully depleted in the batch experiments (Fig. 3B), so the bulk fluid was not characterized by the anaerobic conditions (depleted in both O_2 and NO_3^-) associated with P_i release by PAOs. Notably, only after NO_3^- was fully depleted in the A2 and A2O pre-incubations of the batch experiments did we observe a net release of P_i (Fig. S4). NO_3^- removal efficiencies in woodchip bioreactors depend on residence times and temperatures, and it is common for denitrification to be incomplete and for NO_3^- to be present throughout bioreactors in field conditions (Israel et al., 2023). These results suggest that the lack of complete NO_3^- removal in bioreactors may enhance luxury P_i uptake by denitrifying woodchip biofilms, and only when bioreactors are fully depleted in NO_3^- during low-flow periods in warm weather would it be expected for biofilms to release P_i.

Additional focused experiments are needed to fully elucidate the processes leading to enhanced P_i uptake by woodchips undergoing anoxic-(anaerobic-)oxic cycling. For example, phenotypic identification of PAOs in woodchip biofilms (Wang et al., 2021) is needed to definitively show that these observations can be linked to EBPR-like mechanisms. Our results also do not clearly indicate whether anaerobic vs. anoxic conditions must be reached as part of redox oscillations to stimulate enhanced P_i uptake. For example, enhanced P_i uptake was observed in column experiments even though the bulk fluid was mostly characterized by anoxic (non-NO_3^- - depleted) rather than anaerobic conditions. However, it is likely that anaerobic microenvironments formed within woodchip biofilms (evidenced, for example, by dissolved methane in pore water of DRW columns (McGuire et al., 2021)), so redox zonation within biofilms may play a role in enabling oxic-anaerobic cycling in woodchip media even when the bulk fluid contains NO_3^-. Further research is needed to determine the necessity of anaerobic conditions to promote the enhanced P_i uptake that was observed here.

4. Conclusion

Co-treatment of N and P from nonpoint sources is a challenge but is increasingly important as dual nutrient reduction plans become more common. Prior research has shown that anoxic-oxic cycling of woodchip bioreactors improves NO_3^- removal by enhancing carbon availability for denitrifying microorganisms. Here, we built on these earlier results to show that anoxic-(anaerobic-)oxic cycling also improves P_i removal, more than doubling median P_i removal rates across two multi-week experiments. Woodchips from an anoxic-anaerobic-oxic pre-incubation in batch experiments exhibited 55 % greater P_i removal than woodchips from an anoxic-anaerobic pre-incubation, and experiments with autoclaved woodchips confirmed that P_i uptake was driven by a biological process. Further research is needed to determine the specific biological mechanism(s) for enhanced P_i uptake into woodchip biofilms. This result is of practical importance because it demonstrates that deliberate draining and re-flooding of wood-based biofilter systems can be a straightforward approach to improve the co-treatment of both N and P from nonpoint sources.

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CRediT authorship contribution statement

Zihao Zhang: Writing – original draft, Visualization, Investigation. **Philip M. McGuire:** Methodology, Investigation. **Ruth E. Richardson:** Conceptualization. **April Z. Gu:** Writing – review & editing, Conceptualization. **James P. Shapleigh:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Matthew C. Reid:** Writing – review & editing, Visualization, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Metatranscriptomic raw sequencing reads are publicly available on the JGI Genome Portal with JGI Proposal ID 506611.

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Appendix A. Supplementary data

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