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# Competitive sorption of microbial metabolites on an iron oxide mineral

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## ABSTRACT

A large fraction of soil organic matter (SOM) is composed of small molecules of microbial origin. However, the biotic and abiotic cycling of these nutrients is poorly understood and is a critical component of the global carbon cycle. Although there are many factors controlling the accessibility of SOM to microbes, sorption to mineral surfaces is among the most significant. Here, we investigated the competitive sorption of a complex pool of microbial metabolites on ferrihydrite, an iron oxide mineral, using a lysate prepared from a soil bacterium, *Pseudomonas stutzerii* RCH2. After a 24-hour incubation with a range of mineral concentrations, more than half of the metabolites showed significant decreases in solution concentration. Phosphate-containing metabolites showed the greatest degree of sorption followed by dicarboxylates and metabolites containing both nitrogen and an aromatic moiety. Similar trends were observed when comparing sorption of metabolites with an equimolar metabolite mixture rather than a bacterial lysate. Interestingly, ectoine, lysine, two disaccharides and uracil were found not to sorb and may be more bioavailable in iron oxide-rich soils. Additionally, the highest-sorbing metabolites were examined for their ability to mobilize mineral-sorbed phosphate. All phosphate-containing metabolites tested and glutathione released phosphate from the mineral surface within 30 minutes of metabolite addition. These findings of preferential sorption behavior within a complex pool of microbial metabolites may provide insight into the cycling of SOM and specific nutrient availability. Finally, the release of highly-sorptive metabolites may be an underexplored mechanism utilized by microbial communities to gain access to limited environmental nutrients.

**Keywords:** ferrihydrite, sorption, phosphate, soil organic matter, metabolomics

## 1. INTRODUCTION

Terrestrial ecosystems store more carbon than plant biomass and the atmosphere combined with soil organic matter (SOM) being the largest terrestrial pool of organic carbon (Batjes, 1996; Houghton, 2007). Many contradictory predictions exist regarding the effects climate change will have on microbial activity and composition and the role terrestrial ecosystems will play in the overall carbon cycle (Davidson and Janssens, 2006; Giardina and Ryan, 2000; Hopkins et al., 2012; Karhu et al., 2014). A critical step toward developing better terrestrial carbon cycling models is to understand not only the chemical composition of SOM, but factors controlling the stability, behavior and (microbial) accessibility of these compounds. This may ultimately allow us to predict climate-induced alterations in microbial community composition and anticipate changes in carbon mineralization and respiration rates.

SOM is a complex mixture of compounds with a large fraction derived from microbial products (Ludwig et al., 2015; Schmidt et al., 2011). The soluble fraction (dissolved organic matter, DOM) accounts for less than 2% of SOM, but is the most accessible fraction for microbial degradation and therefore DOM turnover is likely an accurate representation of overall SOM cycling (Guigue et al., 2015; Kaiser and Kalbitz, 2012; Kalbitz, 2003). Subsoil DOM consists of microbial products (Kaiser and Kalbitz, 2012), but the specific chemical composition of DOM is just beginning to be more thoroughly characterized and includes carbohydrates, amino acids, organic acids, fatty acids, sterols and nucleosides (Kakumanu et al., 2013; Swenson et al., 2015; Warren, 2015). Microbes rely on DOM as an important carbon source and tend to prefer carbohydrates and amino acids over complex (possibly lignin-derived) aromatic molecules (Amon et al., 2001; Kalbitz, 2003). As a result of substrate preferences such as these, microbial function and community structure are highly influenced by the abundance and the composition

of DOM (Guigue et al., 2015; Heckman et al., 2013a; Hopkinson et al., 1998; Judd et al., 2006). Hence, alteration of the DOM composition can have a drastic affect on microbial community structure.

In addition to microbial processing, many abiotic (*e.g.* sorption, precipitation, dissolution, diffusion) processes affect microbial accessibility and overall cycling of DOM (Kaiser and Kalbitz, 2012; Kalbitz et al., 2000; Zsolnay, 1996). Sorption to mineral surfaces is potentially the most impactful abiotic factor. Specific mineral content (clay, iron oxide minerals, *etc*) and surface area have a strong influence on the solid-solution partitioning and the accessibility of DOM components to microorganisms (Kalbitz et al., 2000). It would follow that the sorption behavior of specific metabolites may influence community structure by changing the bioavailability of DOM components favoring microbes that can best utilize the available components and access adsorbed metabolites. Indeed, recent studies show that soil composition, in terms of mineral and clay content, shapes microbial community structure (Heckman et al., 2013b; Vogel et al., 2014). For example, forest floor DOM sorption dynamics with goethite and gibbsite affect microbial community composition in a time-dependent manner (Heckman et al., 2013b).

There are limited studies aimed at examining the sorption behavior of well-characterized DOM components on minerals. However, there is general agreement that iron (hydr)oxide minerals such as goethite, ferrihydrite and hematite preferentially sorb molecules containing aromatic, nitrogen, phosphate and carboxylate groups (Omoike and Chorover, 2006; Yeasmin et al., 2014; Zhou et al., 2001). These strong, and often irreversible, mineral-nutrient interactions have a drastic influence on biological dynamics. While studies such as these have led to our understanding of sorption-based DOM stability of general classes of compounds, in order to link

DOM cycling with soil microbial community dynamics, there is a need to investigate specific metabolite sorption behavior.

Another essential component of understanding nutrient availability in terrestrial systems is evaluating how mineral sorption of DOM molecules can affect the mobility of vital mineral-restricted nutrients such as phosphorus. Although total phosphorus in soils is generally high, available soluble forms are limited mostly due to phosphate sorption to mineral surfaces, especially iron and aluminum oxides (Arai and Sparks, 2007; Sharma et al., 2013; Wang et al., 2013). Microbes have developed many mechanisms for phosphate mobilization including production of phosphatases, lowering of soil pH and release of organic acids (Sharma et al., 2013). Possibly due to the limited availability of appropriate metabolomics technology, investigations into phosphate mobilization by high-sorbing DOM components, other than organic acids, are limited.

The objective of this study was to examine the sorption behavior of microbial metabolites as they exist in a competitive and complex, biologically-relevant mixture. Most studies to date tend to focus either on simple systems (Cagnasso et al., 2010; Persson and Axe, 2005) or complex mixtures of organics that are only roughly characterized (Heckman et al., 2013a; Kaiser and Guggenberger, 2007). We used lysates prepared from a soil bacterium to represent compounds commonly found in DOM. This mixture of microbial metabolites represents a unique system that is both highly complex and well-characterized (Swenson et al., 2015). Aged ferrihydrite, an iron (oxyhydr)oxide mineral, was used as our model sorbent due to its ubiquitous presence in natural systems and its nanoporous, highly reactive and large specific surface area (Heimstra and Riemsdijk, 2009). Metabolite sorption as a function of mineral concentration was monitored by liquid chromatography/ mass spectrometry (LC/MS). Individual high-sorbing

compounds were then selected to investigate the potential to desorb and mobilize phosphate from the mineral surface. These data may shed light onto exometabolite mechanisms by soil microbes to gain access to limiting nutrients such as phosphate.

## 2. MATERIALS AND METHODS

### 2.1. Materials

LC/MS-grade water and LC/MS-grade methanol (CAS 67-56-1) were from Honeywell Burdick & Jackson (Morristown, NJ). LC/MS-grade OmniSolv acetonitrile (CAS 75-05-8) was from EMD Millipore (Billerica, MA). MOPS (CAS 1132-61-2), HEPES (CAS 7365-45-9), 3,6-dihydroxy-4-methylpyridazine (CAS 5754-18-7), 4-(3,3-dimethyl-ureido)benzoic acid (CAS 91880-51-2), $d_5$-benzoic acid (CAS 1079-02-3), 9-anthracenecarboxylic acid (CAS 723-62-6), ammonium acetate (CAS 631-61-8) , $KH_2PO_4$ (CAS 7778-77-0) were from Sigma (St. Louis, MO). All compounds in the standards mixture were from Sigma (St. Louis, MO).

Ferrihydrite was synthesized according to the procedure of Schwertmann and Cornell (2000) and dry-aged in the dark for eight years. The resulting mineral was characterized by x-ray diffraction and shown to be low crystallinity, 2-line ferrihyrdite. Bacterial lysates were prepared using the soil isolate, *Pseudomonas stutzeri* RCH2, obtained from Romy Chakraborty (LBNL). Bacteria were grown in M9 minimal media containing sodium acetate (0.2%) as the carbon source and lysates prepared according to the protocol in Swenson et al (2015). In brief, cultures were grown in M9 minimal media until an optical density of 0.5 (at 600 nm) was reached. Cells were pelleted by centrifuging at 3220 x *g* for 10 min, washed with cold phosphate-buffered saline (pH 7.4) and re-pelleted. The supernatant was discarded and pellets resuspended in 1 mL cold methanol, sonicated for 2 x 20 s using a Q125 QSonica sonicator then 5 min in a sonicating

water bath (VWR symphony), centrifuged at 2348 x *g* for 5 min and the supernatant dried. Lysates were resuspended in sterile LC/MS-grade water resulting in a total organic carbon (TOC) content of 158 mg/L (Shimadzu TOC-V CSH analyzer, Kyoto, Japan), a pH of 6.8 and 55 (including six putative) metabolites identified by LC/MS analysis (**Supplementary Table 1**).

To explore the relative sorption behavior of bacterial metabolites when present in equimolar concentrations, the ferrihydrite sorption experiment was conducted with an equimolar mixture of standards. Each compound was prepared at 20 μM each (final concentration) in LC/MS-grade water, resulting in a pH of 6.8. The standards mixture contained 44 out of the 55 compounds reported for the bacterial lysates (the remaining 11 compounds were unavailable) plus five additional compounds (nicotinic acid, dodecanoic acid, hydroxy-proline, ornithine, raffinose) that were detected in the preliminary analysis of bacterial lysates (but not included in the final results of that experiment due to small and ambiguous peaks). Maltose and trehalose were included in the mixture to represent disaccharide 1 and 2, respectively. Of the metabolites added, five were not detected (coenzyme A, tyrosine, citramalic acid, glutathione and alanine) indicating that they may be present in higher concentrations in the lysate than in the standards mixture. The TOC of the standards mixture was 1.698 g/L.

### 2.2. Metabolite sorption assay

Resuspended lysates or standards mixture (1 mL) was added to 5 mL Eppendorf tubes containing mineral (0, 0.5, 1, 2, 4, 8, 16 or 32 mg), tilted at 90° and shaken on an orbital shaker (Orbital-Genie, Scientific Industries, Bohemia, NY) at 200 rpm for 24 h at 25°C. For the temperature-effect experiment, samples were shaken for 24 h either at 4°C, 25°C or 37°C. Samples were centrifuged at 3220 x *g* for 15 minutes and 950 uL of the supernatant dried down

with a Savant SpeedVac SPD111V (Thermo Scientific, Waltham, WA) and resuspended in 100% methanol (200 uL for lysates, 300 uL for the standards mixture) containing internal standards (MOPS, HEPES, 3,6-dihydroxy-4-methylpyridazine, 4-(3,3-dimethyl-ureido)benzoic acid, $d_5$-benzoic acid and 9-anthracene carboxylic acid at 5 μg/mL each). Resuspended samples were vortexed and sonicated for 5 min followed by filtration through 0.22 μm centrifugal membranes (Nanosep MF, Pall Corporation, Port Washington, NY). Each sorption condition contained five replicates.

### 2.3. LC/MS analysis

Extracts were analyzed using normal phase liquid chromatography with a ZIC-pHILIC column (150 mm × 2.1 mm, 3.5 μm 200 Å, Merck Sequant, Darmstadt, Germany) using an Agilent 1290 series UHPLC (Agilent Technologies, Santa Clara, California, USA) with an Agilent 6550 quadropole time-of-flight mass spectrometer using both positive and negative polarities. Samples were maintained at 4°C prior to injection. Mobile phase for chromatographic separation was A: 5mM ammonium acetate and B: 90% acetonitrile w/ 5mM ammonium acetate. Upon sample injection, initial conditions of 100% B at a flow rate of 0.25 mL/min were held for 1.5 min after which the following gradient was applied: linear decrease to 50% B at 25 min; hold until 29.9 min, return to initial conditions at 30 min with a total runtime of 40 min for column re-equilibration. Column temperature was maintained at 40°C. Flow was directed to the electrospray MS source which was operated at the following setpoints for both positive and negative polarity ionization: drying gas temperature and flow: 275°C and 14 L/min; sheath gas temperature and flow: 275°C and 9 L/min; nebulizer pressure: 30 psi; capillary voltage: 3500 V; nozzle voltage: 1000 V. Mass spectra were acquired from 50-1700 m/z at 4 spectra $s^{-1}$.

## 2.4. Data analysis

Data were analyzed using Metabolite Atlas (Bowen et al, 2015) and the Python programming language. Metabolite identification was based on two orthogonal data relative to authentic standards: exact mass (± 5 ppm) and retention time (± 60s) in accordance with the metabolomics reporting standards (Sumner et al, 2007). Putative identifications were assigned in the cases where authentic standards were not available (and indicated in figures by parentheses). Instrument performance was monitored by including internal standards in each sample and by running a quality control mixture before and after each sample set. Metabolites were not quantified since the focus of this study was on the sorption behavior of individual metabolites relative to each other in a biologically-relevant ratio (for the bacterial lysates experiment) and relative to non-mineral controls. Instead, peak areas for each metabolite were compared across all samples and sorption results are reported as a percentage (peak area) of the non-mineral control. To determine statistical significance between mineral-containing samples and the control, peak areas were log2 transformed and a two-way ANOVA was performed along with the Dunnett's test to correct for multiple comparisons. A $p$ value less than 0.05 was considered significant. These calculations only reflect significance between metabolite peak areas with and without mineral.

## 2.5. Phosphate release assay

Phosphate desorption from mineral was measured by loading the mineral (10 mg) with phosphate (10 mL of 0.25 mM $KH_2PO_4$, pH 7) by shaking on an orbital shaker at 200 rpm for 24 h at 25°C. At the end of the phosphate loading step, an aliquot (500 µL) was removed to measure

phosphate concentrations. To pellet the mineral (containing sorbed phosphate), samples were centrifuged at 3220 x *g* for 15 minutes and the supernatant discarded. Mineral pellets were resuspended in metabolite solution (10 mL of 2.5 mM metabolite in deionized water, pH 7) and shaken as above for 24 h. A metabolite-free control (deionized water) was included to account for the amount of phosphate released from the pellet during the 24 h incubation. Aliquots (500 μL) were removed 30 min, 60 min, 120 min and 24 h following metabolite addition. Phosphate concentrations were measured in the initial $KH_2PO_4$ solution, 24 h post-phosphate loading and the above timepoints post-metabolite addition using the phosphate colorimetric kit (Sigma-Aldrich, Saint Louis, MO). Each aliquot used for phosphate measurement was cleared of solid phase by centrifuging at 10,000 x *g* for 10 min, plated (20 μL) on a clear 96-well flat-bottom plate with deionized water (180 μL) and phosphate colorimetric reagent (30 μL) and mixed by pipetting. The plate was incubated at room temperature in the dark for 30 min and absorbance read at 650 nm. The background absorbance for each metabolite solution was measured before and after 24 h on the orbital shaker. Each sorption experiment was performed in triplicate. The initial amount of phosphate assumed to be bound to the mineral after the 24 h loading phase was calculated by:

$$P_S(24) = A_{650}(\text{initial}) - A_{650}(24h)$$

where $P_S(24)$ is the phosphate (absorbance potential) bound to mineral after 24 h, $A_{650}(\text{initial})$ is the absorbance at 650 nm of the initial $KH_2PO_4$ solution and $A_{650}(24h)$ is the absorbance of the $KH_2PO_4$ solution after the 24 h loading phase. The percent phosphate desorption was then calculated by:

$$P_D(t) = [A_{650}(t) - A_{650}(0)] / P_S(24h)$$

where $P_D$ is the percent phosphate desorbed as a function of time, $A_{650}(t)$ is the absorbance as described above at a given time point, $A_{650}(0)$ is the absorbance of the metabolite solution without added mineral or phosphate, and $P_S(24h)$ is the initial amount of sorbed phosphate.

## 3. RESULTS

### *3.1. Microbial metabolite sorption on solid phase*

Iron oxide mineral sorption of microbial metabolites (24 h) was investigated with a series of mineral concentrations to encompass a range of mineral:TOC ratios that may exist in natural systems. The metabolite-mineral incubation did not significantly affect the pH of the solutions, which started at 6.8 and after the 24-h incubation ranged from 6.2-7.2 (varying from sample to sample with no obvious trends with mineral concentration). The detected bacterial metabolites included a broad range of compounds commonly found in SOM (Alexander, 1977). The relative aqueous concentration (calculated as a percentage of the non-mineral control) was significantly decreased by the presence of mineral phase for 32 out of the 55 detected metabolites (reaching at least 76% sorption) (**Figure 1**, **Supplementary Table 2**). Metabolites were grouped into five categories based on the presence of sorption-associated structural moieties: 1) phosphate-containing, 2) dicarboxylates, 3) both aromatic and N-containing, 4) both carboxylate- and N-containing and 5) other. For all metabolites, sorption occurred in a mineral concentration-dependent manner (sorption increased with the ratio of mineral:TOC) (**Figure 1**). The most sorptive group was the phosphate-containing compounds. All peak areas within this group were significantly decreased by the presence of mineral surface with 100% sorption occurring at an approximate ratio of 50:1 (w/w) (mineral:TOC). The second most sorptive group was the dicarboxylates (all metabolites with significantly decreased solution concentration) followed by

the aromatic and N-containing group (10 out of 16 metabolites showed significantly decreased solution concentrations). Only a few metabolites did not sorb at all under the conditions tested: ectoine, lysine, disaccharide 1 and 2 (possibly maltose and trehalose) and uracil. Finally, the overall sorption trends were not affected by temperature (*i.e.* 4°C, 25°C and 37°C) with the phosphate-containing compounds as the most sorptive (**Supplementary Results, Supplementary Figure 1**).

### *3.2. Bacterial lysates versus the standards mixture*

Since the absolute concentration of each metabolite from the bacterial lysate was not measured, the same experiment was conducted with a mixture of standards in equimolar concentration (20 μM each) to explore the possibility that the sorption behavior was a function of relative abundance of each metabolite within the bacterial lysates. In total, 44 compounds were measured for sorption. It is important to note that the standards mixture contained almost ten times more TOC than the lysates, but the same range of mineral concentrations as in the first experiment was still tested to examine the sorption behavior of each metabolite as a function of increasing mineral concentration. Overall, metabolites in the standards mixture behaved similarly to the bacterial lysates with the phosphate-containing compounds being the most sorptive group (all decreased significantly) (**Figure 2**). Peak areas were significantly reduced by the presence of mineral (reaching at least 78% sorption) for 18 out of the 44 compounds tested (**Figure 2**, **Supplementary Table 2**). In general, the high-sorbing compounds reached 100% sorption (relative to the non-mineral control for each metabolite) at lower mineral:TOC ratios in the standards mixture compared to the bacterial lysates (*e.g.* phosphate-containing compounds, aspartate, glutamate and succinate). Compounds that did not sorb (but did appreciably in the

bacterial lysates) were 2'-deoxyuridine and 5-methylthioadenosine. These differences may be a result of these compounds being more abundant in the standards mixture compared to bacterial lysates based on comparison of peak areas (although absolute quantities of the metabolites were not measured).

### *3.3. Phosphate desorption from the mineral surface*

A subset of the most sorptive metabolites was analyzed for their ability to desorb phosphate from the mineral surface. After a 24-h phosphate-loading period, on average, 75% of the added phosphate was sorbed to the mineral surface. Once metabolites were added, those that released the most phosphate (more than 10%) over a period of 24 h included most of the phosphate-containing compounds tested (inosine monophosphate, cytidine monophosphate, adenosine monophosphate, uridine monophosphate, glucose 6-phosphate) and the positive control, citrate (not used in our microbial lysate sorption experiments, but shown in previous studies to desorb phosphate (Johnson and Loeppert, 2006)) (**Figure 3**). Others that resulted in detectable phosphate release, but less than 10%, were glutathione and some phosphate-containing metabolites, NADH, coenzyme A and FAD while fumarate, raffinose, adenosine and 5-methylthioadenosine did not cause significant phosphate desorption (**Figure 3**). It is very interesting to note the wide range of phosphate desorption caused by phosphate-containing metabolites. Not all of the phosphate-containing metabolites were highly efficient in releasing phosphate. Deionized water was included as a control and did not release detectable amounts of phosphate, verifying that phosphate release from other samples was metabolite-dependent.

## 4. DISCUSSION

### *4.1. Mechanisms of metabolite sorption*

The focus of this study was on the sorption behavior of low molecular weight soil bacterium compounds (and only those detectable with our LC/MS method and analysis) rather than on high molecular weight compounds or polymers. The undetected metabolites and larger molecules have the potential to behave differently than what we observed here and may affect the sorption of the low molecular weight compounds. However, the general patterns of sorption were similar between the two experimental systems examined (the complex bacterial lysates versus the simpler standards mixture) indicating that the effects of the undetected compounds appear to be minor.

Organic matter-mineral interactions are complex; typically involving a combination and layers of electrostatic interactions, covalent bonding, hydrogen bonding and hydrophobic bonding resulting in inner-sphere and/ or outer-sphere surface complexes (Kleber et al., 2007; Sutton and Sposito, 2005). Characterizing these specific interactions would require extensive further investigation typically achieved by the use of attenuated total reflectance Fourier transform infrared spectroscopy or x-ray absorption spectroscopy (Arai and Sparks, 2007; Persson and Axe, 2005; Yeasmin et al., 2014). However, predictions can be made on mineral surface interactions based on our experimental conditions and observations made in previous studies.

Iron oxides have a variable charge (pH-dependent) surface resulting from the iron metal center and surface hydroxyls. The point of zero charge for Si-free ferrihydrite is 7.6 (Schwertmann and Fechter, 1982) so at our experimental pH $\leq$ 7.2 it can be assumed that the aged ferrihydrite has a net positive charge. The positive charge on the iron oxide surface and negative charge of phosphate-containing microbial metabolites and carboxyl groups would

induce electrostatic attraction. One proposed mechanism is that phosphate-containing compounds in the bacterial lysate may form phosphate-Fe bonds via a ligand exchange mechanism (by displacing surface hydroxyls), a mechanism observed with goethite (Omoike and Chorover, 2006). However, in many cases, this is usually accompanied by a large increase in pH as hydroxyls are released into solution (Parfitt et al., 1977), which was not observed here, suggesting that this is not the primary mechanism of sorption. Our results agree with previous reports of anionic compounds such as carboxylates sorbing more readily to soil and mineral surfaces (Swenson et al, 2015) with the degree of sorption increasing with the number of dissociable carboxyl groups (Yeasmin et al., 2014). This previous study is also in line with our observation of alanine, phenylalanine and lysine not being preferentially sorbed relative to other carboxyl-containing compounds (Yeasmin et al., 2014). Phenylalanine did sorb slightly better compared to alanine and lysine, potentially due to the presence of the aromatic side-chain.

### *4.2. Sorption patterns and behavior of microbial metabolites*

For individual metabolites, three distinct sorption patterns relative to mineral concentration were observed as shown by figures 1 and 2: linear (sorption steadily increased with mineral concentration), saturation (sorption rapidly increased to 100% with mineral concentration) and on-off-on (sorption at low and high mineral concentrations, but not at medium concentrations). A saturation-type pattern was displayed by all phosphate-containing metabolites as shown by a rapid increase in sorption (at low mineral concentrations) then a plateau at medium-to-high concentrations, presumably as all mineral binding sites (capable of binding these particular metabolites) became occupied at a given mineral concentration. The third pattern, observed with 12 out of the 14 detected amino acids, was the on-off-on pattern with

a small amount of sorption occurring only at the lowest and highest mineral concentrations. The detected amino acids represent a nice set of structurally similar compounds that also vary in several key properties, *e.g.* charge and hydrophobicity, and therefore the comparison of the their behavior can provide insight into the experiment as a whole and merit some further discussion. Since our experimental conditions were below the point of zero charge for ferrihydrite, the surface should be slightly net positive initially or at low surface loadings. Not surprisingly therefore, the two amino acids that sorbed 100% at the lowest surface loading of organics, *i.e.* 32 mg ferrihydrite, were aspartate and glutamate which are also the only two that are strongly negatively charged at pH 6.8 with pIs of 2.8 and 3.2, respectively. The two that didn't sorb at all at 32 mg ferrihydrite treatment were arginine and lysine which are also the two that are positively charged at pH 6.8 (pIs of 10.8 and 9.7, respectively). The other 10 amino acids are all slightly negative at pH 6.8 (pIs 5.5-6.3), and sorbed less than the negative ones and more than the positive ones, *i.e.* between 20-80% at 32 mg ferrihydrite. This range shows that charge is not the only factor impacting sorption, but is likely a key one. At the highest surface loading of organics (0.5 mg ferrihydrite), the mineral surface is expected to be increasingly less positive or potentially negative and more hydrophobic because of more organics on the surface (Weng et al., 2006), and the result is that the two positive amino acids, lysine and arginine, exhibit sorption (4% and 13%, respectively). Many of the slightly negative amino acid also sorb under these conditions, potentially due to the ferrihydrite surface also being more hydrophobic at high surface loading of organics. For example, tryptophan and phenylalanine, two of the most hydrophobic amino acids, sorb 8% and 9%, respectively. The fact that so many of the amino acids show a minimum at intermediate ferrihydrite concentrations is likely a result of the changing importance of competitive sorption and changing surface characteristics, *e.g.* charge

and hydrophobicity as a function of surface loading of organics. These results highlight the situational dependence of organic sorption on minerals surfaces and emphasize the potential of microbial strategies that could change surface behavior in a way that benefits an organism.

Overall, our results suggest that in iron oxide-rich soils, metabolites such as ectoine, lysine, uracil and disaccharides (*e.g.* maltose and trehalose) may be readily available for microbial processing and cycling. Many of these compounds are known to be commonly utilized by a variety of microorganisms (Fredrickson et al., 1991). The availability of these favored metabolites is often central to the survival of terrestrial microbes especially in harsh environmental conditions. For example, in addition to being important carbon sources, ectoine and trehalose are common microbial protectants against changes in salinity and temperature (Reina-Bueno et al., 2012).

### *4.3. Mechanisms of phosphate sorption and exchange with microbial metabolites*

Phosphorus (both organic and inorganic forms) plays an essential biogeochemical role for all living organisms and therefore an understanding of its behavior and retention in soils is crucial. Phosphorus behavior is complex and depends on many environmental factors including iron and aluminum oxide content, pH and organic matter composition (Arai and Sparks, 2007). Ferrihydrite, compared to other iron oxides (*e.g.* goethite and hematite), possesses larger and more rapid phosphate sorption properties probably due to its higher surface area density (Wang et al., 2013). When the environmental pH is below the point of zero charge (which was the case for our phosphate experiments), the primary mechanism of phosphate sorption, as with negatively-charged metabolites, is via electrostatic interaction (Arai and Sparks, 2007). The mechanism of (inner-sphere bound) phosphate release from iron oxide surfaces by organic acids

391 is known to occur by two main mechanisms: ligand exchange and ligand-enhanced dissolution of
392 minerals (Johnson and Loeppert, 2006). Here we examined the ability of high-sorbing microbial
393 metabolites to control phosphate bioavailability rather than to determine the mechanism.
394
395 ***4.4. Microbial metabolite release as a potential mechanism to mobilize phosphate***
396 Given the observed sorption of many metabolites onto the mineral, we wondered if some
397 of these displace phosphate. While there is much evidence of plants releasing organic acids to
398 obtain access to limiting nutrients such as phosphate (Gahoonia et al., 2000; Johnson and
399 Loeppert, 2006; Vance et al., 2003), it seems that the role of metabolite release for this purpose
400 by microbes is less understood. Phosphate-solubilizing microbes (bacteria, fungi, actinomycetes
401 and cyanobacteria) are being explored as a means to solubilize phosphate for plant nutrition and
402 such strains can constitute as much as 50% of the total soil bacteria population (Johnson and
403 Loeppert, 2006; Sharma et al., 2013). Production of organic acids and chelation of mineral ions
404 by these compounds, rather than acidification, appears to be a more effective mechanism for
405 phosphate solubilization from iron or aluminum minerals (Barea and Richardson, 2015).
406 However, most research to date has primarily focused on microbial production of organic acids
407 such as gluconate, citrate, succinate and oxalate (Barea and Richardson, 2015; Illmer et al., 2003;
408 Rashid et al., 2004; Whitelaw et al., 1999), rather than on other potential phosphate-solubilizing
409 small molecules.
410 Here, we examined the most sorptive metabolites and found that of the compounds
411 tested, the ones that released the most phosphate into solution were the phosphate-containing
412 metabolites. Although this would not result in a net gain in phosphate for the microbe itself, one
413 can hypothesize that microbes that are less phosphate-dependent may release these compounds

for the benefit of symbiotic plants (Zaidi et al., 2009) or microbes. Some non-phosphate metabolites (glutathione and citrate) did displace small amounts of phosphate as indicated in our study. A further area of exploration would be to examine more non-phosphate metabolites such as these for their ability to displace phosphate from ferrihydrite or other minerals. Additionally, exometabolomics studies (Silva and Northen, 2015) would be required to confirm the release of metabolites from microbial isolates in support of possible phosphate- (or other oxyanion-) mobilizing mechanisms.

## CONCLUSION

Understanding SOM turnover and mineral interactions of this complex pool of small molecules is essential to understanding soil carbon and nitrogen dynamics and these processes greatly influence microbial community activity and structure. Exploring single metabolite or general SOM sorption is difficult to translate to *in situ* conditions, hence our focus was on a microbial metabolite mixture with an iron oxide mineral. Ferrihydrite is a common soil mineral and is known for its large surface area and generally high reactivity. Iron oxides, and ferrihydrite in particular, are important in the creation of organic matter-mineral associations that are thought to impact the long-term stability of carbon in many soil systems and in controlling nutrient concentrations in soils. Our findings reveal that phosphate-containing and dicarboxylate metabolites strongly sorb to the mineral and thus are unlikely to be readily available for SOM cycling and microbial processing in iron oxide-rich environments, but important osmolytes such as ectoine and disaccharides were found to be less sorptive and more available. Furthermore, the highly-sorptive metabolites identified here mobilize phosphate from the iron oxide mineral surface and may allow microbes to gain access to limiting oxyanion nutrients. Overall, these

results will aid in our understanding of how specific mineral and chemical factors of a given environment may affect SOM dynamics.


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## FIGURES

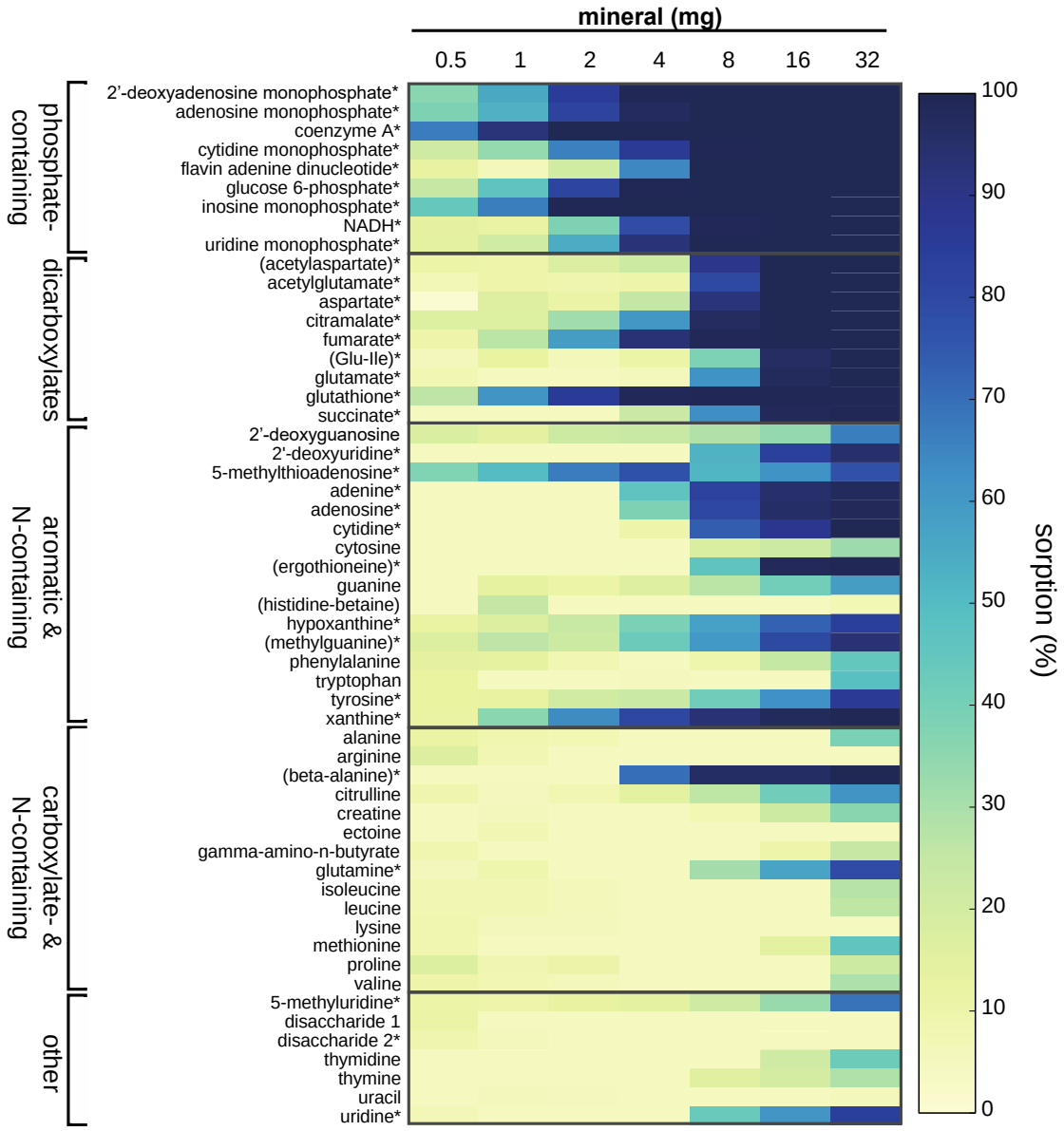


**Figure 1. Mineral sorption of microbial metabolites from *Pseudomonas stutzerii* RCH2 lysate.** For each metabolite, the percent sorption (relative to the non-mineral control) is displayed as mineral concentration is increased from 0.5 to 32 mg. Phosphate-containing metabolites and dicarboxylates were preferentially sorbed. Metabolites not exhibiting any sorptive activity were ectoine, lysine, disaccharides and uracil. Putatively-identified metabolites are indicated by

parentheses. *n*= 5 for each mineral concentration. *$p < 0.05$ for at least one mineral concentration relative to the non-mineral control.

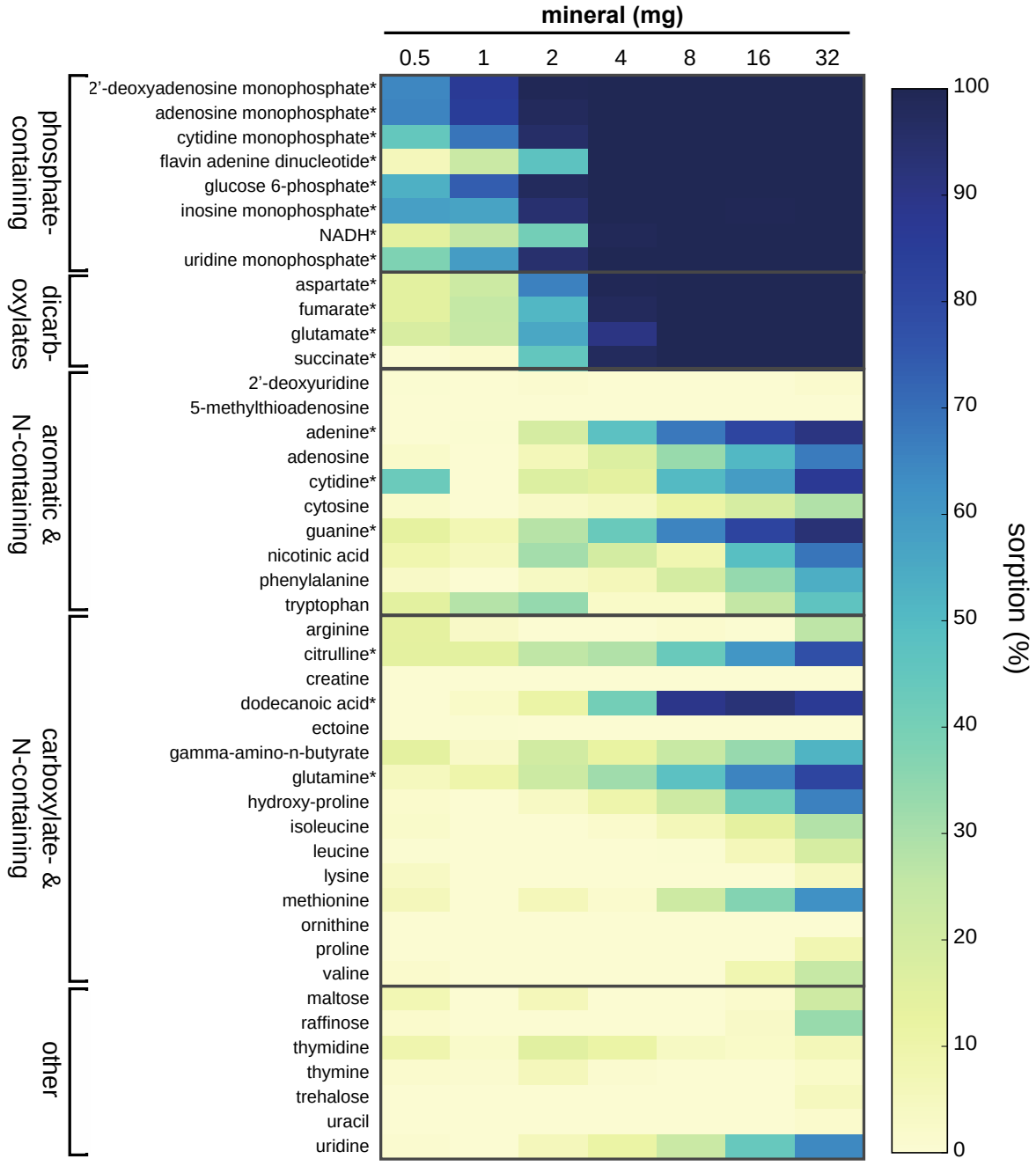

**Figure 2. Mineral sorption of the standards mixture.** For each metabolite, the percent sorption (relative to the non-mineral control) is displayed as mineral concentration is increased (from 0.5 to 32 mg). Phosphate-containing metabolites and dicarboxylates were preferentially sorbed as observed in the RCH2 bacterial lysate experiment. Metabolites not exhibiting any sorptive activity include 2'-deoxyuridine, 5-methylthioadenosine, ectoine and ornithine. *n*= 5 for each mineral concentration. *$p < 0.05$ for at least one mineral concentration relative to the non-mineral control.

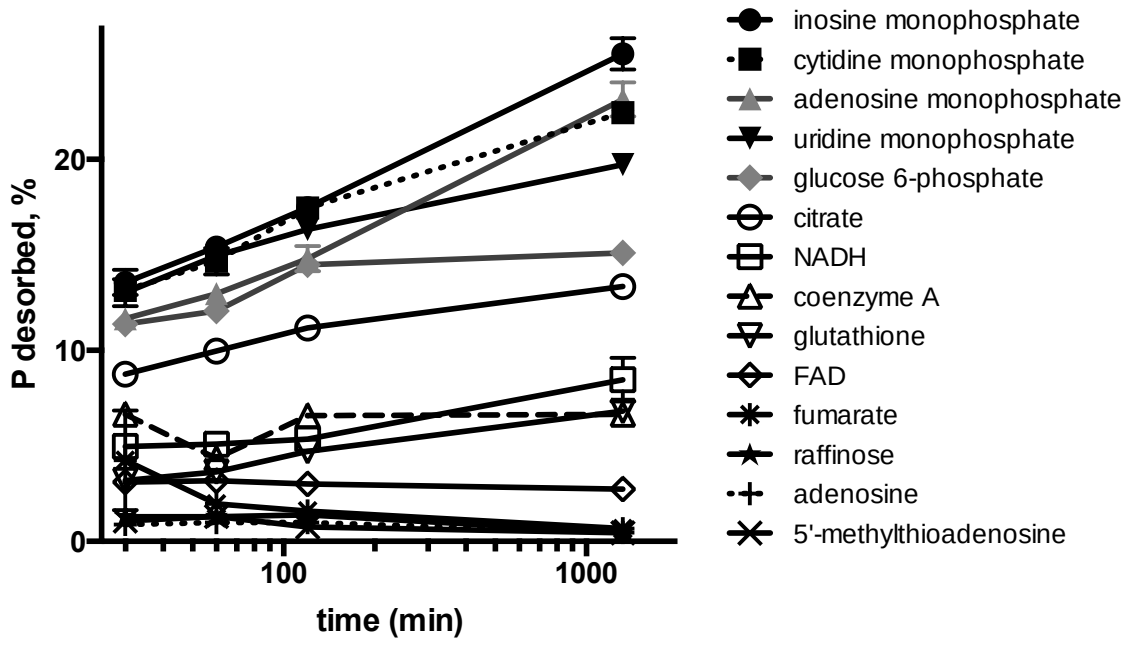


**Figure 3. Metabolite-mediated phosphate desorption from the mineral surface.** The mineral was pre-loaded with phosphate (0.25 mM $KH_2PO_4$) for 24 h. After metabolites (2.5 mM) were added, phosphate desorption was monitored for 24 h. Many phosphate-containing metabolites and glutathione mobilized the most phosphate. *n* = 3 for each metabolite.