Title

Studies on the Technical Challenges of Urine Source-Separation Technology and its Microbial Ecology

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Studies on the Technical Challenges of Urine Source-Separation Technology and its Microbial Ecology

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Executive Summary

Waterless and low-flow urinals offer a way to minimize potable water consumption and collect urine for downstream nutrient recovery; however, its practicality and social acceptability is hindered by technical problems such as pipe fouling. Understanding the biology of the urine drainage systems and factors that influence key reactions will provide insight when developing treatment methods to prevent pipe fouling in urine drainage systems. To understand the biomineral fouling, a four-part study was developed which consists of (1) a literature review, (2) biogeographic microbial ecology study, (3) a multiple regression study to determine the influencers of biomineral urease activity, and (4) an exploratory study on the use of an agricultural urease inhibitor, n-butyl thiophosphoric triamide, to minimize biomineral formation in urine source-separation systems.

A literature review was performed to understand the causes of biomineral pipe fouling and to evaluate current proposed solutions to this issue. By analysing case studies on full-scale urine source-separation projects, it is shown that many currently proposed solutions such as acid and caustic treatments have been embellished by its proponents. Treatments such as electrochemical and pH-dependent urine stabilization techniques had impracticalities or a potential to reduce nutrient recovery efficiencies that were previously undiscussed. On the other hand, the use of n-butyl thiophosphorotriamide (NBPT), which is commonly used on an agricultural scale to inhibit ureolysis in soils, has not yet been investigated in urine source-separation contexts.

That biomineralization and odours are microbially-driven processes has also motivated the microbial ecology component of this study. This study is novel as there have been no previous attempts at studying the microbial ecology of the biomineral precipitates found in

waterless, low-flow, and conventional urinal drainage systems. The objective of this study was to examine the total bacterial community structure and diversity associated with the ureolytic biomineralization from urine drainage systems. In total, 11 California Department of Transportation public restrooms fitted with waterless, low-flow, or conventional urinals were studied in 2019, resulting in 169 biomineral and urine samples collected. By pairing high throughput 16S rRNA Illumina MiSeq sequencing with multivariate statistics, significant differences were found between the bacterial communities and alpha diversities between urinal types. Moreover, it was found that waterless urinal samples were dominated largely by the taxonomic class Bacilli at 86.1% and had the fewest rare (< 2.5% relative abundance) operational taxonomic units at the genera level. It was also shown that waterless urinals, which were the least diverse of the three urinal types observed, also had the greatest average *ureC*/16S gene ratio determined by quantitative polymerase chain reaction. The *ureC* gene is of interest as it is the subunit alpha of the gene encoding urease production. The elevated *ureC*/16S gene ratios in waterless urinals relative to the other urinal types suggests that of the three urinal types, waterless urinals host the largest potentially-ureolytic community by proxy. Future efforts to sequence the *ureC* gene to identify the ureolytic community are needed.

Complementing the microbial ecology study is a physiological component on the urease enzyme activity of 55 biomineral samples collected in 2019. A complete-case analysis, multiple regression model was developed and validated to determine significant influencers of urease enzyme activity of the biomineral samples. It was found that the intrasystem sampling location, or where one sampled within a single urine drainage system, was a significant predictor of biomineral urease activity. More so, the organic/inorganic mass fraction and annual users per rest area were also significant predictors of biomineral urease activity. Conversely, *ureC* gene

abundance, urinal type, and sampling season were not significant predictors of biomineral urease activity. It is concluded that the abundance of the urease gene does not necessarily mean that the gene is being expressed and that there were unmeasured factors in the study that may have influenced gene expression, protein production, and ultimately urease activity. Future studies should incorporate reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) to determine the factors that influence the transcription of genes responsible for urease production.

The urease inhibitor, NBPT, was also investigated for its potential use in urine-source separation settings. In an experiment consisting of batch tests, model glass reactors, and a full-scale component at the Dunnigan northbound rest area, NBPT obtained from commercially produced Agrotain Ultra has exhibited strong inhibitory properties that commercially-available urinal hygiene products lack. More so, field studies yielded evidence that the biomineral accumulation in a sedimentation tank downstream of the waterless urinals decreased compared to a control period when waterless urinals were treated with a custom NBPT urinal cake. Altogether, these findings suggest that novel applications of NBPT in the operation and maintenance of waterless urinals could reduce fouling and odors while increasing nutrient recovery.

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CHAPTER I

Review of Urine Stabilization to Overcome Technical Challenges in Urine Source-Separation and Nutrient Recovery

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Declarations

Availability of data and material

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Authors' contributions

Kahui Lim developed the text and tables. Harold Leverenz developed figures and text.

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Abstract

Waterless urinals and urine diverting toilets are key source-separation technologies for the collection of urine to be used in downstream resource recovery systems. However, waterless urinals are prone to issues such as clogging and ammonia volatilization, which result in low user and maintenance staff satisfaction. While previous small-scale experiments on source-separation maintenance offer insights on preventative measures and treatments, considerations on the technological feasibility of treatments, such as acid treatments and caustic stabilization, are often neglected. The topics discussed in this review are (1) an examination of the technical challenges that detract from overall usability of waterless urinals, and (2) an evaluation of current proposed solutions related to the operations and maintenance of source-separation systems at the user interface. Based on this review, we recommend further research on strategies such as ureolytic inhibitors that minimize odors and clogging of urine source-separation technologie**s** at the point of collection**.**

1. Introduction

When paired with urine nutrient recovery systems, the separation of urine from other wastewater sources using urine diverting toilets (UDTs) and waterless urinals has important implications for urban development, water quality, and sustainable food production systems. However, while nutrient recovery can be a step towards a paradigm shift from disposal to reuse, the preceding step of collecting large volumes of urine via source separation has several challenges. In urine source-separation systems, problems such as clogging caused by biomineral formations and ammonia volatilization complicate maintenance, increase operational costs, and

diminish downstream nutrient recovery efficiency. The objectives of this review are to examine technical challenges that detract from overall usability of waterless urinals using case studies and to critically examine the current proposed solutions related to operations, maintenance, and the design of systems.

The potential benefits of source-separating urine are well documented [1, 2, 11, 3–10], but just as important to discuss are the failures of source-separation technologies using pilot and full-scale projects and the inadequacy of current proposed solutions. If overall user perceptions on source-separation technologies remain low due to technical challenges and if poor user experience diverges with what is advertised, then making source-separation technologies a cultural and household norm will be difficult. Achieving social legitimacy is difficult because the technical and social challenges are often interlinked; technical challenges decrease social acceptance by inconveniencing users and maintenance staff.

Finally, discussing the shortcomings of various urine collection technologies and treatments will yield insight on the scalability and practicality of both. While fouling is commonplace, the current fixture designs and solutions for managing urine chemistry are often inadequate in preventing and removing biomineralization, impractical due to cost and transportation requirements, and pose safety issues. Even if some solutions work in theory, researchers often fail to factor the implications of cost or downstream nutrient recovery losses that can detract from the benefits of urine source-separation.

1.1 Causes of clogging in urine drainage systems

Biomineral pipe fouling and the design flaws of both urinals and drainage systems contribute to negative user perceptions and complicates downstream nutrient recovery [12–14]. Ureolytic bacteria responsible for biomineralization use urease, a nickel-dependent metalloenzyme, to catalyze the hydrolysis of urea into ammonia and bicarbonate [15]. The

production of ammonia subsequently raises the pH and creates conditions favorable of precipitation and ammonia volatilization [15]. Cumulatively, biomineralization is a major cause of clogging and odors.

Biomineralization in urine drainage systems has been described as a viscous sludge that forms at the pipe invert a and solid formations that are caused by precipitation directly on the pipe wall [16–18]. [16–18][16–18]Biomineralization in urine source-separation contexts is likely governed by a combination of multiple mechanisms; medical researchers posited that no one theory of pathogenesis can properly account for urinary stones as they are too varied and their formation too complex for simple understanding [19]. Researchers describe biomineralization in terms of three mechanisms: (1) biologically controlled mineralization, which describes cellular activities that direct the formation of minerals and control crystal nucleation and growth [20], (2) biologically influenced mineralization or passive formation of crystals caused by cell surface organic matter such as biofilms [21, 22], and (3) biologically induced mineralization caused by chemical modification of an environment by biological activity that results in supersaturation and spontaneous precipitation of materials [23]. In general, however, urinary stone formation has been described as a four-step process: (1) supersaturation, (2) nucleation, (3) crystal growth, and (4) aggregation [15]. A figure summarizing key points in urinary biomineralization is shown in Figure 1.

Supersaturation in human urine is made possible by urinary ion content and ureolysis. When supersaturation of stone salts is reached, the ion activity product is greater than the ion activity product at equilibrium (K_{sp}) and spontaneous crystal formation in the bulk fluid becomes possible [24]. For struvite formation, supersaturation is satisfied when there exists a presence of solutes, namely magnesium (Mg^{2+}), phosphate (PO_4^{3-}), and ammonium (NH_4^+) ions for urine, in

a solution greater than its own solubility [24]. Urinary urea is also the primary source of carbonate (CO_3^{2-}) in carbapatite formation [15, 25].

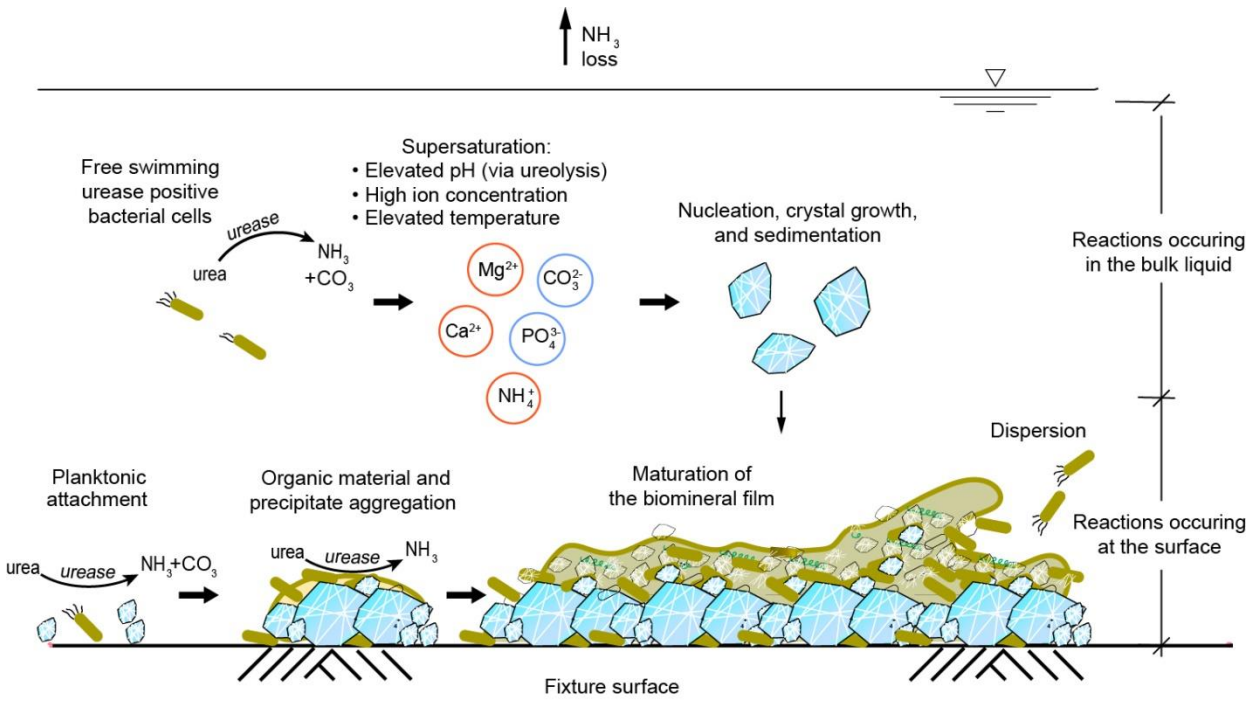

Figure 1: A summary of key biofilm and biomineralization formation processes discussed in this section is shown. Biomineralization in urine drainpipes is a complex interplay between microbial action and urine chemistry. Ureolysis facilitated by the ureolytic community enables conditions favorable for mineral precipitation that eventually clogs pipes.

1.2 Ammonia volatilization of urine

Ammonia loss by volatilization in a urine drainage system is problematic as it reduces the potential yield for downstream nutrient recovery and is a strong contributor of odors associated with urine separation systems. Ammonia volatilization from urine occurs in a high pH environment, especially when the pH is greater than the pKa of ammonium [26]. While the initial pH of fresh human urine is near neutral and nitrogen is primarily in the form of urea, microbially driven ureolysis raises the pH immediately after fresh urine contacts the

biomineralization [27]. Enzymatic decomposition of urea into carbonic acid and gaseous ammonia is initiated when fresh urine contacts ureolytically active cells and biomineral deposits in urine drainpipes or storage tanks. An analogous example of this ureolysis mechanism occurs when fresh urine contacts manure or soil in agricultural contexts [28] The *in situ* rates of ureolysis, pH change, and ammonia volatilization in urine drainage systems have not yet been determined and needs to be further investigated.

Ammonia volatilization coincides with the depletion of titratable alkalinity [NH_3 (aq), HCO_3^-, CO_3^{2-}], which reduced potential for downstream nutrient recovery rates [26]. The depletion of solution alkalinity coincides with volatilization because the rate law of ammonia volatilization is dependent on pH and alkalinity. For example, ammonia air stripping researchers have observed that as alkalinity of the wastewater leachate is decreased, the ammonia recovery rates decreased, signifying a loss in recovery efficiency [29]. Campos et al. (2013) concluded that the treatment of landfill leachate containing high ammonia and alkalinity concentrations is feasible without the addition of an alkali. The treatment of feed urine with depleted or reduced alkalinity would only be feasible if it is externally supplemented with alkali at a cost penalty. The dependence on ammonium and alkalinity concentration poses a challenge for low-flow urine source separation technologies. Minimizing ammonia volatilization is important for maximizing the potential for nitrogen recovery, however, the use of acids (see Section 3.2) to lower urine pH during collection will increase the need for downstream alkali addition to facilitate ammonia stripping.

2. Technical challenges of urine drainage systems observed in field studies

In recent decades, urine diversion has been attempted in settings such as apartment complexes, office buildings, and universities and share the same fundamental challenges [2, 12–14, 16, 30]. The problems contributing to the usability and practicality of urine source-separation are discussed in this chapter.

2.1 Biomineralization in urine drainage fixtures and plumbing

Biomineral formation in urine drain lines presents maintenance complications and leads to poor ratings amongst users in terms of cleanliness. Biomineralization blockages have plagued large-scale urine diversion projects since the earliest projects were studied [2, 10, 16].

In a study involving two apartment complexes in Sweden fitted with UDTs, Lindgren (1999) surveyed tenants of each area and found that 100% of tenants who inhabited each area for at least 6 months saw clogging in the urine traps [12]. Lindgren (1999) observed that biomineralization had reduced the cross-sectional areas of urine drain lines by up to 75% [12]. These blockages require laborious cleaning procedures involving cleaning wires and harsh chemicals such as strong acids and bases that led to the overall dissatisfaction of the toilets for both residents and property managers. More recently, Blume & Winker (2011) documented three years of operating UDTs and waterless urinals at the Deutsche Gesellschaft für Technische Zusammenarbeit (GTZ) office building in Eschborn, Germany [14]. At this office, fifty urine diverting NoMix toilets by Dubbletten were installed to accommodate 400 people, using 1-2 L for the urine flush and 4-6 L for the solids flush. Additionally, 25 waterless urinals were equipped with a flat rubber tube acting as a check valve to prevent odors. Blume & Winker

(2011) also found that to prevent fouling of urine drain valves, overnight organic acid soaks and cleaning were required monthly.

Not only do frequent clogs lead to cleaning difficulties, but the biomineralization in the pipes also contribute to odors that detract from the user experience and subsequent negative perception of waterless urinals. Because the biomineral fouling of the urine drain valves prevented proper sealing in the UDTs, the smell of urine and any ammonia volatilized would then emanate from the toilet. In Blume & Winker's (2011) study, of 88 respondents, 60% found that waterless urinals were worse in terms of odors than conventional systems as did 50% of 218 for the UDTs [14]. Similar results are found in Lindgren's (1999) study of Swedish residences. In Lindgren's (1999) study, most residents concluded that urine source-separation would not be viable until the cleanliness and functionality of the toilets that were compromised by mineral deposits were addressed.

Biomineral clogs pose problems for the efficiency of downstream treatments. The accumulation of urine precipitates, pubic hair, and biofilm deposits in the urine drain valves led to flush water entering the drain lines during flushes in Blume & Winker's (2011) study.
A diluted urine stream would require greater nutrient recovery system energy and processing requirements than would be required when processing a concentrated urine feed.
Biofouling of source-separation technologies also limits system configurations and the use of mechanical devices that might be used in treatment systems. Abeysuriya et al. (2013) noted that pumps are not typically a part of urine diversion configurations as struvite deposits have the potential to seize moving parts [13]. As such, biomineralization is problematic for those who propose to use complex decentralized urine nutrient recovery systems. For example, electrochemical urine stabilization systems, as later discussed in Section 3.4, require pumps,

tubing, membranes, and electrical components that may experience fouling as these components will be in contact with contaminated urine when feeding the system.

2.2 Ammonia volatilization leads to nutrient losses and odors in urine drainage systems

Ammonia volatilization reduces the nutrient recovery potential and presents odor issues in full-scale urine source-separation systems. When studying the source-separation systems at the GTZ headquarters, Blume & Winker (2011) reported that the urine storage tanks had low nitrogen concentrations of 2800 mg/L on an average day, which was two-thirds less than that of expected literature values of 8,000 mg/L [14]. They suspected that ammonia volatilization led to losses through the storage tanks' ventilation systems. Similar observations of nutrient losses and odors due to ammonia volatilization were observed at an office in Dubendorf, Switzerland, where the urine source-separation drainpipes vented to the roof at the offices [18]. At this test site, Goosse et al. (2009) reported minimal odor problems and blockages caused by struvite fouling, but found that ammonia emissions and odors were significant at the roof vent. By measuring the diurnal air flow and ammonia concentrations of the vented air, Goosse et al. (2009) confirmed via mass balance that 44% of the ammonia in the urine was lost through ammonia volatilization [31].

2.3 Ongoing evolution of design and construction experience

Few improvements in drainage system design have been made since the first urine diversion projects debuted three decades ago. Today, waterless urinals in highway rest areas in California clog and have odor concerns despite regular cleaning using manufacturer recommended products and methods [32]. The ineffectiveness of commercial products was also documented in a study described by Lindgren (1999) [12]. These problems remained unsolved in

full-scale systems installed as recently as 2013, further suggesting that the overall design and maintenance procedures of source-separation technologies continue to be inadequate [13, 14].

Standard guidelines of UDTs and waterless urinals have yet to be developed. Pipe system design is an area that needs additional study for several reasons. Factors that can contribute to biomineral accumulations include pipe roughness, pipe material, presence of pipe corrosion, slope, and discontinuities in the drainpipes between fittings, but their effects on urine drainage system operation have not yet been elucidated. For example, discrepancies are reported for pipe slope values in attempts to minimize biomineral clogs. Abeysuriya et al. (2013) recommended a 1.5% slope, while in Palsternackan, Sweden, a 1% pipe drainage slope and minimal 90^{o} bends had zero clogs during 9 years of operation for 51 apartments with urine diverting toilets [13, 33]. Kvarnstrom et al. (2006) found that a 1% slope was sufficient to drain the viscous biomineralization while acknowledging that blockages can still occur. Others believed that a 0.5% slope was sufficient, but acknowledged that crystallization still occurred in a dry sanitation system [34]. Larsen et al. (2007) recommended that pipes that have a steep slope (at least 2-5%), have no tight bends or inaccessible sections, and have a large diameter (65-110mm) would best prevent a gradual accumulation of biomineralization in the drain lines such as that shown in Figure 2 [2]. Various factors such as climate conditions, diets, plumbing practices, maintenance schedules, and usage may influence pipe gradient requirements, but the discrepancies between many studies demonstrates that further research on an optimal drain line slope to minimize biomineralization is required. Moreover, the effects of toilet and plumbing design on long-term performance of UDT systems are also unknown. No urine drainage pipe slopes have been found to be adequate at minimizing biomineralization in all situations, and it would be difficult to develop guidelines and design standards without further research.

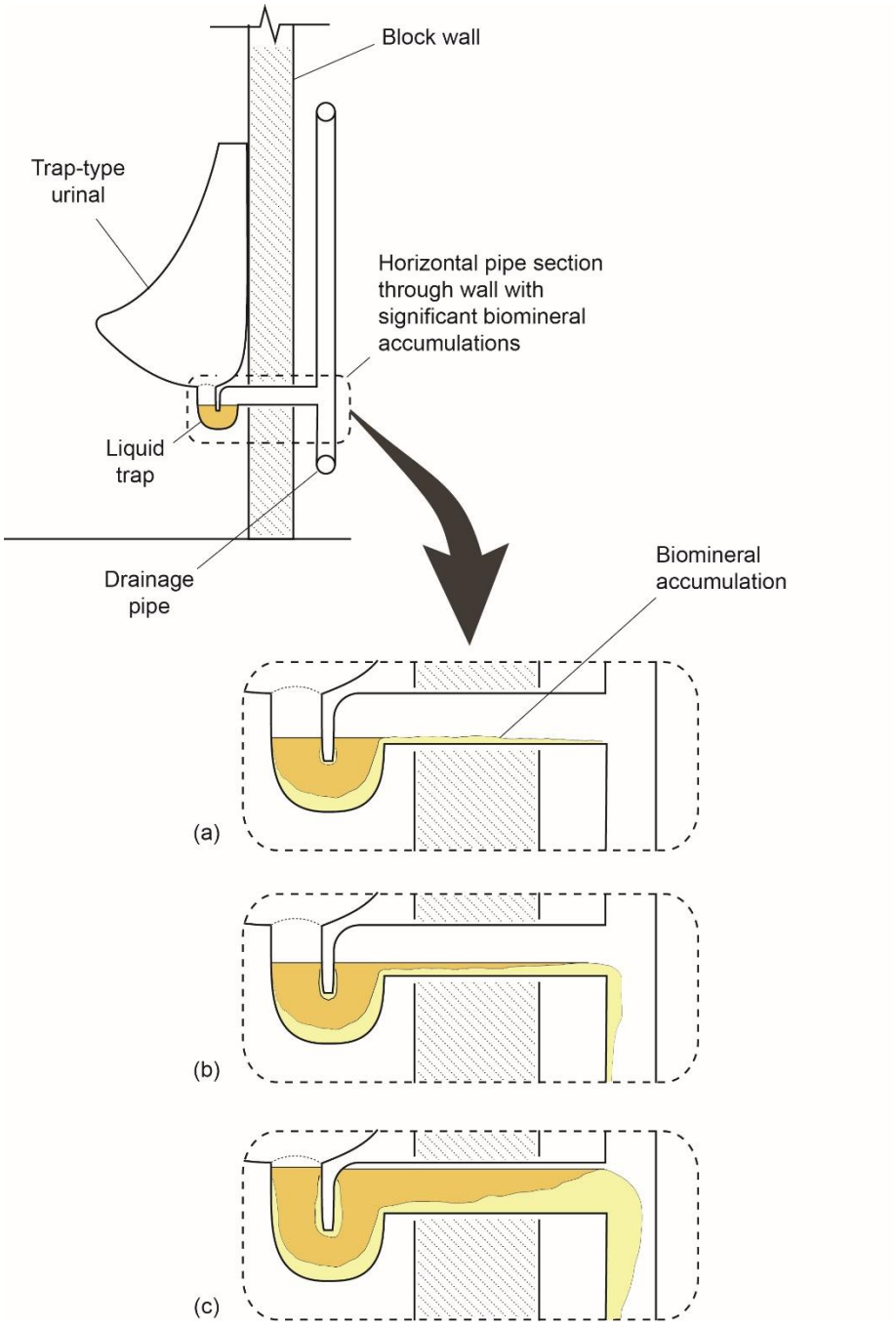

Figure 2: Cross section view of urinal showing progressive buildup at the horizontal pipe section due to surface tension and biomineral dam formation: (a) after initial colonization of new urinal, typically about 1 month, (b) following colonization, fresh biomineral deposits accumulate and are transported to joint where a dam begins to form, and (c) after many months of operation, biomineral dam has nearly clogged drainpipe at downturn.

Lessons for appropriate designs can be found by studying failed urine source-separation projects. Lack of design standards and poor workmanship culminated in the failure of a full-scale urine source-separation project known as the China-Sweden Erdos Eco-town Project (EETP) [35]. Within nine months of operation, residents at the 832-unit complex were complaining of a persistent odors, which were found to be caused by poor installation quality or lack of experience in designing dry sanitation systems [34]. The odors persisted due to improperly sealed urine storage tanks and because some tank inlet pipes were fitted above the surface of the urine, causing volatilized ammonia in the tank to permeate into the bathroom [10]. Zhu (2006) also noted that more than half of the ventilation fans at the EETP were not installed properly [34]. Within three years of operation, the system had experienced mechanical failure of the toilets, unacceptable persistent odors, and biomineralization in the odor traps [35]. Technical complications ultimately led to the rejection of urine source-separation systems by EETP residents, and the management was pressured into retrofitting the system with conventional waterborne sewerage [10]. The original dry toilets were switched to conventional water-based sewage in 2009 after three years of operation [35].

The detraction of the technology's social legitimacy resulting from its technical challenges are best illustrated through community surveys, which show that people expected the convenience, hygiene, and functionality of conventional systems that dry sanitation systems had yet to achieve [12]. Although survey respondents acknowledged the environmental benefits of the dry system and periodic retrofits and upgrades were made, tenants remained dissatisfied [10]. Residents noted that they preferred flush toilets to dry systems during household surveys, and that the dry systems were not acceptable in the long term unless further improvements addressing odors and hygiene were made—92% of those interviewed at the EETP would not recommend the

system and were dissatisfied overall [35]. In 2008, residents boycotted service fees for maintenance of the buildings, and the overseeing committee decided that the dry toilets were to be replaced with conventional flush systems [35].

Even once adequate design standards have been identified, it may take time for users, designers, contractors, and maintenance staff to adapt to installing, using, and maintaining the new technologies. In Abeysuriya et al.'s (2013) study, a Dubbletten UDT was installed incorrectly, but it was not until after 11 months that this was realized and corrected [13]. The plumbers realized that the manufacturer's instructions were not followed precisely and that the problem was attributed to an s-bend odor trap in the pipework required of previous UDTs tested, but was incompatible with the unit being evaluated [13]. The researchers articulate the need to engage closely and respectfully with the plumber who follows a plumbing code not yet updated to accommodate UDT installations [13]. In sum, the technological immaturity of urine source-separation systems marked by the lack of design standards culminates in failed projects and casts negative perceptions on the technology. A summary of observed design problems is shown in Table 1.

Table 1: Summary of Design-related Complications for Waterless Urinals and UDTs

Challenge	Consequences	References
Unintuitive urinal/toilet operation	Toilets not used as intended. For example, NoMix toilets not being sat on results in diluted waste stream	[14, 18, 36]
Lack of cleaning accessibility	Clogs and the inability to efficiently clean out clogged pipes.	[12]
Splashing	UDTs with two compartments often have splashing from the urine to feces compartment, resulting in the need for flushing of both compartments, defeating the purpose of UDTs.	[12]
Lack of Design Standards and General Experience	Optimum configuration yet to be realized. Uncertainties in ideal conduit slopes and material choice to minimize biomineral clogs. Poor building quality contributing to failures of large-scale projects. Lack of ventilation leading to odor issues.	[2, 10, 13, 16]
Hygiene Concerns	Genital contact on the dividing wall in two compartment UDT; odors; perceptions on cleanliness	[12–14]
Inadequate Flushing Power	Weak flushes leaving fecal remnants in UDTs lead to a waste of water due to more flushes used	[12, 14, 18]
High Maintenance	Frequent cleanings and ineffective commercial treatments increase costs and frustration	[12–14]

2.4 Urine dilution and water waste due to toilet design

Efforts to reduce toilet flush volumes can increase the water saving and nutrient recovery potential of urine source-separation systems; yet, poor toilet designs can lead to wasted water and a diluted nutrient stream. Inadequate flushing power has been reported for years and recent studies show that it remains an unresolved problem [12, 14, 18]. One commonly discussed design is the NoMix UDT, which has a toilet bowl divided in two such that one compartment is

dedicated for urine while the rear is for feces. In Lindgren's (1999) survey on apartment complexes fitted with NoMix UDTs, up to 83% of respondents grouped by apartment complex found that the larger of the dual-flush options (4-6 L) was needed to remove the toilet paper used, signifying that the low volume flush (1-2 L) was inadequate [12]. Residents also reported that inadequate flushing power meant that multiple flushes are needed to accommodate large excretions [12]. Moreover, while the small flush is typically reserved for the compartment dedicated to urine, sometimes urine splashing into the feces compartment merited use of the full flush to prevent odors [14]. A 2011 study at the GTZ headquarters fitted with NoMix toilets also found that 39% of users needed two flushes after each use [14]. In other studies, double-flushing occurred in 21% of all uses and has contributed to a final urinary total nitrogen concentration that were 25-50% of expected concentrations [18, 36]. Manufacturers should address the inadequate flush and splashing by iterative design.

Other toilet design factors have also contributed to water inefficiency. Unlike the NoMix toilet, some designs do not have a separate urine drain valve and simply allow flush water to enter the storage tanks [13]. In Abeysuriya et al.'s (2013) study, various models of UDTs including waterless urinals from different overseas manufacturers were retrofitted in a male and female toilet block in a high-use area at the University of Technology Sydney [13]. In this study, the Wostman branded toilets allowed flush-water to enter the urine pipe every time it was flushed, making storage, handling, and collection of the urine more challenging due to dilution [13]. Toilets fitted with urine drain valves are useful in preventing dilution, but some toilet models like the NoMix have valves that divert flush water only when users sit on the toilet [14, 18]. Rossi et al. (2009) noted a low correlation between the total number of flushes per day and the amount of urine collected in the tank in a household experiment. The researchers concluded

that the weak correlation is due to problems caused by toilet design or lack of compliance by the residents because urine does not flow to the collection tank when the user does not sit on the toilet [36].

Proper cleaning procedures of urine diverting toilets may also be unintuitive. Blume and Winker (2011) found that explanations and demonstrations were required before training staff understood that flushing waterless urinals with water was undesirable, suggesting that improper cleaning procedures may have been commonplace [14]. The extra effort required to train staff is reflective of the greater cleaning efforts required to clean UDTs. In the China-Sweden EETP, 88% of users were found to have been adding water to the urinals and urine holes after every use to minimize odors [10]. In this case, the improperly trained users resorted to improper use of the toilets to quell the odor issues caused by poor ventilation and other installation flaws of the source-separation system.

3. Evaluation of proposed urine stabilization methods

The purpose of this section is not to identify all possible solutions to problems that urine source-separation systems face, but to evaluate the viability of urine stabilization solutions Urine stabilization generally is referred to as chemical treatments that minimize or delay ureolysis, and can be used to alleviate ammonia volatilization and precipitation in the drain lines. Urine stabilization is needed to maximize the recovery of nutrients, particularly nitrogen and phosphorus from urine, and to prevent odors and maintenance issues [37]. That few, if any, commercially available solvents and cleaners have any major effect on urinary deposition has prompted researchers to investigate various urine stabilization techniques [12]. Urine stabilization in source-separation contexts usually entails inhibiting the ureolytic reaction using reversible inhibitors, inactivating the urease enzyme, or modulating the pH of the urine enough such that ureolysis does not proceed. Although these techniques may be effective in laboratory studies, their feasibility in on-site applications is largely undiscussed. A summary of proposed urine stabilization techniques is shown in Table 2.

Table 2: Summary of Current and Proposed Urine Stabilization Methods Grouped by Technical Challenges

Proposed Solutions	Mechanism	Complications with Proposed Solutions	References for Mechanisms	References for Complications
Urease inhibitors	Enzyme inhibition	Limited research in urine source-separation contexts; Limited research on dosing method; Proof-of-concept demonstrated, but unproven in larger scale applications; Possibility of long-term development of microbial resistance.	[38]	[39]
Chemical cleaning with acids	Crystal dissolution	Clogs from fouling are still inevitable; laborious cleaning procedure; cleaning staff may not be willing or trained to safely handle hazardous substances	[40]	[12, 14]
Stabilization by pH depression with acids	pH dependence of ureolysis	Complicates cleaning procedures, increases alkalinity needed for downstream nutrient recovery; hazardous substance handling concerns	[41, 42]	[41]
Stabilization by pH elevation with wood ash	pH dependence of ureolysis	Stabilized/unhydrolyzed urine needs to be hydrolyzed for downstream treatment; strong odor of unprocessed concentrated urine liquid fertilizer; fertilizer susceptible to ammonia losses	[37]	[30, 37, 43]
Electrochemical systems	Variable: chlorine production; acidification; reduction of ion concentrations contributing to crystallization	No research using concentrated human urine as urine stabilization methods; Susceptible to fouling on electrodes.	[44]	[45–51]

3.1 Urine stabilization using urease inhibitors

Urease inhibitors have potential applicability in urine drainage systems as they have been used in analogous environments such as urinary catheters, where they were used to minimize biomineralization. Ureolytic inhibitors are mechanistically distinct from the acid and base inactivation of urease that depend on pH-induced enzyme inactivation. Urease inhibitors are generally classified as competitive or non-competitive [38]. Inhibitors such as hydroxyurea, acetohydroxamic acid, and phosphotriamides are classified as competitive substrate-like analogs while inhibitors such as phosphorodiamidates and imidazoles that affect the reaction mechanism are said to be non-competitive [38, 52].

3.1.1 Urease inhibitors are proven in clinical and agricultural applications to minimize clogging and ammonia volatilization

Urease inhibitors have unfoundedly been said to be ineffective due to the complex nature of urine or have been called impractical due to health risks, but this is an overgeneralization as urease inhibitors are a broad class of chemicals and are used in clinical and agricultural applications [38, 43, 53, 54]. The wide variety of urease inhibitors and their current applications warrant further investigation as potentially viable solutions to minimize biomineralization.

In clinical settings, urease inhibitors are used in catheters to minimize urinary pH rise and to prevent salt and biofilm deposition [55]. Hydroxamic acids (HXA) such as acetohydroxamic acid (AHA) and salicylhydroxamic acid, have been used for the treatment of infection-induced renal stones [56, 57]. Jones et al. (2006) observed that an acetohydroxamic acid treatment reduced catheter encrustation by over 93% over the control [58]. Others found that *E. coli* and *B. pasteurii* calcite precipitation was inhibited in the presence of AHA, confirming that the urease

activity is essential for microbially-induced calcite precipitation [59]. Future studies should investigate the usage of urease activity as a predictor for precipitation potential in urine.

Where medical clinicians saw the need to minimize catheter encrustation, agricultural researchers studied urease inhibition applications to maintain air quality, control odor, and prevent nutrient loss in fertilizer applications [60, 61]. N-butyl thiophosphoric triamide (NBPT) is a common agricultural ureolytic inhibitor used to delay the conversion of urea to ammonia, but its mechanism is not completely understood. Researchers explain that the oxo-analogue of NBPT (NBPTO), which forms after NBPT is aerated or contacts soil oxido-reductases, [38, 62], is the actual competitive inhibitor that binds to the active site [38, 62]. Other researchers observed that NBPT does not need to be converted to its oxo-derivative to be inhibitory [63]. Some suggest that hydrogen peroxide can activate NBPT [64], but this observation is difficult to verify because hydrogen peroxide has also been found to effectively reduce ureolytic rates in human urine and urea solutions likely due to the strong oxidative effects of hydrogen peroxide acting on the urease [65, 66]. Commercial NBPT blends such as Agrotain already come activated due to its proprietary blend of detergents that solubilizes NBPT and activates it [67].

Despite uncertainties in its mechanism, the application of NBPT and its commercial equivalents in source-separation contexts is worth exploring because of its widespread use in agriculture to minimize ammonia volatilization from fertilizer application. In 2016, of the 14 metric tons (Mt) of inhibitor-treated fertilizers sold, NBPT blended fertilizers accounted for 53% of fertilizer sales worldwide [68]. Germany also passed legislation requiring that by 2020, all urea fertilizers used in country must be supplemented with inhibitors such as NBPT due to its environmental benefits [68]. The proven performance of NBPT in increasing food crop

production suggests that it should be safe for use in urinals, where there is limited direct human contact with restroom surfaces.

In addition to its ubiquity, NBPT could be logistically practical due to its potency when compared to previously discussed stabilization methods. Compared to Randall et al.'s (2016) caustic stabilization method requiring 20 kg $Ca(OH)_2$ per m^3, the Agrotain Ultra label suggests that only 3.1 L of the 26.7% m/m NBPT blend is required to treat 3,048 kg (3 imperial tons) of urea—a magnitude's difference in efficiency compared to stabilization by caustic treatment. Others demonstrated that an 80 mg dose of NBPT applied per L of swine slurry can temporarily inhibit urea hydrolysis for 6-10 days [69].

3.1.2 Urease inhibitors from natural sources are not suitable for large-scale applications

Aside from synthetic urease inhibitors, others have explored the use of natural alternatives, but with limited practicality. Mathialagan et al. (2017) performed a soil urease inhibition study using an allicin liquid solution prepared from commercially available allicin powder [70]. They found that compared to the recommended application rate of 3.3 mL Agrotain per kg of urea, a 15% ratio of allicin to urea mass application rate was still 75% less effective than NBPT from Agrotain at steady state. While some inhibition occurred, using allicin is currently not considered to be a cost-effective option due to its low relative efficacy. Others who used garlic juice as a source of thiosulfates found that 5.6 g/L was required for 50% inhibition of 0.5 mg/mL jack bean urease acting in a buffered urea solution [71]. Though some inhibition can be achieved using naturally available compounds, obtaining plants and isolating these compounds at volume might be difficult when their availability is limited in nature [72]. The lack of scalability and low potency of plant-based urease inhibitors make it inaccessible for larger

applications. Therefore, plant-based urease inhibitors may not be practical for urine source-separation projects.

3.1.3 Considerations for future research on urease inhibitors for urine source-separation settings

Even though the performance of ureolytic inhibitors in medical settings involving urine and agricultural settings involving fertilizers is known, the dosing requirements and its behavior remains unknown in source-separation settings [41]. It is possible that urease inhibitors can be incorporated into urinal cakes as an active ingredient, but no publicly available research exists on this dosing method yet. A web search reveals that many commercially available urinal cakes designed for waterless urinals are not available in US markets and have proprietary ingredient lists. The performance of commercial urinal cakes, their mechanism, and whether the product performs as the manufacturers claims is not publicly documented. Urinal cakes are commonplace in men's restrooms and should be further explored as a chemical dosing system for ureolytic inhibitors.

Another challenge of using ureolytic inhibitors could be microbial adaptation [39]. To alleviate ureolysis in lamb rumens, researchers have shown that AHA, NBPT, and bismuth compounds retard the production of ammonia, but their efficacy decreases over time [39]. Thus, one should investigate both short-term and long-term efficacy of ureolytic inhibitors in urine drainage systems. Because urine drainage systems are not practically constrained by dosage limits that would need to be considered when dosing live subjects, more research should be done to test whether urease inhibitors can be dosed largely enough to overcome long-term microbial adaptations.

Of special note, future studies should also reconsider the use of plant-based ureases when trying to simulate microbially-driven ureolysis in urine drainage systems. Though recent studies used jack bean urease in urine source-separation experiments, future source-separation studies should avoid using plant-derived ureases to characterize the performance of a given treatment [41, 73]. Plant ureases are not representative of those found in urine drain lines where ureolysis is microbially induced. Though the pH is elevated by jack bean urease, biofilm and bacteria have shock resistance mechanisms that aid in survival at extremely acidic or alkaline environments that would not be replicated in experiments involving plant-based ureases [74]. Some of these alkaline resistant oral bacteria are commonplace in restrooms or source-separation settings and may persist in urine drainpipes or storage tanks [75, 76]. The microbially community in source-separation systems is diverse [77], and so studies using a urease extract that accounts for bacterial diversity seen in urine drain pipes would be more experimentally sound than those that do not. Consider that the inhibitory capacity of a chemical may not be equivalent between different urease sources. For example, AHA inhibited *Helicobacter pylori* urease more potently than it did with jack bean urease [78]. In another case, Todd & Hausinger (1989) reported a hyperbolic response to increasing AHA concentrations with *Klebsiella aeruginosa* urease while others found that the binding rate for jack bean urease was found to be linear [38, 79]. Munakata et al. (1980), who made similar observations comparing the performance of *Proteus mirabilis* and jack bean urease, attributes the differences between the competitive inhibition of plant and bacterial ureases to different enzymatic properties [80]. These differences illustrate the need for future ureolytic inhibition studies in urine source-separation contexts to use a representative urease for experiments.

3.2 Urine stabilization using acids

As urinary biomineralization and volatilization are largely pH dependent processes, many researchers have recommended increasing the acidity of urine as a preventive and or cleaning measure [12, 14, 16, 37, 41, 81, 82]. Acid treatments of urinals supposedly work by minimizing the urease activity and dissolving the mineral scaling in the drain lines [83]. Enzymes such as urease can be denatured by extremely high and low pH levels by altering the enzyme shape and the degree of ionization of an enzyme's acid or base groups, whereby ionizable side groups located at the active site must have a certain charge for the enzyme to bind to its substrate [84]. Modifying the enzyme via pH adjustments can therefore minimize the ureolysis that urinary ammonia volatilization and biomineralization depend on.

Numerous studies illustrate the inconsistent performance and complications of acid cleaning [18]. At an office building, Goosse (2009) reported that 2 to 3 clogs occurred per year for the urinals used most frequently. Clogging remained problematic despite daily toilet wipe-downs and monthly 30-minute urine drain valve submersions in 10% citric acid. Goosse (2009) also noted that the cleaning effort for the NoMix systems were significantly higher than that for conventional toilets. Including the preparation of citric acid and cleaning time, cleaning all 37 NoMix toilets required 6 person-hours per month. In addition to labor, acid cleanings would incur material costs. Large volumes of acid may be consumed depending on the model of the toilet/urinal and its respective trap volume. The cleaning difficulty of source-separation technologies relative to conventional flush urinals could contribute to cleaning staff discontent. More so, the frequent cleaning needed to prevent clogs and minimize odors is time consuming and has hazards associated with handling of acids and bases [12]. Similar acid treatments and recurring clogs due to biomineralization were observed in 1999. [12]. Lindgren (1999), through a series of interviews, found that property managers too were frustrated that cleaning methods

using wire brushes and concentrated phosphoric acid were painstaking: the cleaning brushes wore out quickly and the use of harsh chemicals detracted from what should be a safe work environment [12]. Even with an acid treatment, source-separation technologies were still perceived as odorous and unhygienic to users, suggesting that its inhibitory properties are short-term at best [12]. In short, acid treatments have been tried with less-than-ideal results as indicated by frequent and recurring clogs reported by residents [12].

In addition to being laborious, pH treatments are also short-term solutions. Blume & Winker (2011), who studied the GTZ offices, recommended a monthly 200 mL organic acid, 24-hour soak of the urine drain valves to prevent and clear blockages [14]. They also found that without regular organic acid treatment, the urine drain valves failed after 2 years—detailed diagrams of the fouling can be found in Blume & Winker's (2011) article [14]. From a practical standpoint, an overnight acid soak is a time-consuming procedure not feasible in busy public restrooms that operate day and night. They note that caustic soda is efficient at dissolving hair and organic residues. Researchers also noted that acetic acid is useful in removing mineral precipitates after having the u-bend pipe fixture sit in the acid overnight. However, overnight acid soaks render the urinal non-functional for the duration of the treatment [12, 16].

Despite regular acid treatments in past field studies, waterless urinals and UDTs still clogged likely due to the protective nature of biofilms [12]. Ureolytic biomineralization stabilizes the biofilm and decreases its susceptibility to antimicrobial treatments. Li et al. (2016) demonstrated that urease positive biofilms with homogeneously distributed biomineral precipitates showed greater survival when exposed to ciprofloxacin compared to a urease negative biofilm that lacked biomineral precipitates subjected to identical conditions [85]. The coinciding crystallization not only explains why *Proteus mirabilis* biofilms harden and obstruct

urine flow in catheters [86], but also contributes to the biocidal resistance of ureolytically active biofilms. In clinical settings, this resistance of the biofilm cells to antibacterial agents largely contributes to the difficulties in eliminating these infections using systemic antibiotics and antiseptic bladder washouts, and it is possible that the protective nature of biofilms and biomineralization also confers upon the microbial community a resistance against acid treatments [87].

The downstream nutrient recovery efficiency can also be negatively affected by acid treatments. Even if effective at unclogging urinals and temporarily inhibiting odors, frequent use of acids would deplete the alkalinity in the urine needed to maximize downstream nutrient recovery processes as discussed in Section 2.2. In conclusion, even though acid treatments continue to be recommended, more research and discussion about its complications is needed [41].

3.3 Urine stabilization using caustics and dehydration

Just as ureolytic rates can be retarded via acidification to pH 3-4.5, it too can be slowed raising the pH above 12 [37, 41]. Some have explored mixing readily available materials like wood ash and slaked lime with fresh urine to produce a dry fertilizer. This would prevent enzymatic ureolysis via high pH. As we have observed with acid treatments, little discussion has been made on the impracticalities of caustic stabilization.

For one, caustic stabilization is limited for use on fresh unhydrolyzed urine; it is not recommended for the storage of already hydrolyzed urine. Caustic stabilization is not suitable for stabilizing hydrolyzed urine because the ammonium concentrations would be elevated. While ureolysis is inhibited by the high pH, any present aqueous ammonium would be converted immediately to gaseous ammonia, posing challenges for nutrient recovery and creating odor concerns. This suggests that slaked lime or wood ash must be added immediately during fresh

urine collection as done by Randall et al. (2016) and Flanagan & Randall (2018), where minimal volatilization would occur as most of the nitrogen is fixed as urea.

The logistical challenges of caustic stabilization were also undiscussed by its proponents. Regular handling of caustics poses a logistical problem in that conveyance is costly and complicates maintenance protocols. Should slaked lime be used at busy rest areas, it would require a large onsite storage facility and incur large transportation costs to accommodate high urine volumes based on the 10 g/L fresh urine rate proposed by Randall et al. (2016). More so, Flanagan & Randall (2018) claimed that using caustic urine stabilization to produce a dry fertilizer is profitable but did not include logistical and labor costs and assumed that there is a market demand for urine-based fertilizers. Flanagan & Randall (2017) used $Ca(OH)_2$ to stabilize fresh human urine collected directly into plastic jugs placed around the University of Capetown, but made no mention the intense labor required to actively manage, collect urine from, and dose their plastic jugs with caustics [30]. Future cost analyses and life cycle assessments must account for all costs to determine the true cost of urine source-separation. Caustic dry fertilizer production also requires large surface areas to achieve evaporation. Dehydration by open-air evaporation relies on passive aeration using the high surface area of the resultant dry fertilizer and air flow but would only work in areas where high enough temperatures can facilitate evaporation [43]. Should dry fertilizer production be implemented at decentralized facilities, a dedicated plot of land would be required and may also pose an odor concern for the surrounding area. Land availability and odor concerns make it unlikely that dry fertilizer production using caustics would be feasible in an urban environment. In addition to transportation and storage concerns, handling highly alkaline urine in large volumes could also pose a safety risk for maintenance staff and can lead to a degradation in work environment.

The advantages of caustic stabilization have also been embellished. Flanagan & Randall (2018) suggested that an additional liquid fertilizer could be produced from residual urine resulting from caustic stabilized solid fertilizer production using stripping, adsorption, and precipitation processes described by others [88]. Yet, the resultant liquid fertilizer from fresh human urine treated with $Ca(OH)_2$ would have little ammonium content because urea remains unhydrolyzed—urea cannot be stripped like ammonia can be. To facilitate its conversion to ammonium, the high pH in urine caused by $Ca(OH)_2$ would need to be countered with an acid addition that was not considered in Flanagan & Randall (2018)'s cost analysis. Only then at an appropriate pH range will ureolysis proceed as Flanagan & Randall (2018) proposed. While pH values > 12 have been shown to effectively limit enzymatic urea hydrolysis, Senecal & Vinneras (2017) confirmed that caustic stabilization methods remain susceptible to nitrogen losses [43]. They found that the nitrogen retention for caustic stabilized solid fertilizers were as high as 90% but as low as 64% due to volatilization and non-enzymatic decomposition of urea. This is because caustic stabilized fertilizers may be especially susceptible to ammonia volatilization, as the emission potential is increased in high pH environments [26].

Caustic stabilization may not also be free from pharmaceuticals [30]. Flanagan & Randall (2018) claim that the dry fertilizer is relatively free from pharmaceuticals since 98% of hormones and pharmaceuticals would remain in the liquid phase of urine during struvite precipitation. However, struvite precipitation was not the main mechanism of nutrient recovery in their study, rendering this claim's applicability to liquid fertilizer questionable [89]. Because contaminants in the liquid phase would not be removed during their evaporation process, their resultant fertilizers would likely not be pharmaceutical-free.

3.4 Electrochemical urine stabilization

A variety of studies have used urine as an anode substrate of microbial electrochemical systems to produce energy and to remove and recover nutrients [45–51]. Whether electrochemical urine treatment is a viable nutrient recovery technique is beyond the scope of this review. Instead, this section focuses on electrochemical systems as a urine stabilization method to minimize clogging at the user interface and increase operational reliability of the urine drainage system.

One configuration of electrochemical treatment of human urine for its storage and reuse as flush water was reported to work by urease inactivation via chlorine production. By examining urease unfolding using tryptophan fluorescence, Ikematsu et al. (2007) showed that both sodium hypochlorite and electrochemically produced chlorine can irreversibly inactivate urease given enough incubation time and chlorine dose [44]. They suggest this urease inactivation resulted in a reduction in odors associated with ureolysis [44, 90].

More recently, Paepe et al. (2020) proposed applying electrical energy to reduce oxygen at the cathode to release OH^- and increase the pH of the urine while water is oxidized at the anode, resulting in an acidic urine effluent [73]. The high pH in the cathodic chamber also results in supersaturation conditions with respect to magnesium and calcium, leading to precipitation [73]. They theorize that a decrease in pH , magnesium, and calcium concentrations should then reduce the downstream scaling potential, but future research is needed to characterize the potential scale reduction resulting from the acidified urine stream [73]. Further, no significant increase in electrical conductivity or ammoniacal nitrogen occurred when unhydrolyzed urine was stabilized at pH 11 for the first 7 days, but there followed a gradual decrease to pH 9.25 after 7-10 days [73]. This suggests that Paepe et al.'s (2020) electrochemical urine stabilization method is a temporary measure and that hydrolysis may eventually proceed given enough time.

Source-separated urine has attracted the interest of electrochemical researchers, but the corrosion and fouling associated with ureolysis are impediments to electrochemical processes [90]. Because no pilot or full-scale electrochemical urine stabilization treatment exists yet, the practical feasibility of these systems has been overlooked. No studies have mentioned how the urine needed to feed the electrochemical systems would be collected outside of laboratory experimentation. To apply an electrochemical treatment, fresh urine must be first collected in sufficient quantity; this is a chicken and egg problem. Because it is difficult to collect large volumes of urine in sterile conditions, the collection system preceding treatment, pumps, electrodes, and membranes may be susceptible to ureolytic fouling. Luther et al. (2015) noted that while no corrosion was observed over their 10-day study, precipitation was present on the electrodes, which may compromise the performance of the system [91]. Long term studies to assess the integrity of the electrodes and cation exchange membranes should be conducted because material and energy consumption are currently hindering the technology's scalability [90, 91]. Once electrode and membrane longevity are determined, operations and maintenance costs may be determined. Additionally, trained maintenance staff may be required if electrochemical systems were implemented at a rest area or office building. Finally, one must consider whether an electrochemical system is robust enough to withstand vandalism and chemical dumping if implemented in a public setting such as a highway rest area.

The scalability of electrochemical systems has also not been discussed. Both Ikematsu et al. (2007) and Paepe et al. (2020) propose that electrochemically stabilized urine can be used as flush water, but this is only feasible if the electrochemical system can treat the urine at a sufficient rate to match urine collection rates [44]. Apart from scalability, while many studies describe the electrochemical treatment and recovery of diluted urine or wastewater; yet, no pilot

scale electrochemical urine stabilization systems (as opposed to electrochemical nutrient recovery systems) have yet been tested using concentrated urine [45–51, 92]. As such, it remains uncertain if electrochemical urine stabilization solutions are suitable for the treatment of concentrated urine from waterless urinals.

3.5 Various urine stabilization approaches for odors

Methods to control odors that result from the volatilization of ammonia and volatile organic compounds have included dilution with water, cooling, and applications of chemical masking agents [93]. Various commercial products that are available online claim to have digestive enzymes, microbial cultures, and masking agents that prevent odors, but because of trade secrets, their effectiveness and mechanisms remain unclear. Masking, disinfecting, and oxidizing agents can provide short-term control of malodor, but the capacity of these additives is finite and require frequent reapplication. Though temperature control of flush water may work to control odors in laboratory batch testing, refrigeration may be cost prohibitive and impractical in urine drainage systems [94]. Odor control by dilution, as proposed by Hashemi & Han (2017), are also counter-productive, as dilution would hinder nutrient recovery efficiencies.

4. Opportunities and needs in future work

Urine source-separation is a promising water and nutrient conservation technology. However, the high nutrient density of urine and the water saving benefits apparent in studies discussed in this review are often overshadowed by poor user experiences with urine separating toilet facilities due to odors and laborious maintenance. We saw that trials in office buildings, universities, and apartment complexes encountered similar problems regarding clogs, odors, and user dissatisfaction. The urinals and UDTs and drainage systems tended to clog or not function as intended due to design flaws. Treatments such as acid cleanings tested by the researchers

tended to be short-term and laborious and others such as caustic stabilization and electrochemical treatment had impracticalities that were overlooked. On the other hand, urease inhibitors, a broad class of chemicals already used in clinical and agricultural settings to prevent clogs and or ammonia losses, have been wrongly dismissed by previous source-separation researchers. We conclude that treatments such as urease inhibitors should be tested in urine source-separation settings. Future researchers should investigate the effects of urease inhibitors on reducing pipe fouling, and ammonia volatilization in urine storage tanks and drainage systems.

Ultimately, a combination of solving the technical problems of urine source-separation, finding a market for the resultant fertilizer, and making proactive marketing efforts are needed for the social legitimacy of urine source-separation systems. We realize that aside from the need to address technical challenges, the adoption of urine source-separation also hinges on public relations work as the widespread adoption of water recycling did. Like in the case of recycled water, public opposition could be a barrier to successful implementation of urine source-separation and subsequent fertilizer production for fear of it being unsafe and unsanitary. Proven recycled water marketing strategies can be translated to establish legitimacy for urine source-separation technologies. Scholars have conceptualized the success of Orange County Water District (OCWD) using a legitimacy framework. Through various marketing and public relations strategies, the OCWD implemented a high-profile recycled water project with high public acceptance by demonstrating pragmatic, moral, and cognitive legitimacy [95]. Legitimacy entails creating widespread trust in an innovation. It depends not only on targeting individual psychology, but also on the compatibility between the innovation and societal norms [95]. Potential urine source-separation public relation strategies are shown in Table 3 and are

juxtaposed with proven water recycling public relation strategies that garnered its widespread acceptance.

Table 3: Summary of legitimization strategies

Legitimacy	Subtype	Definition[a]	Proven Recycled Water PR Strategies[a]	Potential Urine Source-Separation PR Strategies
Pragmatic	Exchange	Support for an innovation based on its perceived value to the user	Outreach and education campaigns	Outreach and education campaigns, YouTube channel on nutrient recovery topics, information booths at public events, fairs, etc.
	Influence	Support for an implementing organization because it shares decision-making power with users	Elicited feedback from established Citizen Advisory Board	Elicit feedback from stakeholder advisory boards such as growers, farmers, and consumers.
	Dispositional	Support for an implementing organization based on a belief that the organization is acting in the user's best interest	Demonstrated utility trustworthiness by disclosing NDMA concerns	Researchers and implementing organization should acknowledge common concerns of fertilizer quality such as salt, odor, and pharmaceutical residuals. Disclose what is being done to fix it during outreach.
Moral	Consequential	Support based on perceptions of implementing organization's accomplishments	High water quality track record	Use university reputation to market source-separation project. Implement urine-derived fertilizer pilot studies on university project farms and involve the media.
	Procedural	Support based on perception of implementing organization's specific procedures concerning safety and reliability	Emergency intervention and water quality monitoring plans	Implement fertilizer quality monitoring plans and reports. Incorporate findings in public outreach events and pamphlets
	Structural	Support based on evaluation of physical entity contributing to safety and reliability	State-of-the-art technology, on-site laboratory	Use a third-party laboratory for fertilizer quality analysis to enhance transparency. Unbiased, peer-reviewed research that is convincing to the public [96].
	Personal	Support enhanced by managerial charisma and involvement	Management involvement in outreach	Management and researchers should be proactive in outreach efforts.
Cognitive	Comprehensibility	Support because an innovation fits in with user daily life experiences and expectations	Serving tour visitors bottled recycled water	Provide fertilizer samples at outreach events and interested stakeholders to demonstrate product usability. Free samples can also be advertised at hydroponics shops and well-frequented online forums such as /r/freebies on reddit.com.
	Taken-for-grantedness	Support deriving from compatibility between the innovation and subconscious, unquestionable user expectations	Framing potable reuse as recycling and environmental protection	Frame urine source-separation as nutrient recycling and environmental protection. Emphasize water and energy savings and the unthinkability of wasting a nutrient dense resource.

[a] Recycled water public relation strategies and legitimacy definitions adapted from Harris-Lovett et al. (2015).

Conflicts of interest

The authors declare no competing financial interest. Any trade names or products mentioned were for descriptive purposes only and does not imply endorsement by the authors.

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CHAPTER II

A biogeographic 16S rRNA survey of bacterial communities of ureolytic biomineralization from California Public Restrooms

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Abstract

In this study, we examined the total bacterial community associated with ureolytic biomineralization from urine drainage systems. Biomineral samples were obtained from 11 California Department of Transportation public restrooms fitted with waterless, low-flow, or conventional urinals in 2019. Following high throughput 16S rRNA Illumina sequences processed using the DADA2 pipeline, the microbial diversity assessment of 169 biomineral and urine samples resulted in 3,869 reference sequences aggregated as 598 operational taxonomic units (OTUs). Using PERMANOVA testing, we found strong, significant differences between biomineral samples grouped by intrasystem sampling location and urinal type. Biomineral microbial community profiles and alpha diversities differed significantly when controlling for sampling season. Observational statistics revealed that biomineral samples obtained from waterless urinals contained the largest *ureC*/16S gene copy ratios and were the least diverse urinal type in terms of Shannon indices. Waterless urinal biomineral samples were largely dominated by the Bacilli class (86.1%) compared to low-flow (41.3%) and conventional samples (20.5%), and had the fewest genera that account for less than 2.5% relative abundance per OTU. Our findings are useful for future microbial ecology studies of urine source-separation technologies, as we have established a comparative basis using a large sample size and study area.

1. Introduction

Source-separation technologies such as waterless urinals and low-flow urinals save water and, in the case of waterless urinals, require less plumbing than conventional flush systems. Most urine drainage/collection systems are susceptible to microbially-induced precipitation, or biomineralization. Additionally, lower flow rates associated with water conserving urinals have been implicated in drainage and aesthetic problems. Biomineralization in urine drainage systems has been described as both a viscous sludge that forms at pipe inverts and storage tanks , or as solid formations that are caused by precipitation directly on the pipe wall [16–18]. Ureolytic biofilm and biomineral formations are persistent complications in cleaning and maintenance measures for urine source-separation systems. These biofilm and biomineral formations are why waterless urinals continue to clog despite strong acid and caustic treatments tried in past field studies [12].

Biomineralization is typically a mixed composition of struvite, calcium phosphate, calcium oxalate, calcium carbonate, and organic matter. Its occurrence in source-separation fixtures compromises the social acceptability of the technology due to associations with clogging, odor, and overall user dissatisfaction [12]. While biomineralization in urinals is due to a combination of various mechanisms, a key contributor to its formation is the hydrolysis of urea, or ureolysis, catalyzed by the microbial enzyme, urease. The prevalence, mineral composition, and formation of ureolytic crystallization is a function of urine chemistry such as the pH and ionic strength, modulators and inhibitors of crystallization, and the bacterial community [15, 24, 97–100]. Though ammonification of proteins and dissimilatory nitrate reduction to ammonia contributes to pH increases, ureolysis has been considered the most efficient pathway to raise pH into the range required in biomineral formation [23, 59, 101–103].

Ureolysis involves the production of ammonium and carbonate from the breakdown of urea, leading to an increase in saturation indices that coincide with the pH shift that culminate as biomineral formation [15]. Over time, as the biomineral hardens and accumulates, the pipes will clog in a manner similar to urological catheters [15, 104, 105]. Due to its role in biomineralization, the activity of urease was of particular interest in this study.

This is the first study on the microbial ecology of the biomineralization taken from dedicated urine drainage systems behind urinals. Whereas struvite precipitates studied by Lahr et al. (2016) were produced *in vitro*, the biomineral precipitates examined in this study were taken directly from urine drainage systems in operation along busy California highways. Earlier, Lahr et al. (2016) attempted to characterize the microbial community in urine collected from public events using portable toilets, and found that aged, hydrolyzed urine has less OTU richness than does freshly collected urine (< 24 hours). Lahr et al. (2016) also made observations that bacterial communities associated with artificially induced struvite precipitates did not differ from liquid associated communities. However, no statistical analysis was performed on this empirical observation. Our large sample size study also builds upon the research of Lahr et al. (2016), exploring how differences in urine collection systems, geography, and seasonality can influence the composition of the bacterial communities that are associated with biomineralization. Of note, this novel study also builds upon our previous regression study from the same sampling periods and used the same dataset [27]. Whereas our previous regression study focused solely on predicting the influence of environmental variables on biomineral urease activity, this study substantially differs as it draws new conclusions on the microbial ecology of biomineral samples using different statistical techniques applied to sequencing data [27].

Through a state-wide biogeographic survey using high-throughput 16S rRNA gene sequencing, the objectives of this microbial ecology study were to (a) assess the alpha and beta diversities of biomineral and liquid associated bacterial communities taken from urine drainage systems across California, (b) determine the average bacterial community structure with regard to urinal type (waterless, low-flow, and conventional), and (c) observe the effects of seasonality and intrasystem sampling location on the bacterial community diversity of biomineral samples, and (d) posit the relationship between alpha diversity metrics and the abundance of the *ureC* gene estimated using quantitative polymerase chain reaction (qPCR).

2. Materials and methods

In 2019, ureolytic biomineralization and urine from 11 public restrooms owned by the California Department of Transportation (Caltrans; funding agency) were sampled for a microbial ecology study using high throughput sequencing of the 16S rRNA marker. These public rest areas were chosen because they were known by Caltrans to be frequently clogged due to ureolytic biomineral precipitates. Permission to sample these rest areas was obtained via the funding contract (Agreement Number 65A0734) with the sponsoring agency. All environmental metadata including sampling dates can be found at the Dryad repository (DOI: 10.25338/B82906). Nucleotide sequences were deposited at the National Center for Biotechnology Information (NCBI) Sequencing Read Archive (SRA) under the BioProject Accession number PRJNA699694.

2.1 Sample collection

A total of 2 conventional, 4 low-flow, and 5 waterless urinals located at public safety roadside rest areas (SRRAs) were observed in this study as mapped in Fig 1. Conventional rest areas are those fitted with urinals producing ~ 1 gal/flush, low-flow ~0.125 gal/flush, and

waterless no flush. If there was sufficient mass available, biomineralization deposits were scraped into sterile 50 mL conical tubes from fouled fixtures, cartridges, drain traps, screens, and drain lines such as those shown in S1 Fig from the S1 File.

All samples were stored in an ice chest immediately after collection and processed immediately after return to campus. Previous work monitoring the ureolysis rate in soils have found that a distinct slowdown in ureolytic rate was not detected until 8 months of storage and is consistent with a past study on the effects of storage on soil urease activity [106]. Regarding the effects of storage condition on soil microbial community compositions, others have demonstrated that neither storage time nor storage temperature substantially altered overall communities relative to more than 500 previously examined soil samples [107]. DNA sequencing reads are shown to be consistent for samples stored at both 4^{o}C and 20^{o}C for 0, 2, and 5 days prior to freezing as shown in the S2 Fig from the S1 File. As such, the sampling preservation measures were deemed adequate. Therefore, we demonstrated adequate storage techniques during the sampling trips by evaluating the effects of time and temperature on the observed community structure.

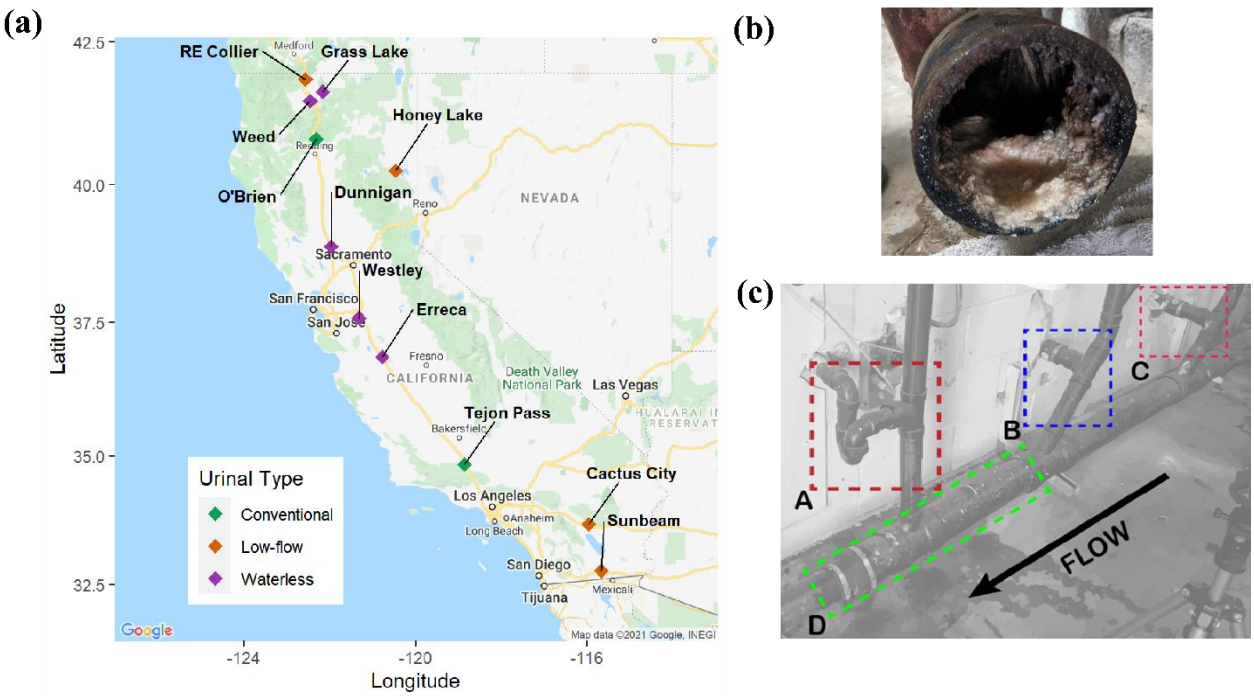

Figure 1. Sampling sites, characteristic sample, and representative intrasystem sampling locations in the plumbing gallery.

In total, (a) 11 separate rest areas owned by the California Department of Transportation were sampled for this study, (b) characteristic biomineral formation on the invert of a gallery main drain at Erreca northbound rest area. (c) the gallery drainpipes directly succeeding the urinals such as that shown from Erreca northbound rest area were typical sampling sites. As depicted are: (A) sink (not sampled), (B) American Disability Act (ADA) drain line, (C) standard urinal height drain line, (D) main drain.

2.2 Biomineral ureolytic enzyme activity characterization

Biomineral ureolytic enzyme activity was measured for correlation analyses with alpha-diversity scores. To determine the enzymatic activities of biomineral sample, a known wet mass of the biomineral samples was suspended and mixed in a 100 mL volume of 7.3 pH 200 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) buffer containing 2.5% urea m/m. The rate of increase in conductivity is proportional to that of urea hydrolysis and can be used as a

surrogate measure for enzymatic activity [108]. As a comparative basis between samples, one unit of specific activity is defined as the uS cm^{-1} per gram of volatile solids (VS). Volatile solids were determined using standard methods for examination of water and wastewater [109].

2.3 Quantifying ureC and 16S gene abundance using real-time polymerase chain reaction (qPCR)

To examine the relationship between the biomineral bacterial community structure, diversity, and ureolytic genes, the genomes representing the presence of urease genes were examined by qPCR. A similar protocol was previously described [110, 111]. The urease associated gene and 16S assays were designed on the urease alpha subunit encoding gene (*ureC*) and the 16S rRNA gene, respectively. Primer sequences were obtained from the literature [110, 111].

Biomineral samples were kept frozen at -20°C prior to DNA extraction. DNA was manually extracted from 0.25 g of wet mass using a commercially available kit following manufacturer's recommendations and eluted in 100 µL of diethylpyrocarbonate (DEPC)-treated water (Qiagen DNeasy Power Soil Kit, cat # 12888-50). Each 12 µL reaction contained 6 µL SYBR master mix (Applied Biosystems SYBR Green PCR Master Mix, cat # 4309155), 0.48 µL of a primer-water mixture (primers at final concentration of 400 nM), 4.52 µL of DEPC-treated water, and 1 µL of extracted DNA. qPCR was performed using an automated fluorometer (ABI PRISM 7900 HTA FAST, Thermo Fisher Scientific). Standard amplification conditions were used: 95°C for 3 min, 40 cycles of 95°C for 15 s, 52°C for 30 s, and 72°C for 30 s, with a melting curve at 95°C for 15 s, 52°C for 15 s, and 95°C for 15 s. Data was analyzed using Applied Biosystems SDS software, version 2.4. Fluorescent signals were collected during the annealing phase and C_q values extracted with a threshold of 0.2 and baseline values of 3–10 for both *ureC* and 16S assays. Amplification specificity was verified using the dissociation

temperature (T_m) of the qPCR amplicons specific to each gene. Acceptable T_m ranges were determined to be +/- 2% of the positive controls. For *ureC*, the acceptable T_m range was 80.8°C - 84.1°C and for 16S it was 81.8°C - 85.2°C. Samples with detectable amplification but with T_m's outside of the acceptable ranges were considered false positives and were deemed negative for the gene of interest. The absolute copy numbers were also normalized in terms of volatile solids (VS) mass present in the biomineral samples.

The sensitivity of ureC-F (5'-TGGGCCTTAAAATHCAYGARGAYTGGG-3') and ureC-R (5'-SGGTGGTGGCACACCATNANCATRTC-3') was < 4,000 copies/qPCR reaction and the efficiency was 80.6% (R^2 = 0.9974). Poor sensitivity and low efficiency for *ureC* is expected due to the nature of SYBR degenerative primers. The 16S assay, 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 533R (5' -TTACCGCGGCTGCTGGCAC-3'), yielded a sensitivity of < 10 copies/qPCR reaction and efficiency of 102.2% (R^2 = 0.9981).

2.4 16S rRNA Illumina high throughput sequencing

The microbial community was assessed using high throughput 16S rRNA sequencing using an Illumina MiSeq platform. First, the DNA was extracted from biomineral and liquid samples using Qiagen DNeasy PowerSoil kits following the manufacturer's instructions. Primers 319F (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG(spacer)GTACTCCTACGGGAGGCAGCAGT) and 806R (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG(spacer)CCGGACTACNVGGGTWTCTAAT were used to amplify the V3-V4 domain of the 16S rRNA using a twostep PCR procedure. ureC-F and ureC-R primers were the same as that described in Section 2.3.

In step one of the amplification procedure, both forward and reverse primers contained an Illumina tag sequence, a variable length spacer (no spacer, C, TC, or ATC for 319F; no spacer,

G, TG, ATG for 806R) to increase diversity and improve the quality of the sequencing run, a linker sequence, and the 16S target sequence. Each 25 mL PCR reaction contained 1 Unit Kapa2G Robust Hot Start Polymerase (Kapa Biosystems), 1.5 mM $MgCl_2$, 0.2 mM final concentration dNTP mix, 0.2 mM final concentration of each primer and 1 uL of DNA for each sample. PCR conditions for 16S rRNA amplification were: an initial incubation at 95°C for 3 min, followed by 25 cycles of 95°C for 45 s, 50°C for 30 s, 72°C for 30 s and a final extension of 72°C for 3 min. In step two, each sample was barcoded with a unique forward and reverse barcode combination using forward primers (AATGATACGGCGACCACCGAGATCTACACNNNNNNNNTCGTCGGCAGCGTC) with an Illumina P5 adapter sequence, a unique 8 nt barcode (N), a partial matching sequence of the forward adapter used in step one, and reverse primers (CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTCTCGTGGGCTCGG) with an Illumina P7 adapter sequence, unique 8 nt barcode (N), and a partial matching sequence of the reverse adapter used in step one. The PCR reaction in step two contained 1 Unit Kapa2G Robust Hot Start Polymerase (Kapa Biosystems), 1.5 mM $MgCl_2$, 0.2 mM final concentration dNTP mix, 0.2 mM final concentration of each uniquely barcoded primer and 1 uL of the product from the PCR reaction in step one diluted at a 10:1 ratio in water. PCR conditions were: an initial incubation at 95°C for 3 min, followed by 9 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s and a final extension of 72°C for 3 min.

The final product was quantified on the Qubit instrument using the Qubit Broad Range DNA kit (Invitrogen) and individual amplicons were pooled in equal concentrations. The pooled library was cleaned utilizing Ampure XP beads (Beckman Coulter) then the band of interest was further subjected to isolation via gel electrophoresis on a 1.5% Blue Pippin HT gel (Sage

Science). The library was quantified via qPCR followed by 300-bp paired-end sequencing using an Illumina MiSeq instrument in the Genome Center DNA Technologies Core, University of California, Davis.

2.5 Bioinformatics and statistical analyses

All statistical work and data visualization was done using R version 4.0.2. Sequenced reads were demultiplexed and primers were trimmed using dbcAmplicons [112]. The fastq files were processed using DADA2 using the parameters described in the referenced workflow [113, 114].The protocol was modified to accommodate a new truncation length (truncLen) argument of 265 for forward and 165 for reverse reads.

For all post-sequence processing, overlapped reads were denoised, summarized to amplicon sequence variants (ASVs), and filtered for chimeric sequences using DADA2. Taxonomic assignment was done using the DADA2 implementation of the naive Bayesian classifier method using the Silva SSU database version 138 [115]. Samples with less than 10,000 reads were filtered from further analysis. Rarefaction curves were calculated for each sample using vegan and plotted using ggplot2. Various other exploratory plots involving diversity metrics were produced using phyloseq and then plotted with ggplot2 [116, 117].

Taxa diversity and evenness were assessed by calculating the Shannon index, Chao1, and observed richness using phyloseq as was taxa bar plot percentages. PERMANOVA (permutational multiple analysis of variance) was performed to compare the total sum squared dissimilarities among objects belonging to different groups. PERMANOVA testing was performed whereby demultiplexed reads were also screened for PhiX sequences, overlapped, screened for reads containing Illumina adapter sequences, filtered to exclude any reads containing uncalled bases, and filtered for a minimum overlapped length of 380 bp using HTStream [118]. PERMANOVA analyses were conducted using the function adonis2 in the R

package vegan, version 2.5-6 and the function adonis.II in the R package RVAideMemoire, version 0.9-78. These analyses were based on Bray distances between log-transformed relative log expression (RLE) normalized taxon counts.

To understand the effect of geographic distances and environmental variables on the bacterial community within biomineral samples, a Mantel test was performed [119]. The environmental parameter distance matrix was generated using a Euclidean distance, the geographic coordinates matrix using the Haversine distance, and the community compositional differences using the Bray-Curtis distance. Mantel tests were performed to evaluate Spearman rank correlations between each two distance matrices or between one single factor and a matrix. Finally, non-metric dimensional scaling (NMDS) using Bray-Curtis dissimilarity was performed to visualize the community differences between samples grouped by intrasystem sampling location, seasonality, and or urinal type. Sequencing reads were deposited to NCBI's sequencing read archives under the BioProject accession number PRJNA699694.

3. Results and discussion

After the DADA2 pipeline, the microbial diversity assessment of 169 biomineral and liquid samples obtained from 11 different California state-owned rest areas resulted in a total of 3869 reference sequences aggregated as 598 OTUs. There were a total of 10,454,960 raw reads from which yielded 8,791,330 filtered reads and 8,550,615 merged reads. Of the filtered reads, 7.6% were chimeras and were removed. After DADA2 filtering, the average read lengths were 422 bp. Approximately 76.5 % of the 598 OTUs were classified at the genus level, 92 % at the family level, and 100 % at the phylum level. However, that 23.5% remains unclassified at the genus level points to the importance of future isolation and characterization studies of microbes from urine drainage and other sanitation systems.

3.1 The influence of sample type, urinal type, and sampling season on alpha diversity scores

Alpha diversity scores within samples grouped by urinal type and by sample type (biomineral and liquid) are summarized in Fig 2. Alpha diversity describes the diversity that exists within an ecosystem and is measured by the number of species or the richness of OTUs within that ecosystem [120]. Non-parametric Kruskal-Wallis testing suggests that the alpha diversity Shannon indices for biomineral and liquid samples grouped by sample type (biomineral and liquid) do not significantly differ ($p = 0.072$). This indicates that sample type may not be significant influencer of within-sample diversity. Kruskal-Wallis testing also showed that the within-sample diversity grouped by sampling seasons differed significantly for both biomineral and liquid samples ($p < 0.001$).

While the sample size of conventional urinals is too small to draw conclusions from, we found that waterless urinals generally exhibit a lower alpha diversity score than does low-flow urinals in terms of observed richness, Chao1, and Shannon indices as shown in Fig 2. Kruskal-Wallis and pairwise Wilcoxon rank sum testing suggests that waterless urinal alpha diversity scores depart from the other two urinal types ($p < 0.001$). Our observations on lower relative Shannon indices for waterless urinals as shown in Fig 2 may be because highly ureolytic and alkaline environments characteristic of waterless urinals are more selective of alkaliphilic bacteria that can survive a high ammonia and pH environment [75, 121, 122]. This is reasonable as ammonia, which is a product of ureolysis, is known for its cytotoxic effects on bacteria at sufficiently high concentrations [121, 123]. A rapid increase in pH and ammonia may also be preventing a diverse bacterial community to develop as existing communities may not be given sufficient time to acclimate [102]. Similar diversity studies by Lahr et al. (2016) confirmed that low species richness is associated with high pH (> 9) and ammonia (> 5 g/L) characteristic

of aged, hydrolyzed urine similar to the actively ureolytic environment found in urine drainage systems [75]. Others noted that ureolytic stimulation similarly decreased the community diversity in soil studies, likely due to the same pH selectivity mechanisms [102]. Previous researchers demonstrated that dilution rate is a key factor in reducing pathogenic microorganisms in urine, as high ammonia concentrations associated with waterless urinals may be correlated with greater inactivation (un-survivability) [123, 124]. The decrease in diversity demonstrated in Fig 2 may coincide with a decrease in pathogenic microorganisms found in concentrated urine.

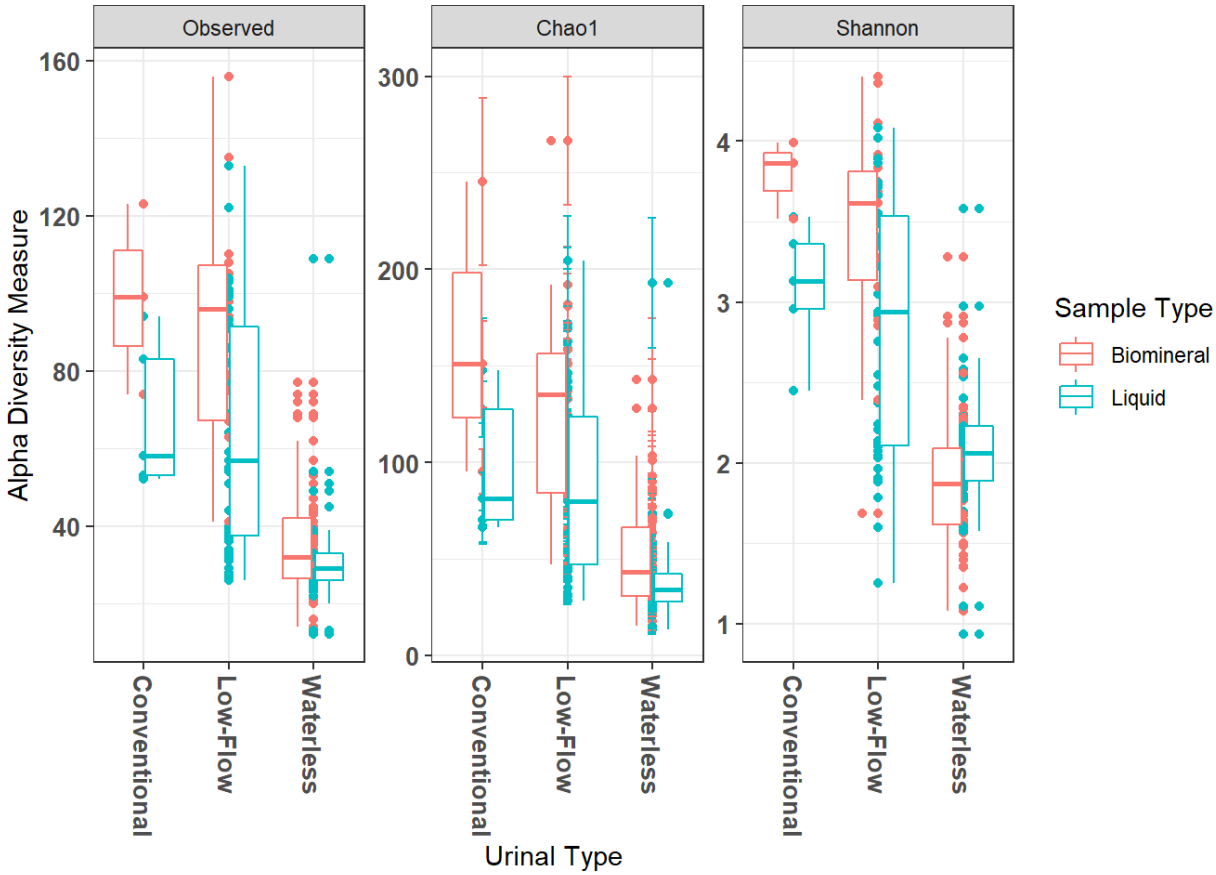

Figure 2. Alpha diversity metrics of all biomineral and liquid associated bacterial samples grouped by urinal type.

3.2 Waterless urinals are the least diverse of the urinal types but likely have the largest ureolytic bacterial community

Based on the results shown in Fig 2, waterless urinal samples are the least diverse of the urinal types; however, biomineral samples obtained from waterless urinal fixtures exhibit the greatest *ureC*/16S gene copy ratios compared to those obtained from flush-type urinals. The mean *ureC* copy number per g VS is 6.22×10^7 for waterless urinal biomineral samples and is 1.95×10^6 for those from flush-type urinals. The mean 16S rRNA (copy number per g VS) is 9.97×10^9 for waterless urinal biomineral samples and is 6.06×10^9 for flush-type urinals. A t-test on the normalized, natural logarithmically transformed data shown in Fig 3 shows that the *ureC*/16S rRNA gene copy ratios grouped by urinal type differed significantly ($p < 0.001$). The large relative abundances of the *ureC* gene in waterless urinal samples suggest a possibility that there is a greater relative abundance of a potentially ureolytic community in waterless urinal samples than in low-flow samples. High concentrations of urea in concentrated urine collected from waterless urinals could lead to highly ammoniacal and alkaline conditions that select for a bacterial community exhibiting greater abundances of the *ureC* gene. The selection of the *ureC* gene due to urea supplementation has been previously reported in a cow rumen study [110]. We hypothesize that a similar phenomenon is occurring in the waterless urinals observed in this study and that there is also an environmental selection of bacteria that have the *ureC* gene. Others found that nitrogen metabolizing bacteria such as ammonia-oxidizing bacteria (AOB) increased not only in absolute numbers but also in relation to other bacterial groups investigated in urea-amended plots [125]. The relationship between ammonia concentrations in urine drainage systems and nitrogen metabolizing bacteria can be verified by future sequencing and microbial ecology studies targeting the *ureC* gene and other genes driving the microbial nitrogen cycle by building upon past studies in ureolytic environments [110, 111, 126, 127].

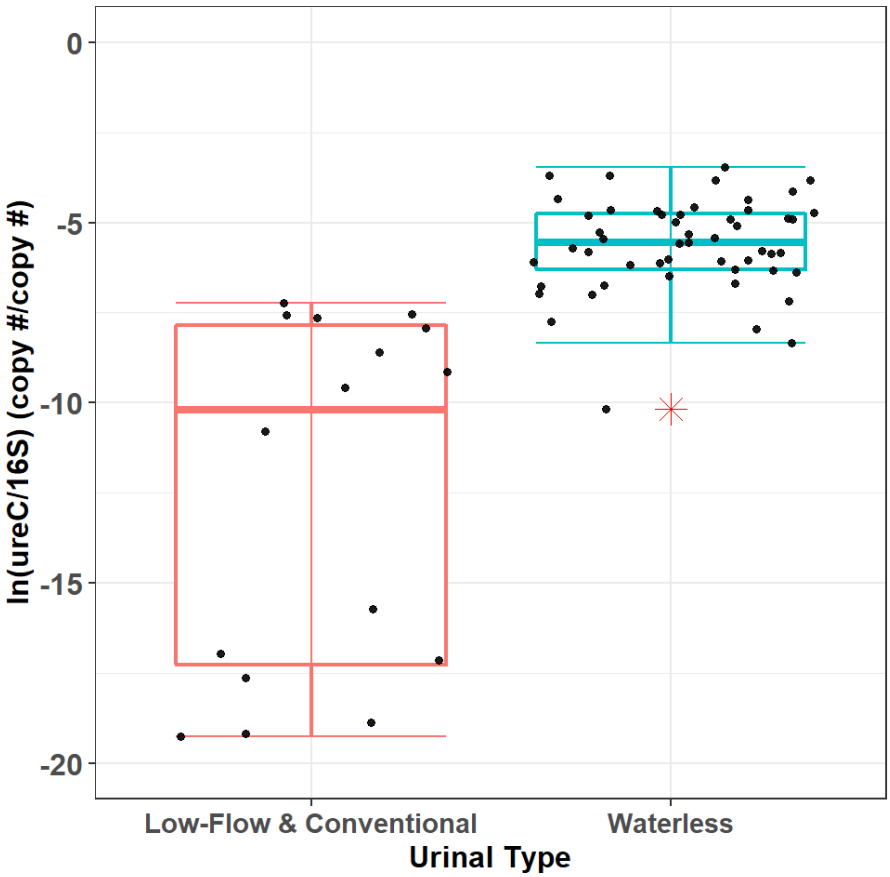

Figure 3. Natural logarithmic transformations of *ureC*/16S gene ratios grouped by urinal type. Low-flow and conventional urinal measurements were aggregated as one group in a comparison with waterless biomineral natural log transformed *ureC*/16S copy number ratios.

3.3 Low correlations between alpha diversity and biomineral urease activity

The activity of urease was of particular interest due to its role in biomineralization. A lack of correlation between diversity scores suggests that richness and evenness alone cannot linearly predict the ammonia producing capacity associated with a given biomineral sample. Simple linear regression between Shannon indices and biomineral ureolytic activity yielded a poor goodness of fit as shown in S3 Fig from the S1 File (R^2=0.014). Therefore, the community

composition may not be a strong predictor of the urease activity associated with a given biomineral sample, even if there may be differences in the abundances of the potentially ureolytic community. The differences in abundances are indicated by proxy of the *ureC*/16S gene ratios for samples grouped by urinal type. Cumulatively and in direct continuity of our previous study focusing solely on the effects of environmental variables from the same sampling periods on biomineral urease activity, we conclude that the abundances of a ureolytic bacterial community and the within-sample diversity does not strongly influence biomineral activity. Our previous study suggests through a multiple regression analysis that an increase in *ureC* gene abundances does not necessarily suggest stronger urease activities [27].

In addition to quantifying gene abundances as done in this study, future studies should assess whether the transcriptional activity of *ureC* gene is influenced by urinal type and intersystem sampling locations using reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) to quantify biomineral *ureC* mRNA levels. Quantifying the mRNA transcripts can provide insight into how the ureolytic community may be upregulating *ureC* transcriptional activities to produce more urease in response to differing conditions specific to the habitat. Future microbial ecology studies on urine drainpipe environments should also monitor nutrient concentrations to determine community structure relationships with mRNA transcripts.

3.4 Microbial community composition in biomineral samples vary by urinal type

Fig 4 summarizes the abundances of taxa at the phylum, class, and genus level associated with the biomineral samples. These bar plots agree with the decrease in the alpha diversity scores based on the amount of flush water associated with each urinal type. Fig 4 suggests that the composition of conventional and low-flow biomineral samples are more similar to each other than either are to waterless biomineral samples.

At the phylum level, Firmicutes dominated the compositional structure for waterless urinals and accounted for 95.6% of all OTUs, whereas 3.4% were Proteobacteria and the remaining 1% of phyla were rare (< 2.5% relative abundance). Conversely, low-flow urinals are more diverse and were composed of 17% Actinobacteria, 54.7% Firmicutes, 24.8% Proteobacteria, and the remaining 3.5% phyla exhibited less than 2.5% relative abundance. In conventional urinals, the relative abundances of 39.6% Actinobacteria, 28.1% Proteobacteria, 26.7% Firmicutes, and 3.5% Bacteroidetes were observed. The dominance of Firmicutes in the waterless urinal biomineral samples is consistent with past metagenomic studies on saline and alkaline soils, which is sensible as the urinal environment is expected to be more saline and alkaline compared to that of conventional urinals [128]. The pluralities of Firmicutes, Actinobacteria, and Proteobacteria in samples obtained from conventional and low-flow urinals also match observations made in past restroom microbial ecology studies [129]. Firmicutes and Bacteroidetes have been reported to have a key role in organics hydrolysis and in secondary fermentation, and may thrive in environments with organic content such as the undiluted urine found in waterless urinals [130]. Though it is unclear why Firmicutes can tolerate high ammonia concentrations, that Firmicutes can may explain why they encompass the largest fraction of the community in waterless urinals, which have more concentrated urine and thus more ammonia when compared to flush-type urinals [131–133]. In biogas reactors, Firmicutes dominated the community composition in environments with total ammoniacal nitrogen concentrations (TAN) ranging from 2.4 to 4.2 g/L [133].

At the class level, waterless urinals also exhibited an increase in relative abundances of the Bacilli (86.1 %) class compared to that found in low-flow (41.3 %) and conventional (20.5 %) biomineral samples. The presence of Bacilli has been previously observed in ureolytic

microbially induced calcite precipitation soil studies [102]. Gat et al. (2016) cites Burbank et al. (2012) where it was found that 7 of 10 isolates of indigenous ureolytic soil bacteria were classified as Bacilli, which are members of the Firmicutes phyla known to be ammonia resistant as mentioned previously [134]. Gat et al. (2016) also found that a significant increase in Bacilli following ureolytic treatment coincided with an increase in culture media pH. It can then be inferred that greater urease activities observed in waterless urinal biomineral samples in our past regression study also coincide with the abundance of Bacilli bacteria [27]. Future studies can include high throughput Illumina sequencing of the *ureC* gene to determine the relative abundances of various taxa containing the gene.

Conventional urinal biomineral samples have bacterial communities distinct from both low-flow and waterless urinal samples, but the availability of ureolytic biomineralization present in the drain lines demonstrates that biomineral formation can occur in all urinal types despite differences in bacterial communities. Of the three urinal types, conventional urinals have the lowest relative abundance of Firmicutes (26.6 %), but the greatest percentage of Actinobacteria (39.6 %). It is possible that where Bacilli drives biomineralization in low-flow and waterless urinals, Actinobacteria could be the driver of precipitation in conventional urinals. In other ureolytic biomineralization studies on cave moonmilk formation, researchers have proposed that Actinobacteria promotes calcium carbonate precipitation by creating locally favorable conditions with the bacterial cell walls serving as the crystal nucleation sites [101, 135]. That ureolytic organisms and biomineralization found in conventional urinals persist as they do in waterless urinals suggests that increasing flush water volumes may not be enough to prevent ureolytic pipe fouling. Future studies should compare the composition of the biomineralization between each urinal type and determine the influence of the bacterial community on the mineral composition.

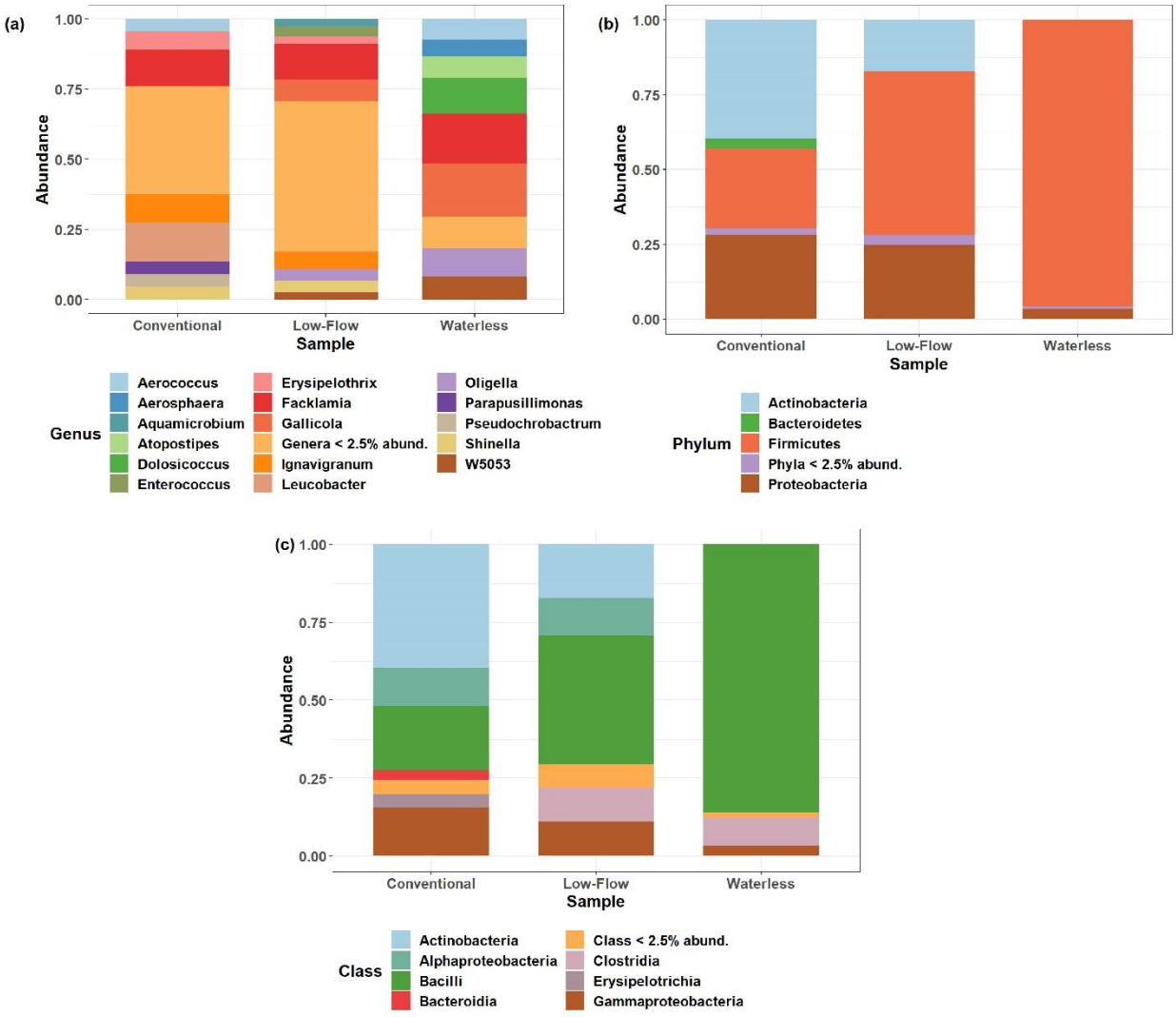

Figure 4. Taxonomic relative abundance bar charts grouped by urinal type. Bar charts showing relative abundances of the bacterial community grouped by urinal type are depicted at the (a) genus, (b) phylum, and (c) class level. Results were summed and averaged for all samples respective of their urinal types.

Cumulatively, our results confirm that restrooms host a diverse microbial community [129]. We found that the relative abundance of rare OTUs (< 2.5% relative abundance) at the genus level is 11.5% for waterless urinals compared to that observed for conventional (48.7%)

and low-flow (53.9%) urinals. The abundances and richness of rare OTUs is consistent with the alpha diversity hypothesis testing results. This marked difference between the abundances of rare OTUs found in waterless and flush-type urinals is likely attributed to the differences in ammonia concentrations imparted by flush water dilution. As discussed previously, elevated ammonia concentrations and pH may lead to more a selective environment in waterless urinals where the selected alkaliphiles can tolerate elevated pH and ammonia levels. Of note, several of the genera observed can be ureolytic, but this is inconclusive unless species level identification is obtained. These genera may include *Shinella, Morganella, Tissierella, Thauera, Parapusillimonas, Pseudomonas,* among the more commonly observed *Oligella* [136–140]. Additionally, some genera identified in this study could be potentially pathogenic. These include genera such as *Morganella, Tissierella, Erysipelothrix, Atopistipes*, and *Facklamia* [137, 141–143]. Past research is consistent with our observations that potentially pathogenic genera are present in most conventional, low-flow, and waterless urinal biomineral samples [75]. Through our findings, we concur with Lahr et al. (2016), who highlighted the limitations of solely relying on enteric indicator organisms to assess bacterial risks involved in freshly (< 24 hours) collected urine from social events [75].

It was also expected that the microbial communities present in urinals are also influenced by the surrounding environment and its users. From Fig 4, the presence of *Facklamia* and *Corynebacterium*, which are typically associated with the urinary tract, is not surprising given that all sampling took place in men's restrooms [144]. Other observed genera such as *Enterococcus* have been identified as common colonizers of human skin and gut [129]. OTUs associated with manure, compost, and soil were also identified and include *Shinella, Georgenia, Gallicola, Allorhizobium, Thiopseudomonas, Leucobacter, Tissierella, Atopisiptes,*

Corynebacterium, and *Erysipelothrix* [138, 142, 145–149]. It has also been established that microbial transport can take place by air currents. Similarly, toilet and urinal flushing propels aerosols from toilet bowls into the air, and can subsequently settle on surfaces including other fixtures in the room [129]. Given the dynamic nature of busy public restrooms, it is possible that there could be cross-contamination between microbes found on the ground and those in the fixtures.

3.5 Beta diversity analysis demonstrates the community compositional shifts influenced by intrasystem sampling location, urinal type, sample type

Community (dis)similarity was estimated using the Bray-Curtis metric. From the GUSTAME multivariate statistics guide, NMDS plots can be interpreted as such [150]: objects that are ordinated closer are likely to be more similar than those further apart. The scale of the axes is arbitrary as is the ordination of the plot. Tight clusters of points that are well-separated from other clusters indicate sub-populations in the data. Fig 5 shows strong similarities between bacterial communities grouped by intrasystem sampling location indicated by strong clustering for biomineral associated communities by their respective groups. There is also suggested a goodness of fit as indicated by a low NMDS stress value of 0.06 [150]. In general, communities from gallery drain lines appear distinct from communities from waterless urinal cartridges, but some adjacent clustering is also apparent. PERMANOVA was used to test if there was a significant difference between groups of interest [150].

The community compositions significantly differ when grouped by urinal type, intrasystem sampling location, and sample type. Clustering by urinal type is apparent, whereby the bottom left side of the NMDS diagram is dominated largely by waterless samples clusters while other regions represent biomineral communities found in flush-type urinals.

PERMANOVA testing for biomineral samples with at least three samples per factor also confirms that the community structure strongly differed by intrasystem sampling location (Pseudo-F = 32.56, $p = 0.001$) and urinal type (Pseudo-F = 33.03, $p = 0.001$). There also appears to be an interaction between the two main effects (Pseudo-F = 32.67, $p = 0.001$). This interaction may indicate that the observed effects on biomineral microbial communities from sampling locations depend on the urinal type. From Fig 5, a pronounced shift between communities is clear when comparing the samples taken at the user interface from the waterless urinal cartridges and the main drains, which contacts urine diluted by sink water. It is possible that the presence of water and its effects on nutrient concentrations could be influencing the bacterial community as has been observed in past soil microbial ecology studies [151]. Further PERMANOVA tests suggest that the community structure differs when grouped by biomineral and liquid sample types (Pseudo-F = 12.16, $p = 0.001$). When also controlling for urinal type in the PERMANOVA model, there were weak interactions between sample type and urinal type as variables (Pseudo-F = 2.54, $p = 0.019$).

Our statistical test suggests that the community structures differ by sample types, but this observation disagrees with Lahr et al.'s (2016) empirical observations on the similarity of liquid and struvite associated communities. Communities may differ between the liquid and biomineral samples for several reasons. [152]. For one, the biofilms present in biomineralization may confer a resilience that contributes to a localized microbial community. In dental plaque studies, researchers found that the production of an adequate amount of ammonia generating capacity in ureolytic oral biofilms is essential for the stabilization of microbial communities [153]. Li et al. (2016) demonstrated that urease positive biofilms with homogeneously distributed biomineral precipitates showed greater survival when exposed to ciprofloxacin compared to a urease

negative biofilm that lacked biomineral precipitates subjected to identical conditions [85]. More so, the age of the microbial communities also differs between sample types and may affect community composition. Liquid samples found in drain traps or waterless urinal cartridges are constantly flushed out as people urinate in them. Conversely, communities found in biomineral samples are more likely established and stable as they are protected by the biofilm that prevents them from being washed away.

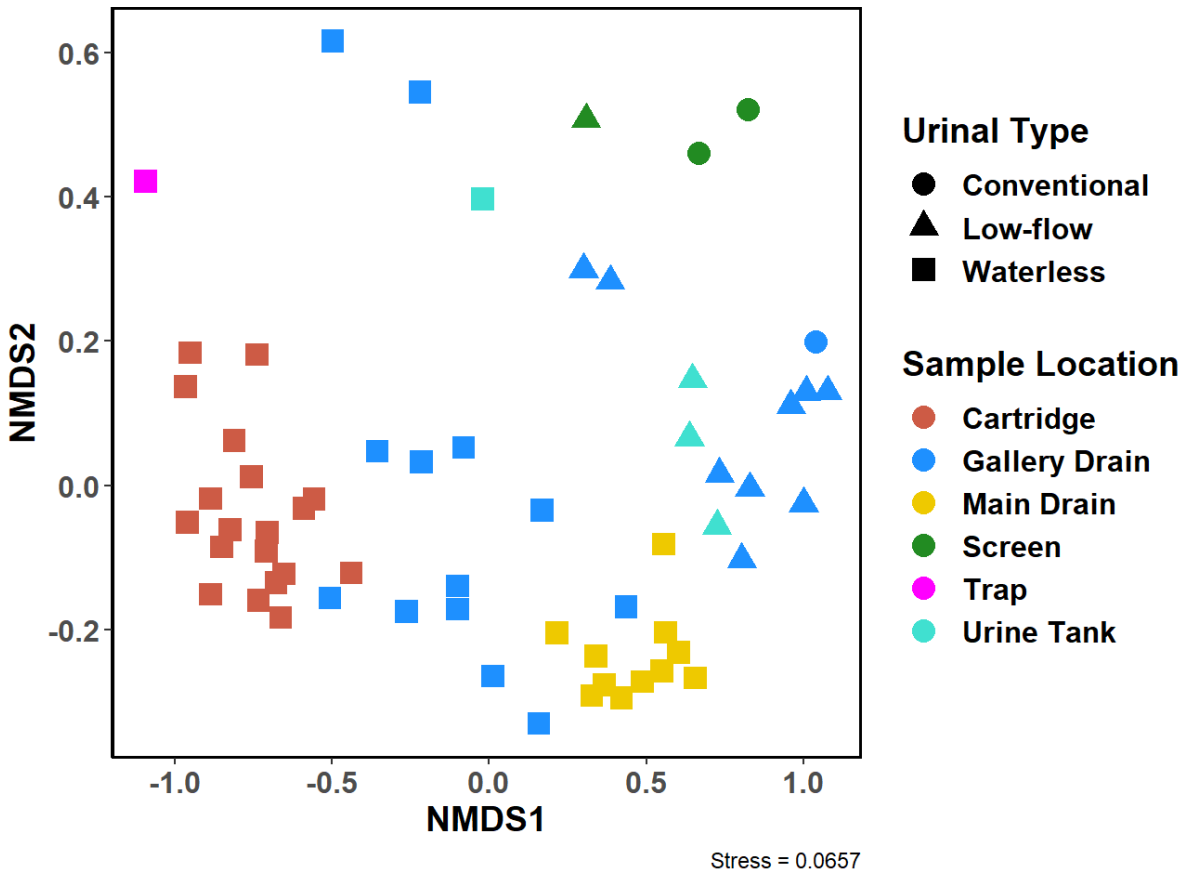

Figure 5. NMDS diagram of bacterial communities for all biomineral samples grouped by intrasystem sampling location.

The influence of when the samples were taken with respect to season (summer or winter) on the biomineral bacterial community structure is inconclusive—this is based on comparing PERMANOVA results using all biomineral samples and those only obtained from Westley. Based on PERMANOVA results, there were no significant large effect size differences in bacterial community composition in biomineral samples obtained from Westley between those sampled in summer and winter months (Pseudo-F = 0.973, $p = 0.337$). For Westley biomineral bacterial communities, there was also no observed interactive effects between sampling season and intrasystem sampling location (Pseudo-F = 2.06, $p = 0.097$). However, PERMANOVA testing for all sample sites including Westley suggests that the bacterial compositions can vary by sampling season (Pseudo-F = 5.26, $p = 0.014$) with interactive effects between sampling season and intrasystem sampling location (Pseudo-F = 3.59, $p = 0.007$). Community differences due to sampling location, but not sampling season are also illustrated by distinct clustering between the three sample locations in the NMDS diagram describing Westley biomineral samples (Fig 6). Notably, our previous multiple regression study suggests that sampling seasons were not a strong and significant predictor of the biomineral urease activity [27]. Because this is the first report on ureolytic biomineral communities from urine drainage systems, future microbial ecology studies on urine drainage systems should continue to describe the effects of seasonality for comparison. Inconclusive observations on the (dis)similarity between biomineral bacterial communities grouped by seasonality was unexpected, as temperature and seasonality is a common distinguishing factor between communities [154, 155].

Conversely, the biomineral bacterial communities associated with Westley (Fig 6) followed similar trends observed for all sample sites (Fig 5). Biomineral communities from

Westley significantly differed based on intrasystem sampling locations within the urine drainage system (Pseudo-F = 31.56, p = 0.001).

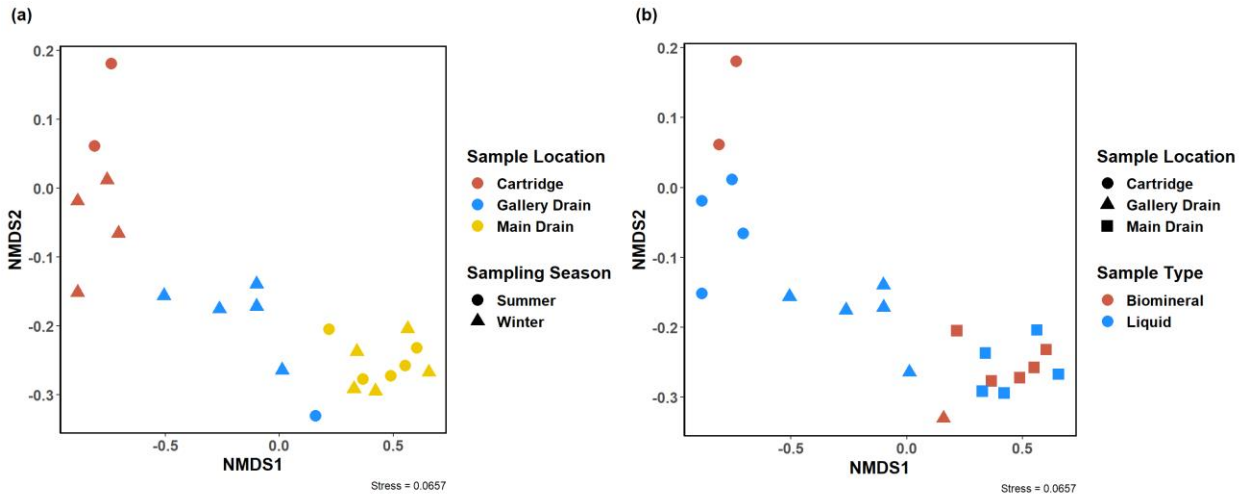

Figure 6. NMDS diagram of bacterial communities for biomineral samples from Westley rest area (a) grouped by sample location and sampling season, and (b) grouped by sample type and sample location.

3.6 Limitations of the study

Due to cost and scheduling constraints, one limitation of this microbial ecology study is that some locations were more well-sampled than others. For example, only two conventional urinal sites were studied, but this leaves many opportunities for future microbial ecologists to explore. Having more evenness in the sampling distribution would have conferred greater statistical power when drawing conclusions about conventional urinal biomineral samples.

Moreover, certain rest areas were more well sampled than others due to the availability of biomineral samples in the pipes and urinal cartridges. Cleaning, at times, affected the availability of biomineralization in the drainpipes, and so sufficient amounts may not have been available during certain sampling days. Consider that some rest areas may have been recently cleaned by

maintenance staff prior to sampling while other sites may have established biomineral microbial communities in the drain lines. Some rest areas were also designed to be more accessible than others, as clean-out ports were not installed in every plumbing gallery. This meant that obtaining biomineral samples from certain locations at each rest area may not have been feasible. In addition to making it difficult to study some of the facilities, the lack of clean-out ports is a design flaw, as it would make removing clogs difficult and costly should they occur. In the future, engineers should incorporate clean-out ports in waterless urinal plumbing fixtures where possible.

It is possible that the environmental metadata used in the NMDS was incomplete as suggested by Mantel testing. A Mantel test was used to estimate the degree of geographic distance influencing the compositional differences between various rest sites. It was found that the microbial community is more strongly correlated with geographic distance than with the environmental factors (Mantel statistic r: 0.685, $p < 0.001$). The correlations in the Haversine distance matrix suggest that geographically closer samples are compositionally more similar to each other than those further apart. This is expected as environmental conditions found in regions grouped closer together are also expected to be more similar. Mantel testing also demonstrated that the measured environmental factors are moderately correlated with biomineral microbial community (Mantel statistic r: 0.351, $p < 0.001$). Samples which are similar in terms of moisture content, biomineral ureolytic activity, *ureC* gene copies/VS (g), and VS per total solids (TS) ratios tend to be more similar in terms of their microbial community composition. It is possible that the environmental factors are less correlated to the community composition than geographic distance because the environmental dataset is incomplete. Had we included pH, nutrient, and ion concentrations in our study, then there could have appeared stronger

correlations between environmental variables and the community composition indicated by the r value in the Mantel test.

Finally, the greater influence by sampling sites on community composition than the environmental variables measured in this study indicates another possible limitation of the study. Because rest areas are highly dynamic environments, there are uncontrolled factors such as differences in cleaning products as well as the frequency and intensity of cleaning performed at each rest area. Differences in cleaning procedures and frequency can also affect the age of the biomineral samples within the pipes and cartridges. Subsequently, the age of the samples was uncontrolled in the study, which could affect the physical and community composition of the samples. Though controlling for sample sage would be ideal, we note that it would have been impractical to impose a strict cleaning schedule on rest area laborers statewide. Future studies on the effects of biomineralization age on the microbial community structure and on urease activity should be conducted.

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CHAPTER III

A Multiple Regression Assessment of the Biomineral Urease Activity from Urine Drainpipes of California Public Restrooms

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Declarations

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Conflicts of Interest: The authors declare no competing financial interest.

Data/Code availability: The R Markdown HTML output containing the script and results can be found in the Supplementary Information section. The raw data can be found in the Dryad repository (DOI:10.25338/B82906). The preprint can be found on BioRxiv (DOI: 10.1101/2021.02.18.431895).

Author Contributions: Kahui Lim performed the statistics and field sampling. Harold Leverenz provided editorial review and performed the field sampling. Samantha Barnum and Cara Wademan performed qPCR.

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Abstract

Clogging and odor is strongly associated with ureolytic biomineralization in waterless and low-flow urinal drainage systems in high usage settings. These blockages continue to hinder widespread waterless and low-flow urinal adoption due to subsequent high maintenance requirements and hygiene concerns. Through field observations, hypothesis testing, and multiple regression analysis, this study attempts to characterize, for the first time, the ureolytic activity of the biomineralization found in alternative technologies located at 9 State-owned restrooms. Multiple regression analysis (n = 55, df = 4, R^2 = 0.665) suggests that intrasystem sampling location ($\hat{\beta}$ = 1.23, $p < 0.001$), annual users per rest area ($\hat{\beta}$ = 0.5, p = 0.004), and the organic/inorganic mass fraction ($\hat{\beta}$ = 0.59, p = 0.003), are statistically significant influencers of the ureolytic activity of biomineral samples ($p < 0.05$). Conversely, *ureC* gene abundance (p = 0.551), urinal type (p = 0.521) and sampling season (p = 0.956) are not significant predictors of biomineral ureolytic activity. We conclude that high concentrations of the urease alpha subunit, *ureC*, which can be interpreted as proxy measure of a strong, potentially ureolytic community, does not necessarily mean that the gene is being expressed. Future studies should assess *ureC* transcriptional activity to measure gene expression rather than gene abundance to assess the relationship between environmental conditions, their role in transcription, and urease activities. In sum, this study presents a method to characterize biomineral ureolysis. This study establishes baseline values for future ureolytic inhibition treatment studies that seek to improve the usability of urine collection and related source separation technologies.

Keywords: ureolysis, urine source-separation, biomineralization, ureolytic activity

1. Introduction

Waterless and low-flow urinals reduce water consumption, improve hygiene with touchless operation, and can be used for source separation of urine; additionally, waterless systems require less plumbing than conventional systems. However, these source-separation technologies are susceptible to biomineralization [12, 16]. Biomineralization, usually of a mixed composition of struvite, calcium phosphate, calcium oxalate, and calcium carbonate, has plagued urine diversion projects since the earliest projects were studied, leading to clogging, odor, and overall user dissatisfaction [12, 15, 16, 156].

Researchers have described the formation of biomineralization in terms of (a) cellular activities, (b) passive formation of crystals caused by biofilms, and (c) biological and chemical facilitation of crystal supersaturation conditions [20–22]. Biomineralization in urine source-separation contexts is likely governed by a combination of mechanisms.

Urease and its ureolytic activity are measures of biomineralization potential because the rate of precipitation is dependent, in part, on the rate of increase of media pH, which depends on the rate of ureolysis. The elevated pH resulting from ureolysis plays a critical role in the supersaturation crystal formation process. Because urinals are subject to intermittent supplements of a urea and an ion source, urinals and urine drainage traps become a selective breeding ground for ureolytic organisms that cause an increase in the pH of collected urine and facilitate mineral precipitation as has been observed in urological devices[75]. Ureolytic bacteria responsible for the biomineralization use the nickel-dependent metalloenzyme, urease, to catalyze the hydrolysis of urea into ammonia and bicarbonate which in turn raises the pH and creates conditions favorable of precipitation [15]. In a past catheter study, researchers have demonstrated that rates of calcium and magnesium encrustation caused by various ureolytic

bacteria isolates is correlated with an increase in ureolytic activity [104] An elevated pH promotes calcium phosphate and oxalate stone formation due to a shift in phosphate speciation from HPO_4^{2-} to PO_4^{3-} and the decomposition of ascorbic acid into oxalate—both cases represent an increase in ion concentrations that lead to elevated encrustation rates found in catheters [24]. Ureolysis also results in carbonate and bicarbonate ion formation which can further contribute to biomineralization as the urine becomes supersaturated [98]. Researchers similarly showed that greater ureolytic rates from bacterial urease are correlated with greater rates of calcium carbonate precipitation [103, 157, 158]. Studies using *Proteus mirabilis* have shown that urease defective mutants fail to form crystalline biofilms in laboratory models, demonstrating the key role of pH and urease activity in crystal formation [152]. In dental plaque studies, researchers suggest that ammonia generating capacity in a mixed-species model of ureolytic oral biofilms is essential for the stabilization of microbial communities in ureolytic environments [153]. Losses of sufficient quantities of urease resulted in the acidification of biofilms and a decrease in community diversity [153].

Through multiple linear regression modelling, this study will be the first of its kind to: (a) model biomineral enzyme activity in terms of both categorical and quantitative predictors, (b) examine biomineral enzyme activity from urine source-separation technology, and (c) do so on a geographic scale with a sufficiently large sample size. This study also builds upon previous works describing soil or biofilm ureolytic activity that (a) use small sample sizes in parametric hypothesis tests (n=6) or multiple regression (n=4), (b) neglect discussion of model validation beyond the coefficient of determination (R^2), (c) do not discuss whether their data fits assumptions required for application of a statistical test, and (d) mention statistical significance, but not practical significance, i.e. the magnitude of effect [159–162].

Finding a link between environmental parameters such as intrasystem sampling location, usage frequency, seasonality, gene abundance found through qPCR, and urinal types with the enzymatic activity of the biomineral samples will be useful in understanding the effects of restroom configuration on ureolytic activity. Understanding the effect of seasonality and sampling locations within a urine drainage system where ureolytic activity is highest may be insightful when predicting locations and times of year where the components of the urine collection system are most susceptible to biomineral fouling.

2. Materials and methods

The coming subsections will describe the sampling procedures and locations followed by methods used in downstream analyses to quantify the environmental variables used in the statistical analyses. The R Markdown HTML output containing the script can be found in the Online Resources section. The raw environmental data can be found in the Dryad repository (DOI:10.25338/B82906) as an .RDS file.

2.1 Sample collection

In the summer (July-August) and winter (December) of 2019, a total of 9 of public rest areas owned by the California Department of Transportation were sampled for ureolytic biomineralization. Due to poor pipe gallery design and lack of access points, no biomineral samples were collected from Honey Lake. The Honey Lake rest area, however, was assayed using the *in-situ* urease test later discussed.

These rest areas are situated throughout California along rest areas and had varying usage frequencies as estimated by California Department of Transportation using highway ramp volume counts [32]. Rest areas were categorized by the types of urinals installed: conventional ~

1 gal per flush (3.78 L per flush), low-flow ~0.125 gal per flush (0.473 L per flush), and waterless (no flush).

Biomineralization deposits were scraped into sterile 50 mL conical tubes from fouled fixtures and drainage systems when available. A total of 2 conventional, 2 low-flow, and 5 waterless public restrooms along California highways, also known as rest areas, were observed in this study. The men's restrooms were typically fitted with two urinals at two different heights to conform to the American Disability Act (ADA).

All samples were stored in an ice chest after collection and processed within three days of sample collection. Previous work monitoring the ureolysis rate in soils have found that a distinct slowdown in ureolytic rate was not detected until 8 months of cooled storage [106]. As such, the sampling preservation measures were deemed adequate.

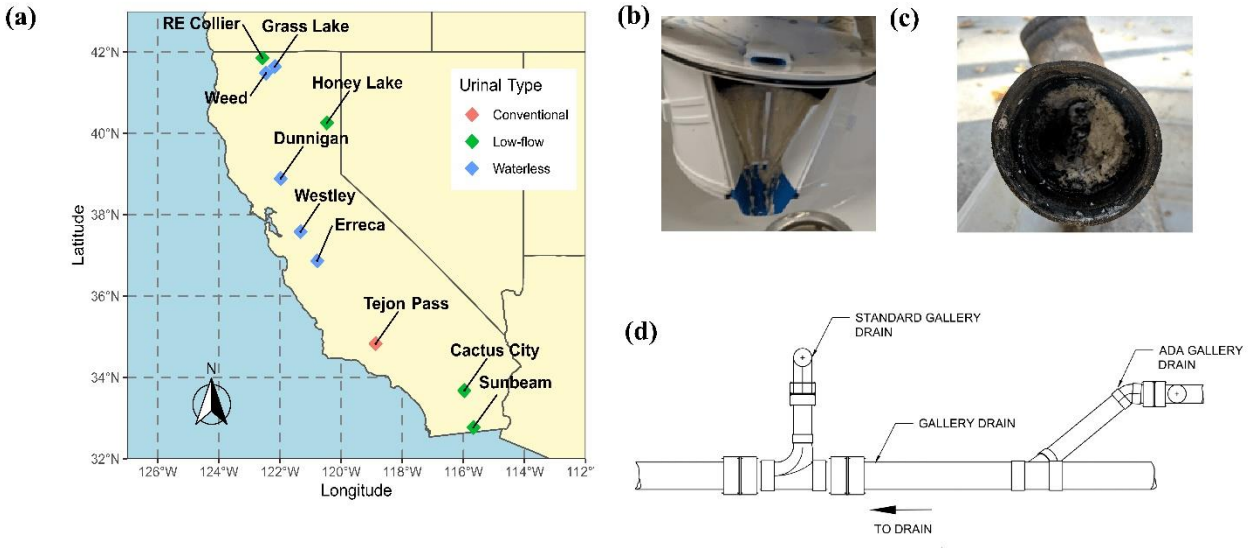

Figure 1 A summary of sites, drainpipe configurations, and characteristic samples are shown in Figure 1 as: (a) location of sampling sites with respect to urinal type used in this study, (b) biomineralization formation on a waterless urinal cartridge at Erreca on 16 Sep 2019, (c) a view of reduced internal pipe diameter by biomineralization in a urine drainage pipe at the

Dunnigan northbound oriented public rest area on 12 Dec 2019, and (d) general drainage system layout consisting of the drains directly connected to the urinals, which collectively flows into a main drain also connected to the sink drains.

2.2 Biomineral ureolytic enzyme activity characterization

To compare enzymatic activities of biomineral samples between various sites *in vitro*, a known wet mass of the biomineral samples was suspended and mixed in a 100 mL volume of 7.3 pH 200 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) buffer containing 2.5% urea m/m. The rate of increase in conductivity is proportional to that of urea hydrolysis and can be used as a surrogate measure for enzymatic activity [108]. As a comparative basis between samples, one unit of specific activity is defined as uS cm^{-1} min^{-1} per gram of volatile solids (VS).

Gravimetric analyses followed standard methods for the examination of water and wastewater [109]. A mass balance was performed by comparing the wet solid mass with the dry mass following 105^{o}C desiccation and fixed mass after 550^{o}C ashing. Volatile solids can then be determined and represents the organic matter in a given sample. Each biomineral sample was analyzed in triplicate and then averaged.

2.3 Quantifying gene abundance using real-time polymerase chain reaction (qPCR)

To examine the relationship between *in vitro* ureolytic activity and the genetic predispositions for ureolysis, the genomes of phylotype representatives for the presence of urease genes were examined by qPCR. A similar protocol was described previously [110]. The urease associated gene were designed on the urease alpha subunit encoding gene (*ureC*). Primer

sequences were obtained from the literature [111]. Sensitivity and efficiency were established from the y-intercept and slope of the standard curve, which was created by running triplicate, 10-fold serial dilutions of plasmid DNA containing the ligated amplicon of each gene (Eurofins Genomics LLC, Louisville, KY). The sensitivity of ureC-F (TGGGCCTTAAAATHCAYGARGAYTGGG) and ureC-R (SGGTGGTGGCACACCATNANCATRTC) was <4,000 copies/qPCR reaction and the efficiency was 80.6% (R^2 = 0.9974). Poor sensitivity and low efficiency for *ureC* is expected due to the nature of SYBR degenerative primers. Biomineral samples were kept frozen at -20°C prior to DNA extraction. DNA was manually extracted from 0.25 g of sample using a commercially available kit following manufacturer recommendations and eluted in 100 µL of diethylpyrocarbonate (DEPC) treated water (Qiagen DNeasy Power Soil Kit, cat # 12888-50). Each 12 µL reaction contained 6 µL SYBR master mix (Applied Biosystems SYBR Green PCR Master Mix, cat # 4309155), 0.48 µL of a primer-water mixture (primers at final concentration of 400 nM), 4.52 µL of DEPC-treated water, and 1 µL of extracted DNA. qPCR was performed using an automated fluorometer (ABI PRISM 7900 HTA FAST, Thermo Fisher Scientific). Standard amplification conditions were used: 95°C for 3 min, 40 cycles of 95°C for 15 s, 52°C for 30 s, and 72°C for 30 s, with a melting curve at 95°C for 15 s, 52°C for 15 s, and 95°C for 15 s. Data was analyzed using Applied Biosystems SDS software, version 2.4. Fluorescent signals were collected during the annealing phase and C_q values extracted with a threshold of 0.2 and baseline values of 3–10 for the *ureC* assay . Amplification specificity was verified using the dissociation temperature (T_m) of the qPCR amplicons specific to each gene. Acceptable T_m ranges were determined to be +/- 2% of the positive controls. For *ureC*, the acceptable T_m range was 80.8°C - 84.1°C. Samples with detectable amplification but with T_m's outside of the

acceptable ranges were considered false positives and were deemed negative for the gene of interest. The absolute copy numbers were also normalized in terms of volatile solids (VS) mass present in the biomineral samples.

2.4 Statistical analyses

All statistical work and data visualization was done using R version 4.0.2. An a-priori power analysis was first used to inform the design of this study, suggesting that a linear model can sufficiently capture a large effect size (f=0.35) at a level of significance of 0.05 for a power of 0.8 using 1 tested dependent variable and 5 total predictors with a minimum sample size of 25 [163]. After excluding sample rows missing data from low quality qPCR reads and samples that did not have enough mass for gravimetric analysis or biomineral enzyme activity, this randomly sampled, complete case analysis included a sample size of 55 from 9 different facilities. In the regression analysis, conventional urinals were aggregated with low-flow urinals because both urinal types include flush water. A stepwise forward variable selection method was used. A corrected Akaike information criterion (AICC) was also used to validate model selection [164]. The ordinary least squares (OLS) multiple regression analysis was performed assuming that a natural log-log transformed linear model is an adequate descriptor of the system, whereby normality was verified in the Supplementary Information section. A natural log-log transformed dataset (as represented in Supplementary Figure 3) enables for a practical interpretation of the effect size as a percent change, or in this case, the elasticity between two biological variables [165]. Regression coefficients were interpreted as natural log-level for categorical variables. For the coming subsections, unless specified, variables will be discussed in terms of natural logarithms.

2.5 Characterizing the ureolytic activity in urinal traps in situ

In situ urinal trap testing was conducted to characterize the ureolytic rate at the time of sample collection within the urine drain trap. *In situ* biomineral ureolytic activity was used to support the regression analysis derived from *in vitro* urease assays. The project team developed a method using pH and conductivity meters to characterize the baseline ureolytic rates. A description of the trap testing is shown graphically in Figure 2.

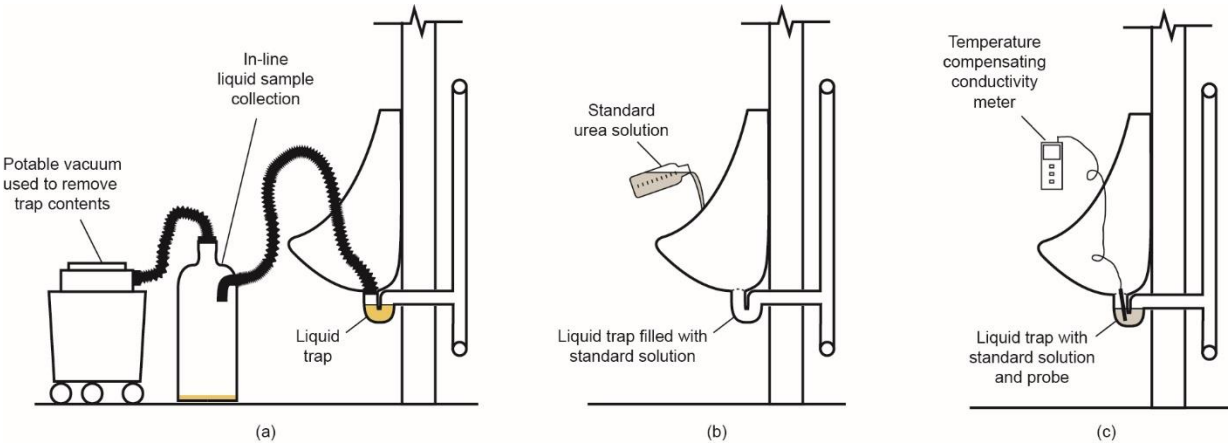

Figure 2 Schematic of *in situ* trap activity test procedure: (a) using portable vacuum and in-line liquid sample collector to remove trap contents, (b) application of standard urea solution to empty trap, and (c) testing of urinal liquid trap to determine relative activity

The in-situ urinal trap procedure was conducted as follows:

First, the urine drain trap is vacuumed out as shown in Figure 2. Once emptied, a 200 mM 7.3 pH HEPES buffer containing 2.5% m/m urea is added until the drain trap is full. Logging pH and EC meters were submerged in the trap opening and recorded for a total of 10 minutes from which the ureolytic rate could be estimated using the rate of EC formation.

The in-situ urinal trap procedure was conducted as follows:

3. Results and discussion

After evaluating and selecting the most parsimonious multiple linear regression model composed of categorical and quantitative environmental variables, the observed influence, or lack thereof, of these variables will be discussed in the context of biomineral ureolytic activity.

3.1 Multiple linear model and validation

The multiple linear model composed of 55 observations is described in Tables 1 and 2. As shown in correlation heatmaps and residual analysis from Supplementary Figures 1 and 2, the linear model is in agreement with the Gauss-Markov OLS regression assumptions, which require that: a) the expected value of the regression residuals tends towards zero, b) the residuals are homoscedastic c) there is no autocorrelation between the regressors and the residuals such that exogeneity is upheld, d) the predictors are not multicollinear, and e) the residuals are also normal [165]. The residuals shown in Supplementary Figure 1 do not appear to have a trend based on the index plot, do not exhibit any correlation with each other from the autocorrelation plot, and appear homoscedastic from the fitted values vs. residuals plot. Finally, the residuals also appear normally distributed from the quantile-quantile plot in Supplementary Figure 1. As such, it was concluded that the natural log-log linear model appropriately describes natural logarithmically transformed data and that the model fits well with the data. The AICC model selection results are shown in Supplementary Table 2, suggesting that the most parsimonious and probable model is Model 3 [164, 166].

The regression results describing the most probable model (Model 3) is shown in Table 1 and 2, which also depicts the regression results from other tested models. The results presented in Tables 1 and 2 suggest that *ureC* gene concentrations (Model 4, $p = 0.551$), sampling season (Model 5, $p = 0.956$), and urinal types were statistically insignificant predictors of ureolytic

activity ($p > 0.05$) and of low practical significance as indicated by the relatively small regression coefficients (see Table 6). From Table 1, the strongest predictor of biomineral ureolytic activity was the sampling location, namely, those sampled from the main urinal drainage pipes exhibited the greatest enzymatic activity. In Model 3, the second strongest predictor was the organic to inorganic fraction. Annual number of users at a given rest area also positively influenced urease activity likely due to the increased loading and usage frequency resulting in a semi-constant stream of nutrients and salts necessary for a strong ureolytic community to develop and thrive.

Table 1 Summary of effect sizes of significant predictors on biomineral ureolytic activity

Significant Predictor Variables	$\widehat{\beta}$	**CI (95%)**	**Effect on Biomineral Activity per g VS as Elasticity[a]**
Annual Users per Rest Area	0.5	0.17, 0.82	A 25% increase in annual users per rest area corresponds to a 11.7 (3.9, 20.1) % increase in biomineral activity
VS/TS (g/g)	0.59	0.21, 0.97	A 25% increase in VS/TS (g/g) corresponds to a 14.1 (4.8, 24.2) % increase in biomineral activity
Intrasystem Location: Main Drain	1.24	0.83, 1.64	Compared to samples obtained from cartridges, those obtained from the gallery main drain had a 245 (129, 416) % larger geometric mean in biomineral activity

[a] Parenthetical contents represent effect sizes at limits of confidence intervals

Table 2 Multiple regression summary of model predicting biomineral ureolytic activity

	Model 1	**Model 2**	**Model 3**	**Model 4**	**Model 5**	**Model 6**
Predictor Variables	Estimates	Estimates	Estimates	Estimates	Estimates	Estimates
Intercept	**-7.35 (-12.80 – -1.90)****	-2.18 (-6.92 – 2.57)	-0.55 (-5.05 – 3.94)	-0.46 (-5.00 – 4.08)	-0.56 (-5.12 – 3.99)	-0.36 (-4.92 – 4.20)
Annual Users per Rest Area	**0.96 (0.56 – 1.37)*****	**0.57 (0.22 – 0.92)****	**0.50 (0.17 – 0.82)****	**0.47 (0.14 – 0.81)****	**0.49 (0.16 – 0.83)****	**0.49 (0.16 – 0.82)****
Intrasystem Location: Gallery Drain		-0.28 (-0.62 – 0.07)	-0.19 (-0.52 – 0.13)	-0.15 (-0.51 – 0.21)	-0.19 (-0.53 – 0.15)	-0.14 (-0.50 – 0.22)
Intrasystem Location: Gallery Main Drain		**1.02 (0.61 – 1.43)*****	**1.24 (0.83 – 1.64)*****	**1.26 (0.85 – 1.68)*****	**1.24 (0.83 – 1.65)*****	**1.23 (0.82 – 1.64)*****
VS/TS (g/g)			**0.59 (0.21 – 0.97)****	**0.57 (0.19 – 0.96)****	**0.58 (0.17 – 0.99)****	**0.56 (0.18 – 0.95)****
ureC Concentration (copy #/g VS)				0.01 (-0.02 – 0.04)		
Sampling Season					0.01 (-0.31 – 0.33)	
Urinal Type						-0.12 (-0.49 – 0.25)
Observations	55	55	55	55	55	55
R^2 / R^2 adjusted	0.299 / 0.286	0.595 / 0.571	0.662 / 0.635	0.665 / 0.630	0.662 / 0.628	0.665 / 0.631

[a]
Significance codes: 0 ‘ *** ’ 0.001 ‘ ** ’ 0.05 * ‘ . ’ 0.1 ‘ ’ 1

3.2 The influence of organic matter on ureolytic activity

That the organic content is significant ($p = 0.003$) and of sizeable effect ($\hat{\beta} = 0.59$) in predicting ureolytic activity, as shown in Table 1, is consistent with past findings from soil research that found correlations between organic matter concentrations and urease activity [103, 158, 167]. Others also observed that increased carbohydrate availability at neutral pH was correlated with increased *Actinomyces naeslundii* and *Sporosarcina pasteurii* urease activity [158, 159]. Liu et al. (2008), however, noticed that carbohydrate availability had no effect on *ureC* gene expression marked by through reverse-transcriptase quantitative real-time PCR (RT-qPCR) mRNA transcripts [159]. Liu et al. (2008) hypothesizes that these observations were due to carbohydrate availability and pH modulation affecting the expression of genes other than *ureC* responsible for urease synthesis or apoenzyme activation [159].

Increasing the biomass of the inoculum by providing a carbon source in microbial induced calcite precipitation studies has been reported to promote the ureolytic activity [158]. Tobler et al. (2011) concluded that molasses supplementation selected for a larger microbial community that obtains their nitrogen from ureolysis, though there is no nitrogen limitation in urinals [158]. Others, who studied the environmental factors affecting microbially induced calcium precipitation concluded that increasing biomass may also increase ureolytic activity as there could be more active cells present [168]. Extracellular urease has also been suggested to be stabilized by adsorption to soil colloids, particularly organic matter, which may be similar to that observed in biomineral samples obtained from urine drain pipes [161].

One limitation of this study is that it is unclear what component of the organic fraction is correlated with increased ureolytic activity as VS is a bulk measurement encompassing any organic mass. Within the biomineral/stone matrix is also an organic fraction composed of

carbohydrates, proteins, lipids, and dead cell mass that binds the mineral fraction of the precipitate [15]. Therefore, future research could evaluate different organic components such as proteins and exopolysaccharide substances.

3.3 The non-effect of urinal type and seasonality on ureolytic activity

In addition to the linear regression results, Kruskal-Wallis testing for biomineral ureolytic activity between waterless and low-flow urinals provides evidence that waterless and low-flow are likely identical in population in terms of biomineral activity ($p = 0.47$). While urinal type is not a statistically significant predictor of ureolytic activity, biomineral samples from waterless urinals have exhibited a greater maximum ureolytic activity than any biomineral sample obtained from low-flow urinals in this study, as shown on Figure 3.

Finally, sampling season (as shown in Table 2) demonstrated no statistical ($p = 0.956$) or practical significance ($\hat{\beta} = 0.01$) in predicting biomineral activity. This may explain why fouling is a year-round phenomenon, as the biomineral ureolytic activity remains unaffected by seasonality, as the high urease activities year-round facilitate conditions necessary for precipitation to occur. Because seasonality does not seem to impact biomineral activity, future observations on the ureolytic activity of urine drainpipes may be performed without temporal confounding effects. Though, future microbial ecology studies are needed to understand the bacterial community structure of the biomineral samples and should include sampling events from different seasons.

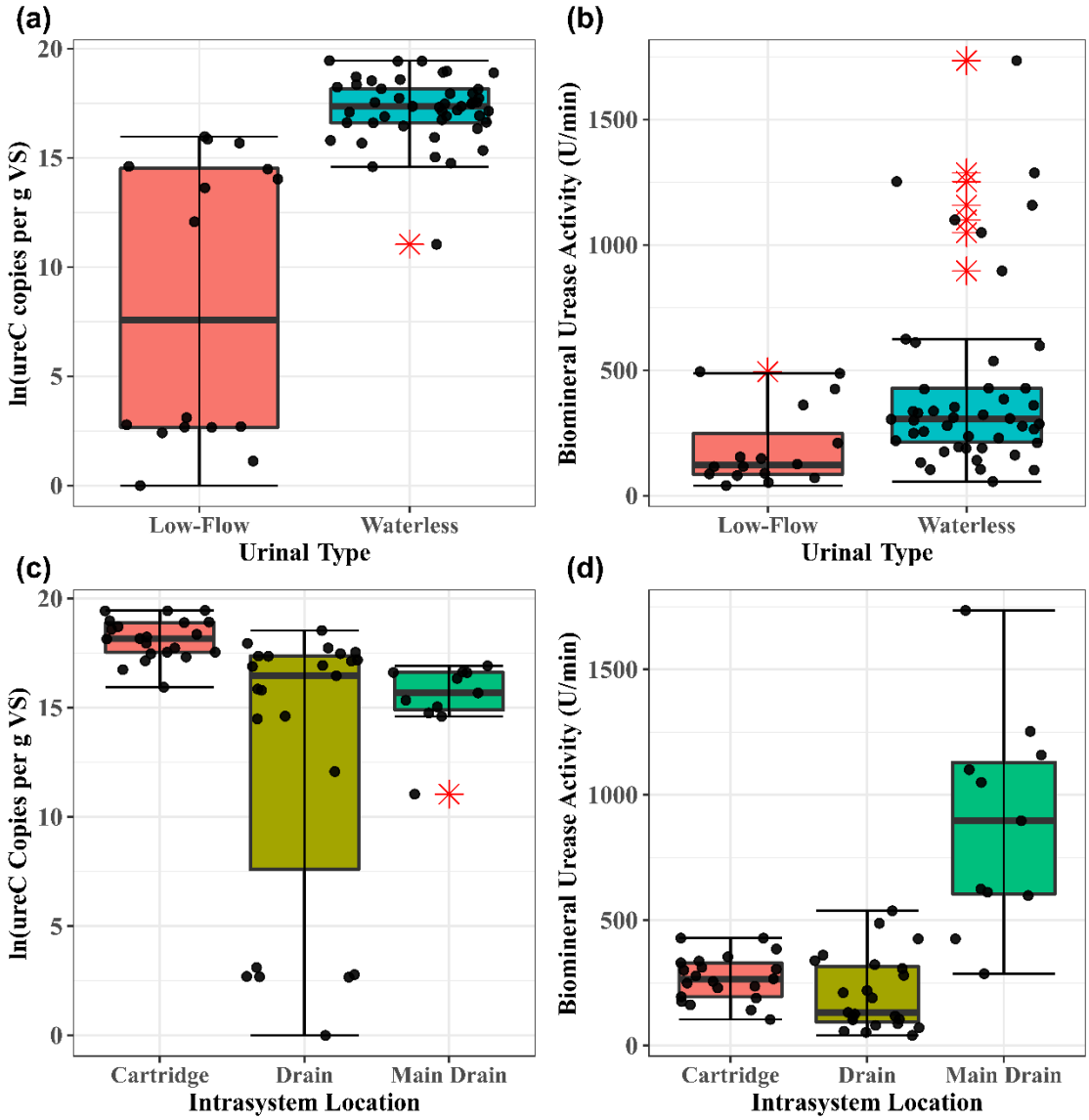

Figure 3 Descriptive statistics on the effects of urinal type on natural log-transformed *ureC* gene copies and biomineral activity

3.4 Effects of intrasystem sampling location on ureolytic activity

While the ureolytic activity of biomineral samples obtained from the drainage pipes immediately following the drain traps were not significantly different from those corresponding

to samples obtained from waterless urinal cartridges (Pairwise Wilcoxon Rank Sum: $p = 0.053$), samples taken from the main drain lines which contacts handwashing water were significantly non-identical in terms of ureolytic activity (Kruskal-Wallis: $p < 0.001$; Pairwise Wilcoxon Rank Sum: $p < 0.001$). Within one system, cartridges and gallery drain lines immediately succeeding the urinal are exposed to the same urine feed without mixing with potable water and thus face similar environmental conditions that influence ureolytic activity [103]. Because drain line samples directly follow cartridge samples and are exposed to the same urine, the relative similarity in environmental conditions between cartridge and drain line samples may explain their different ureolytic rates compared to main drainpipe samples but not with each other.

3.5 Biomineral ureolytic activity may be predicted by transcriptional activity more than by urec gene abundance

Kruskal-Wallis testing results suggest that the *ureC* abundance between low-flow and waterless urinals are significantly nonidentical ($p < 0.001$), but there was no detected significant effect on biomineral ureolytic activity as suggested by the multiple regression results shown in Table 2. The lack of statistical significance describing the relationship between *ureC* gene copies and ureolytic activities disagrees with bivariate correlation studies done by Fisher et al. (2016) and Sun et al. (2019), where it was found that soil ureolysis rates were significantly correlated with *ureC* gene copies [169, 170]. Notably, neither studies discussed effect size and used a small sample size ($n < 12$) for analyses describing individual soil horizons [169, 170]. Conversely, other soil urease studies have also found that ureolytic activities are correlated with total nitrogen (TN), total carbon (TC), and soil organic carbon (SOC) concentrations, but not the abundance of *ureC* genes as in agreement with our study [171]. The regression results suggest that ureolytic gene abundance is insufficient in predicting ureolytic activity in a linear model.

Greater abundances of potentially ureolytic bacteria indicated by proxy of sample *ureC* gene concentrations, may not be correlated with biomineral ureolytic rates as suggested by the regression results. That *ureC* was detectable indicates that part of the bacterial community in the biomineral samples has the urease-positive genotype, but not all bacteria with the *ureC* may be displaying a urease-positive phenotype [101]. This is because urease activity may not be expressed under the growth conditions found in urine drain pipes, and may explain why urease activities did not differ significantly when grouped by urinal type [101]. Expression of the urease-positive genotype and the eventual translation into the urease protein is regulated at the transcriptional level rather than at the genomic level [172–174].

That *ureC* gene abundance is not a statistically significant predictor of biomineral ureolytic activity is likely due to the need for environmental conditions that would induce certain microbial transcriptional responses that cause an increase in urease activity. When comparing *ureC* copies per g VS, values grouped by intrasystem sampling location differed significantly between cartridge vs. gallery drain (Kruskal-Wallis: $p < 0.001$; Wilcoxon Rank Sum: $p < 0.001$) and cartridge vs. gallery main drain (Kruskal-Wallis: $p < 0.001$; Wilcoxon Rank Sum: $p < 0.001$). However, Figure 3 reinforces hypothesis testing results in that samples from the main drain with the lowest functional gene concentrations exhibited maximal ureolytic activity of all samples as predicted by the multiple regression model. One possible explanation is that the main drains and low-flow urinal drain lines are exposed to flush and sink water, which leads to a decrease in nitrogen concentrations in the stream contacting the biofilm due to dilution. In response, the ureolytic ammonia oxidizing bacterial community may be upregulating *ureC* transcription to produce more urease to convert the urea into ammonia at a faster rate for pH regulation or to acquire ammonia for biomass production or energy generation [175].

Further regression testing (Supplemental Figure 4) by including the 16S rRNA gene concentration in a model also suggests that it is not a strong ($\hat{\beta} = 0.13$) or significant ($p = 0.127$) predictor of ureolytic activity. This suggests that a greater bacterial load within a sample, estimated by proxy of gene concentration) may not correspond to greater ureolytic rates in a given biomineral sample. Our observations on the lack of correlation between 16S rRNA gene abundance and ureolytic activity disagrees with Wang et al.'s (2018) study, where they found a statistically significant correlation between urease activity and 16S rRNA copies via automatic linear modelling [167]. However, such discrepancies in results may be due to distinct environmental conditions between soil samples and ureolytic biomineralization from drain pipes which could influence the expression of the urease gene and its eventual translation.

From Figure 4, the observation that conventional and low-flow urinals can have similar *in situ* ureolytic rates with those from waterless urinals is consistent with the regression results where it was found that urinal type is neither a significant ($p = 0.521$) and practical ($\hat{\beta} = -0.12$) predictor of the *in vitro* biomineral ureolytic activity. While low-flow urinals constitute most fixtures described in Figure 4 due to drain trap inaccessibility for other urinal types, Figure 4 demonstrates that Dunnigan northbound, a waterless urinal site, exhibited the greatest maximum *in situ* ureolytic rates of all drain traps tested. Conversely, the standard urinal at Tejon Pass ranked 2nd of all sites screened for *in situ* ureolytic rate. The Tejon Pass urinal exhibited a similar rate (115 uS cm^{-1} min^{-1} per g VS) compared to that of the Dunnigan northbound standard height urinal (118 uS cm^{-1} min^{-1} per g VS). Urinals in the same study sites also appear to exhibit different urea conversion rates. One possible explanation is that there simply may be less ureolytically active biomineral mass in one drain trap compared to the urinal adjacent to it at the time of sampling. It cannot be guaranteed that there is sufficient biomineral mass within a given

drain trap at any given time, which could be affecting the *in situ* ureolytic rates. Ideally, a larger sample size for the *in-situ* tests could alleviate any ambiguity from this confounding factor, and so further research with increased sample size is needed. Regardless of this confounding factor, the *in-situ* tests demonstrate that it is possible for the ureolytic activity of biomineral samples from urinals with high flush water volumes to match that from waterless urinals. Raw urease activity values grouped by sampling sites can be found in Supplementary Table 2.

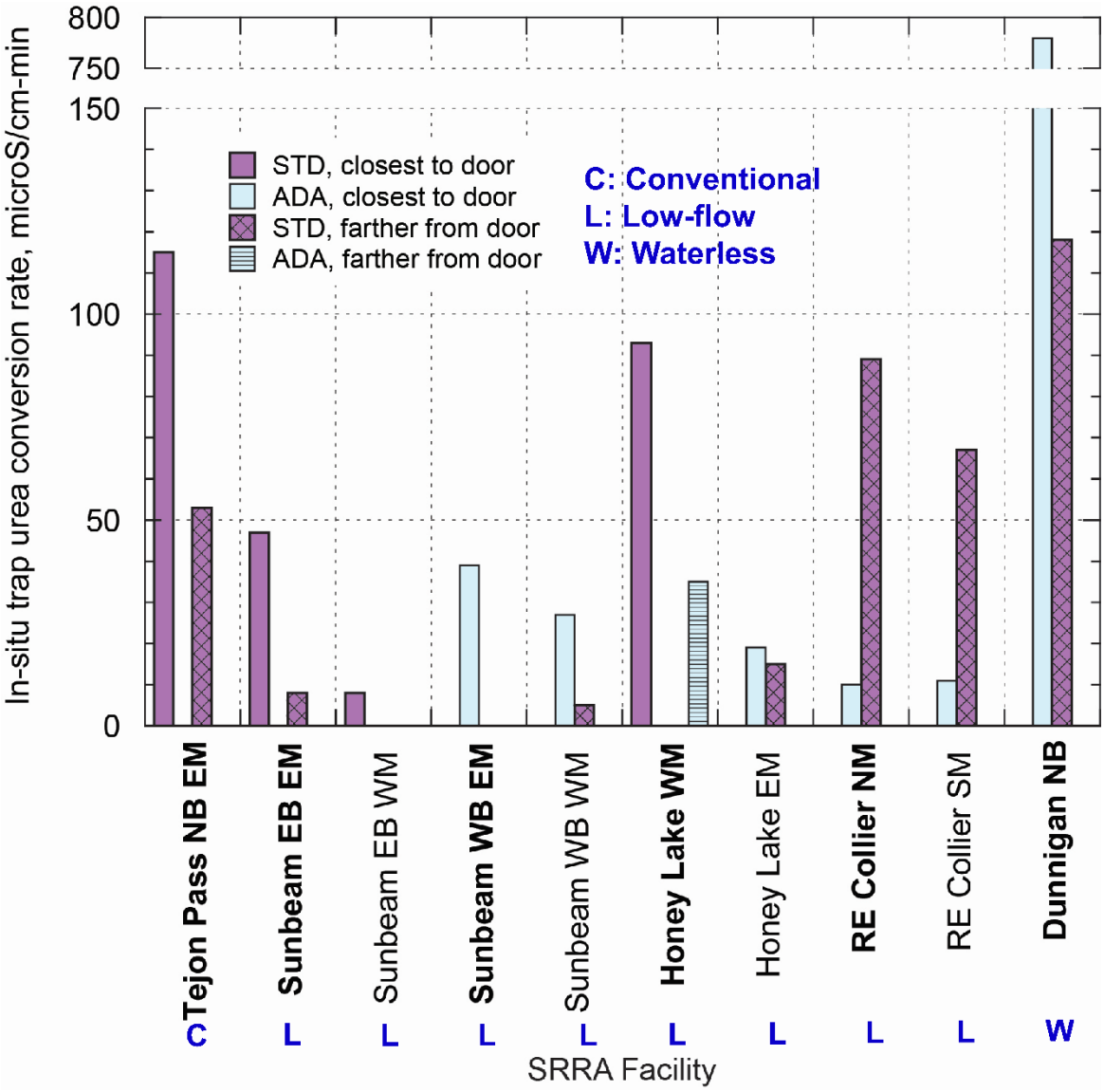

Figure 4 Comparison of *in situ* trap urea conversion rate for various rest areas with trap-type urinals

4. Conclusion

In conclusion, *ureC* gene abundance was not a strong and significant predictor of biomineral urease activity. More so, the regression model suggests that rest areas with greater user frequencies and organic content represented by volatile solids exhibited greater biomineral urease enzyme activities. Where one samples within a urine drainage system also appears to affect the strength of the enzyme activity. Conversely, urease activities did not appear to differ based on the seasonality of the sampling period or the urinal type. One limitation of the study is that the age of the biomineral samples was uncontrolled largely due to different cleaning and maintenance frequencies at each rest area examined. Though, it would be impractical to impose strict cleaning routines for dozens of laborers who maintain these rest areas statewide. Currently it is unclear how age could affect the strength of the urease activity of biomineral samples, but future studies should explore such effects.

Our findings indicate that flush water alone may not be an adequate preventative measure for preventing ureolytic biomineralization. Urease activity can be as strong in conventional and low-flow biomineralization as it is in waterless biomineralization, even if there is a smaller ureolytic community in flush type urinals as indicated by low relative *ureC* gene concentrations shown in Figure 3. It is also possible that flush water may also influence the precipitation chemistry in drain lines, as flush water containing elevated magnesium and calcium concentrations may contribute to crystallization. While the smaller abundance of *ureC* gene concentrations in low-flow urinal samples is insufficient in accounting for the similar ureolytic activities exhibited by the two urinal types and intrasystem sampling locations, the differences in *ureC* gene concentrations grouped by urinal type shown in Figure 3 may likely be due to a difference in community structures. Future next-generation-sequencing and microbial ecology

studies should visualize the potentially ureolytic microbial community structure by sequencing the *ureC* gene in addition to 16S rRNA to visualize the total bacterial community to find relationships between the bacterial community, environmental factors, and ureolytic activity.

In conjunction with measuring bulk parameters such as pH, future studies should incorporate RT-qPCR to determine the effects of nutrient concentrations, sampling locations, and urinal types on urease gene expression at the transcriptional level. A future RT-qPCR experiment on ureolytic biomineral samples can reveal how the effects of varying dilution rates between low-flow and waterless urinals affects the transcriptional activity of a gene of interest and its relationship with ureolytic activity.

CHAPTER IV

Exploratory Evaluation of N-butyl Thiophosphoric Triamide and Commercial Products for the Minimization of Ureolytic Activity in Urine Drainage Systems

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Declarations

Availability of data and material

All references can be found in the references section of this article.

Competing interests

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1. Introduction

Urine source-separation systems are prone to clogging caused by ureolytic biomineralization. Ureolytic bacteria responsible for the biomineralization use the nickel-dependent metalloenzyme, urease, to catalyze the hydrolysis of urea into ammonia and bicarbonate which in turn raises the pH and creates supersaturation conditions favorable of mineral precipitation [15]. Researchers have demonstrated that the rates of calcium and magnesium encrustation caused by various ureolytic bacteria isolates is directly correlated with an increase in ureolytic activity [104].

Because of the relationship between ureolytic organisms, ureolytic activity, and biomineral encrustation, urease inhibitors have been used in urological applications to minimize catheter clogs caused by ureolytic biomineralization. Historically, clinicians have used classes of inhibitors such as hydroxamic acids and phosphorodiamidate solutions to minimize the activity of microbially-produced urease associated with calcium and struvite precipitation catheters [15, 38].

The use of urease inhibitors can be extended to urine source-separation settings which are analogously plagued by ureolytic biomineralization as catheters are. While researchers have posited that urease inhibitors are not viable for source-separation use due to concerns on toxicity [43], widespread agricultural usage of n-butyl thiophosphoric triamide (NBPT) suggests that urease inhibitors can be safe in some applications. Of the 14 metric tons (Mt) of inhibitor-treated fertilizers sold in 2016, NBPT blended fertilizers accounted for 53% of fertilizer sales worldwide [68].

The use of NBPT in urine source-separation settings remain little explored, and so the objective of the present study is to quantify the effectiveness of inhibiting bacterially produced

urease on the bench scale using batch tests and a model glass reactor to simulate a urine drainage system. Unlike previous studies that use plant-based ureases, this study uses a bacterial urease derived from a waterless urinal public restroom which is more characteristic of conditions encountered in urine drainage systems. More so, this is the first attempt at using NBPT in a urine source-separation context.

2. Methods

To quantify the inhibiting potential and enzymatic activity based on the dose of NBPT added in solution, batch reactors containing a buffered urea solution were dosed with a constant volume of urease and varying concentrations of the active ingredient. Then, a model glass reactor simulating urine drainage system conditions were used to dose various commercial treatments and a custom NBPT urinal cake into the urine drain trap where the pH was tracked over several days. Currently, custom NBPT urinal cakes are being tested at the Dunnigan northbound rest area for its ability to minimize biomineral formation in the urine drainage system.

2.1 Production of whole-cell bacterial urease extract for use in batch experiments

A single batch of a whole-cell urease extract used throughout the batch experiments was produced by inoculating synthetic urine with biomineral obtained from a waterless urinal rest area. A sequencing batch reactor consisting of 1 part urine drainpipe biomineral obtained from the Dunnigan Northbound rest area and 2 parts synthetic urine was drained and filled every 48 hours for three generations at 21^{o}C. The synthetic urine recipe is shown in Table 1 and is similar to formulations used in prior research [41, 52, 176, 176]. After the third generation, the entire batch was stirred, aliquoted, and centrifuged at 4200g (g-force) using nominal 500 mL centrifuge tubes for 20 minutes. The contents were aspirated, and the remaining pellet mass was washed

with Milli-Q water twice, and resuspended in 5 mL of 7.3 pH 0.1 M (molar concentration) phosphate buffer for each centrifuge tube. The contents were from each centrifuge tube were homogenized and used throughout the batch testing experiments.

Table 1: Synthetic Urine Formulation

Number	Reagent	Mass (g) per Liter
1	**$CaCl\ 2H_2O$**	2.60
2	**$MgCl_2\ 6H_2O$**	2.60
3	**NaCl**	18.4
4	**Na_2SO_4**	9.2
5	**KH_2PO_4**	11.2
6	**KCl**	6.4
7	**NH_4Cl**	4
8	**Trisodium Citrate**	2.6
9	**Sodium Oxalate**	0.092
10	**Urea**	100
11	**Creatine**	4.4
12	**Tryptic Soy Broth**	40

2.2 Batch testing development of dose-response curve using NBPT

To quantify the inhibiting potential based on the dose of NBPT added in solution, batch reactors containing a buffered urea solution were dosed with a constant volume of bacterial urease and dosed with varying concentrations of NBPT. To determine the enzymatic activities of biomineral sample, 1 mL of the whole-cell extract was suspended and mixed in 25 mL of 7.3 pH 200 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) buffer containing 2.5% urea m/m. The electrical conductivity was measured continuously for 5 minutes, and the rate of formation is taken as a surrogate measure of ureolytic activity [108].

2.3 Development of a urinal cake dosing method using NBPT as an active ingredient

To formulate a urinal cake used to dose NBPT as an active ingredient, a recipe from an expired patent from Holdt et al. (1987) was used [177]. Table 2 describes the general components of Holdt et al.'s (1987) recipe and specific ingredients used. NBPT was sourced from the commercial product Agrotain Ultra, which is a proprietary agrichemical containing emulsifiers, 26.7% m/m NBPT, and a green dye. The Agrotrain Ultra was used as the dye component of Holdt et al's (1987) recipe. Using the recipe shown in Table 1, the resulting NBPT urinal cake shaped into a 60 g cylindrical mass is shown in Figure 1.

Table 2: Examples of formulations from patents discussing easily sourced chemicals

General Components from Holdt et al. (1987)	**% Weight**
Alkylbenzene sulfonate	30-50
Inorganic alkali metal salts	30-45
Plasticizers	0-10
Disintegration rate regulators	10-15
Perfumes	1-10
Dyes	5-15
Components Based on Example A from Holdt et al. (1987) in this Study	**% Weight**
Sodium dodecylbenzene sulfonate with 20% sodium carbonate	39.5
Sodium sulfate, anhydrous	21
Sodium tripolyphosphate	13
Cocoamide monoethanolamide (MEA)	7
Agrotain Ultra (Dye and active ingredient)	10
Pine oil fragrance	6
Stearic Acid	3.5

Figure 1: A custom NBPT urinal cake was produced using a formulation based on Holdt et al's (1987) patent.

2.4 A relative comparison of commercially available urine hygiene control products and the proposed NBPT urinal cake in a batch reactor

A batch experiment was devised to compare the relative inhibitory potential of commercial products and the custom NBPT urinal cake described in Section 2.3. A 1 cm diameter mass of commercial products and the NBPT urinal cake was swirled for 1.5 minutes in a beaker containing 25 mL of 7.3 pH 200 mM HEPES buffered 2.5% m/m urea solution. The 1 cm mass of product was removed from the beaker and a 1 mL dose of whole-cell extract was

applied to the beaker, and enzymatic activity was determined conductometrically. For each tested substance, the enzymatic activity assay was replicated 10 times and the resulting enzymatic activities were plotted on a box plot for observational comparison.

2.5 Exploratory glass model reactor testing to determine the ureolytic inhibition potential of commercial products and the proposed NBPT urinal cake

A model drain trap as shown in Figure 3 and 4 consisting of 24/40 ground glass fittings and a peristaltic pump was used to quantify the ability of commercial products and the custom NBPT urinal cake to minimize a rise in synthetic urine pH in an environment similar to that of a urine drainage system. In this experiment, 20 g worth of the treatments shaped into ~1 cm diameter masses were placed in a 50 mL soxhlet extractor fed with sterile synthetic urine that dosed approximately 20 times per 24 hour period using a peristaltic pump. To inoculate the model reactor with a bacterial culture, 5 mL of biomineral slurry obtained from the Dunnigan northbound rest area was added to the P-trap. The P-trap shown in Figure 3 was continuously monitored for pH. Reactors were operated for 3-5 days or depending on when incidental contamination occurred in the system.

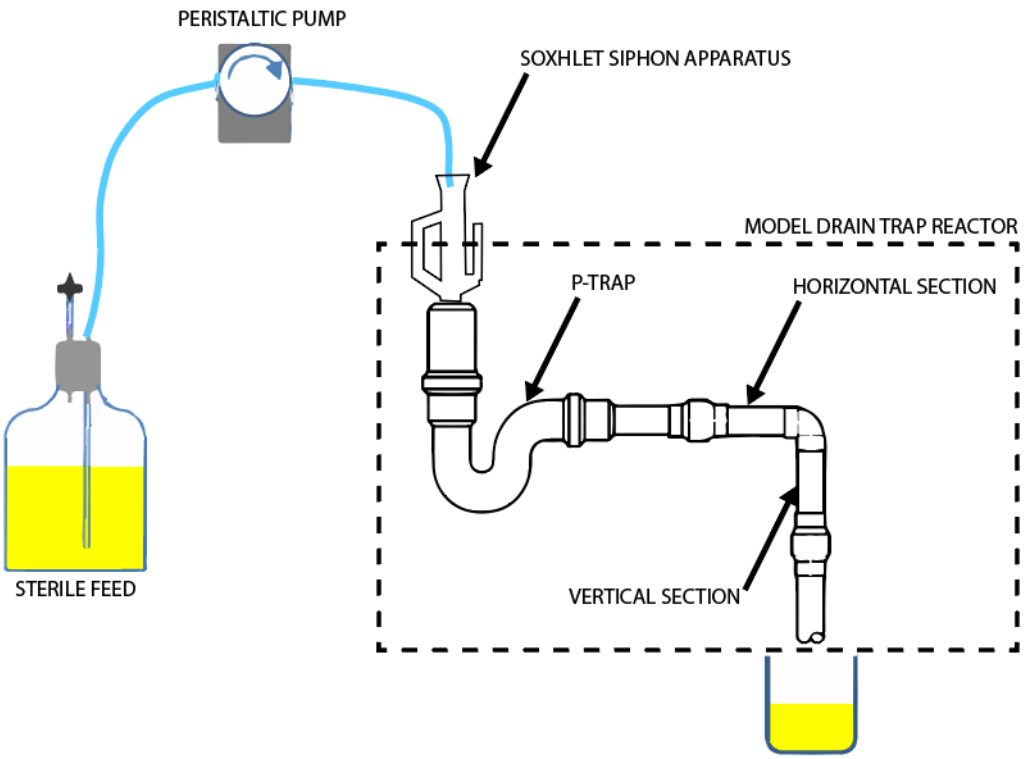

Figure 3: A model drain trap was fed synthetic urine mixed with selected treatments to facilitate biofilm formation and biomineralization.

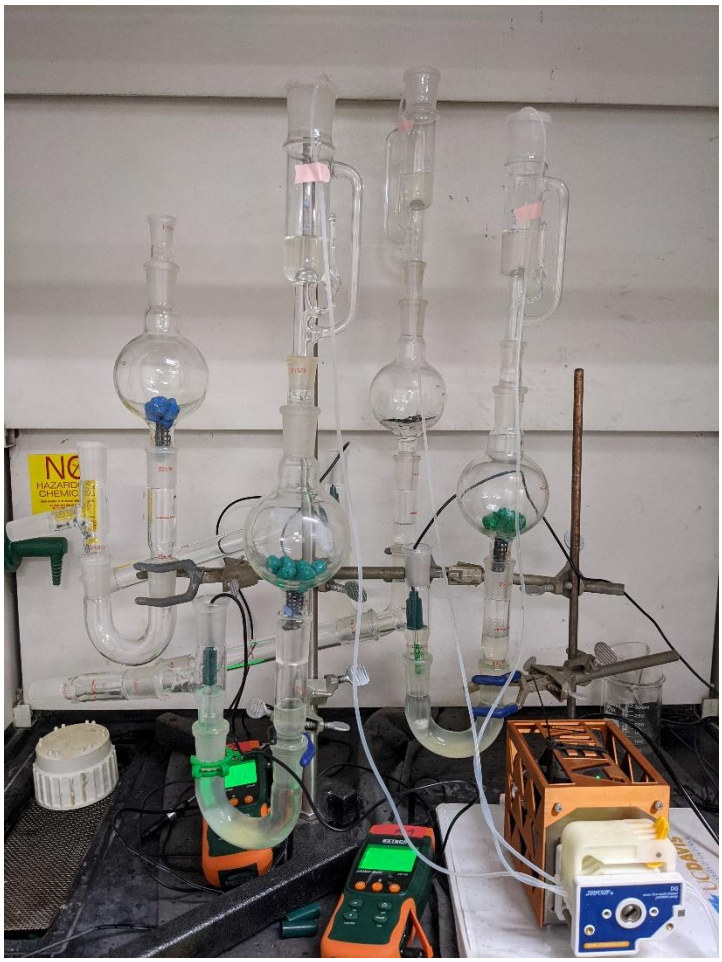

Figure 4: Model drain trap reactors were operated in parallel to compare the effectiveness of various commercial treatments and the custom NBPT urinal cake.

3. Results and Discussion

The results of inhibitor batch testing and inhibitor performance in urine drainage trap glass model reactor is discussed in the following subsections.

3.1 NBPT demonstrates urease inhibitory potential unlike commercial products in batch reactors

Dose-response testing and batch comparison of ureolytic rates associated with various products suggest that NBPT has an inhibitory potential that the commercial products failed to exhibit. In three replicate trials, NBPT has consistently demonstrated an inhibitory potential for a mixed bacterial urease acting in a buffered urea solution as shown in Figure 5.

NBPT is the only substance of the tested variety of products that inhibited ureolysis. Various treatments were tested for enzymatic activity after swirling a 1 cm diameter mass of a chosen product in 25 mL of buffered urea solution for 1.5 minutes and dosing the solution with 1 mL of whole-cell urease extract. This batch comparison of commercial products and the custom NBPT urinal cake suggests that commercial products do not rely on ureolytic inhibition as the key mechanism to prevent clogs and odors that the manufacturers' claim to solve. Figure 6 demonstrates that generic para-dichlorobenzene urinal blocks, Whiffaway, Drainnet Bioblock, and Kohler branded urinal cakes increased the ureolytic activity relative to the control rather than decrease it as originally expected. The resulting increase in ureolytic activity may be due to interactions between the sodium dodecylbenzene sulfonate (SDS) with the electrical conductivity probe or with the urease. Sodium dodecylbenzene sulfonate is a commonly used surfactant in many cleaning products. Surfactants have been known to influence the activity of enzymes, but the relationship between SDS and urease has yet to be elucidated [178]. As shown in Figure 6, dosing the buffered urea solution at a rate of 1 g/L of SDS with the 1 mL of whole-cell urease extracted produced a similar result of an elevated ureolytic activity compared to the control. Despite the presence of SDS in the custom NBPT urinal cake, the NBPT's inhibitory properties overcame any urease enhancing properties observed in other urinal cakes tested.

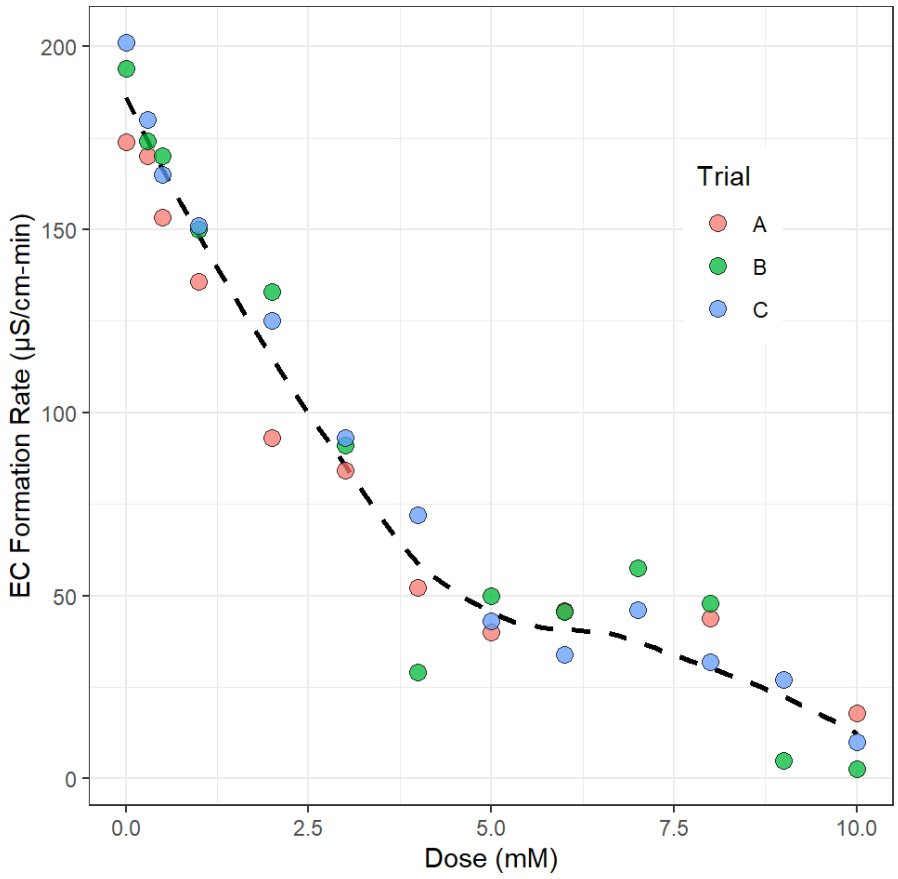

Figure 5: The enzymatic activity as predicted by the EC formation rate in solutions dosed with varying concentrations of NBPT is shown.

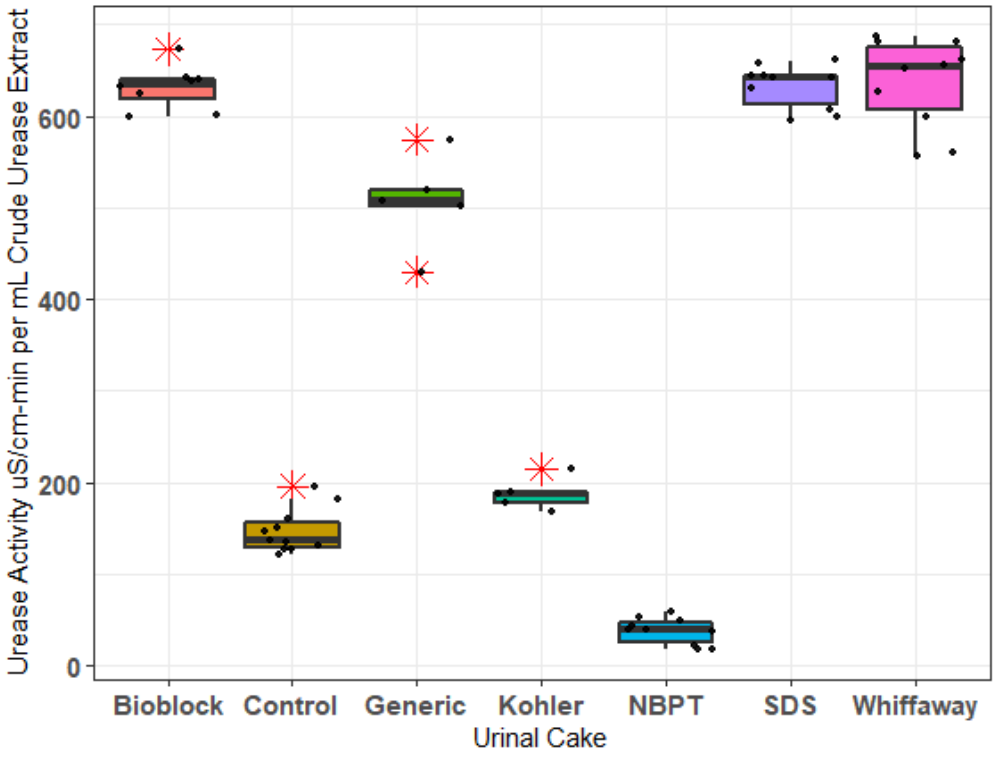

Figure 6: Ureolytic activity assay results with ten replicates per treatment demonstrates the (in)effectiveness of various urinal cakes tested.

3.2 NBPT can inhibit ureolytic activity in a model glass reactor under simulated urine flow conditions

NBPT can inhibit ureolysis in a model glass reactor under simulated flow conditions as shown in Figure 7. Whereas the untreated controls maintain a pH value of approximately 9, the NBPT treated reactors maintain a pH under 7.6 for 4.5 days. NBPT is therefore recommended for future field studies testing its ability to minimize biomineral formation in urine drain lines.

Despite poor short-term urease inhibition using Whiffaway branded urinal cakes in the batch tests described in Section 2, reactors treated with Whiffaway urinal cakes maintained a pH of less than 7.5 in the P-trap (Figure 8). Due to trade secrets, the ingredient list in the urinal cakes

is unknown and it is then difficult to speculate on the mechanism explaining such observations. One problem with the Whiffaway urinal cakes is that the eroded cake tended to accumulate at the bottom of the P-trap as shown in Figure 9. As such, it is possible that a clog may occur from the resulting accumulation of the Whiffaway particles.

Other commercial products such as the generic para-dichlorobenzene urinal cake, DrainNet Bioblock, and Kohler balls were ineffective at lowering the P-traps' pH relative to the control treatments as shown in Figure 10. As such, these products are not recommended for future field trials.

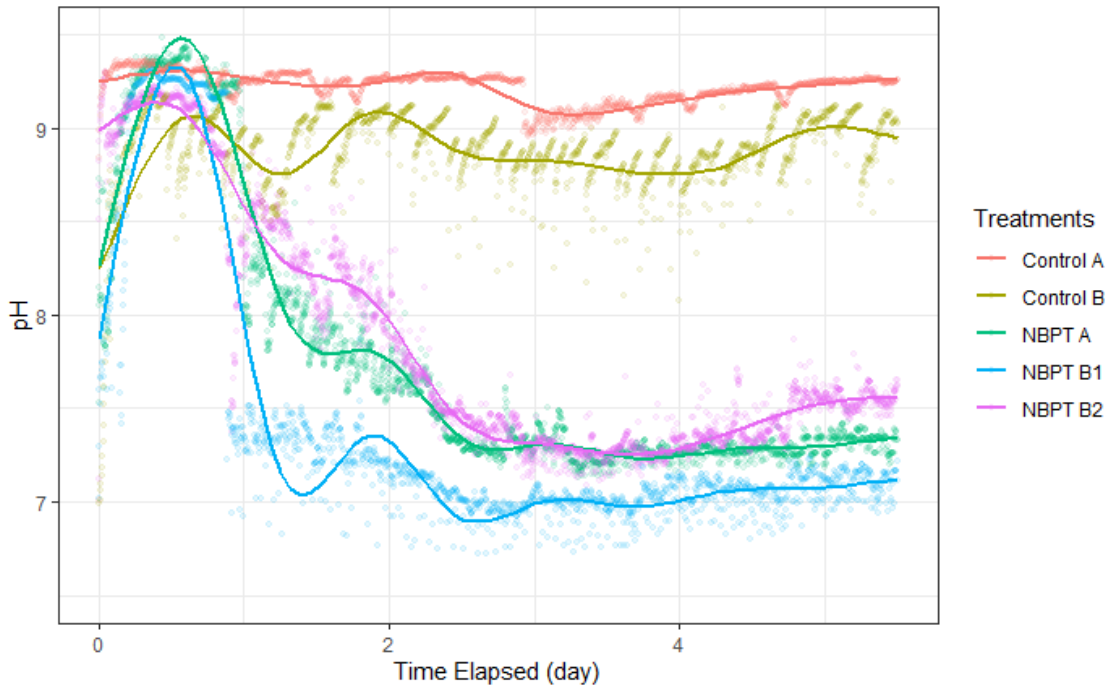

Figure 7: The pH vs. time diagram demonstrates that the pH was kept below pH 7.6 for all three replicates for at least 4.5 days using the NBPT treatment.

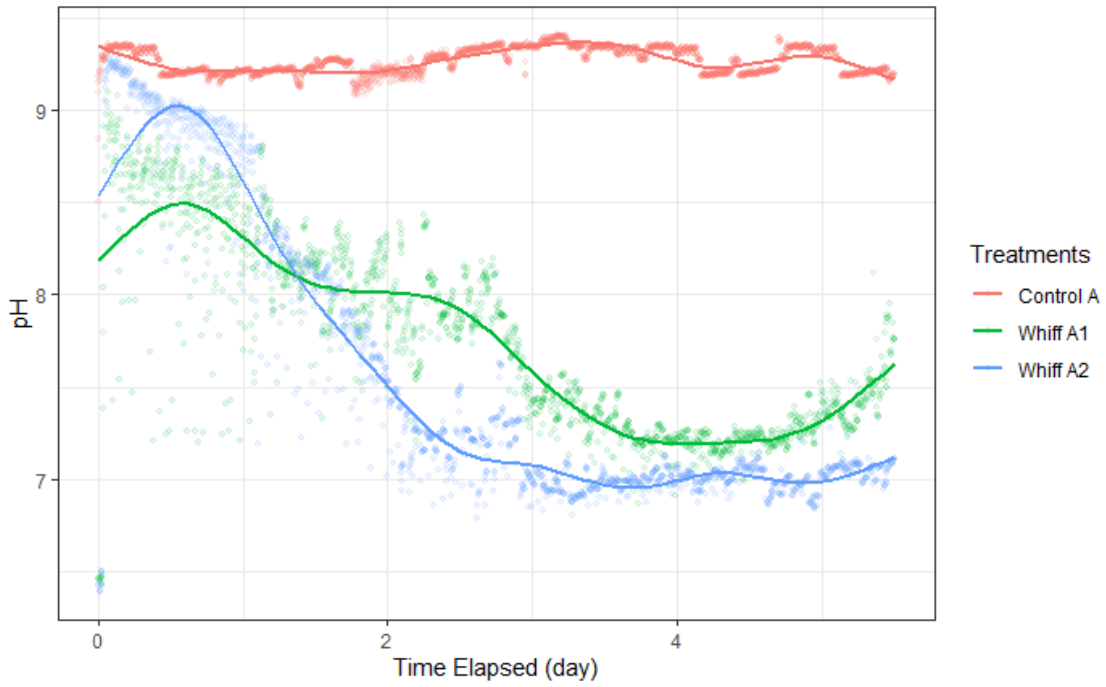

Figure 8: pH vs time results indicate that Whiffaway can minimize the rise of pH as similar seen using the NBPT treatment

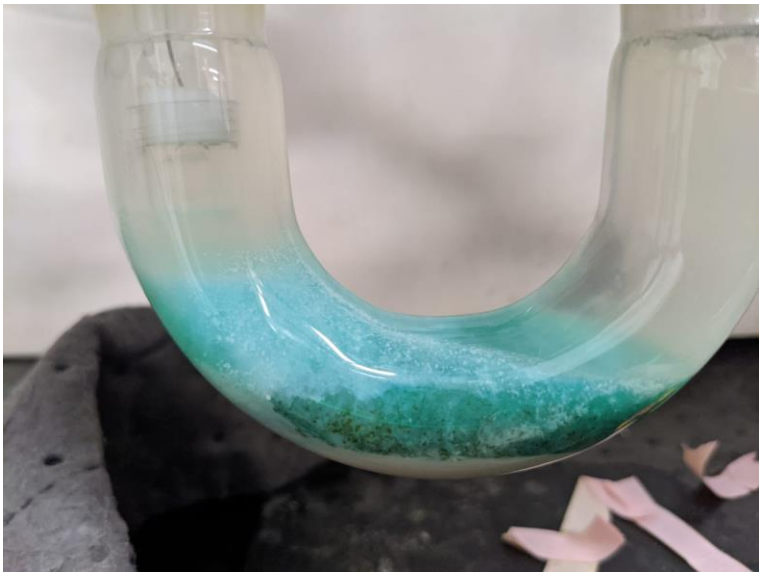

Figure 9: Eroded Whiffaway particles can reduce the volume of the U-bend and potentially cause clogs.

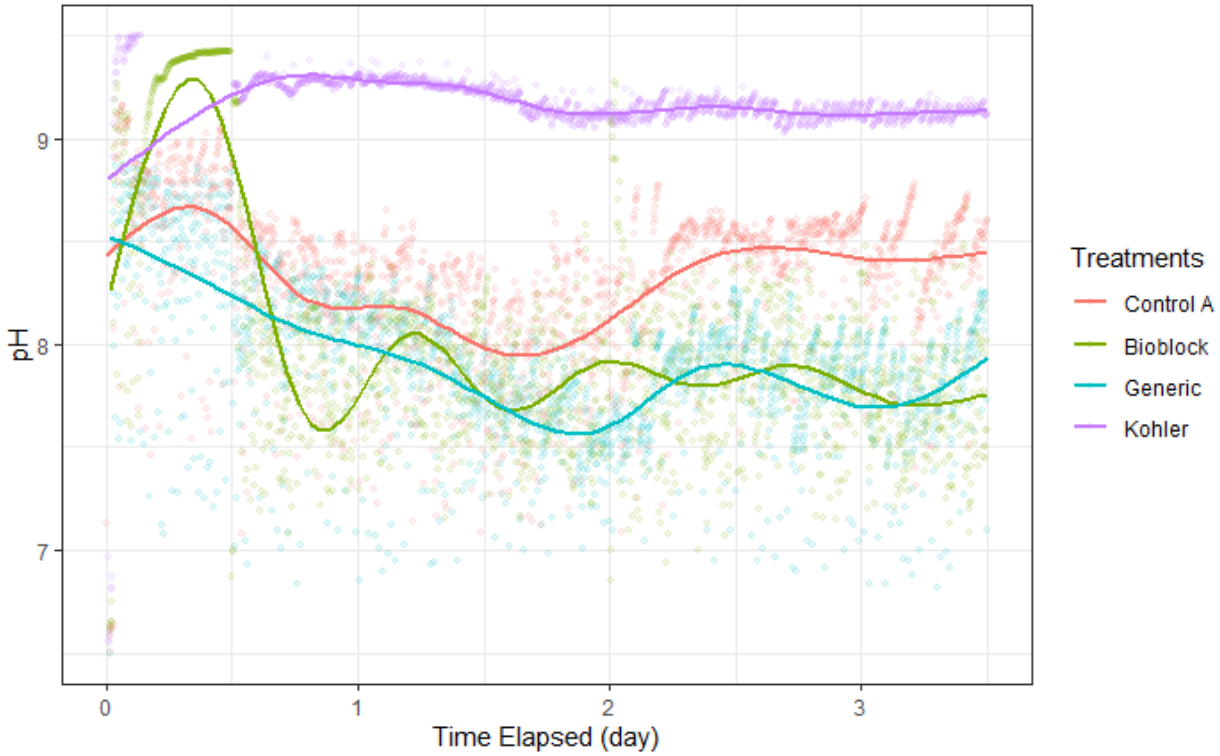

Figure 10: Commercial products did not inhibit ureolysis in the glass P-trap.

4. Current field study in progress

Currently, a field trial is in progress where 60 g masses of NBPT urinal cakes were regularly placed in each waterless urinal at Dunnigan northbound rest area to determine its effects on biomineral production downstream of the urinal. Treatment began on February 12, 2021 after 12 weeks of baseline monitoring to study the weekly biomineral production and the associated ureolytic activity. The urinal cakes were placed in screens as shown in Figure 11.

Figure 11: Waterless urinals tested at Dunnigan northbound rest area.

Downstream of the urinals is a sedimentation tank which has a pump inlet at a predetermined level of the tank to convey urine to a remotely located nutrient recovery system as shown in Figure 12. The liquid is decanted weekly and the weekly biomineral production is quantified using a graduated cylinder as shown in Figure 13. Biomineral samples from the sedimentation tank are also conductometrically assayed for urease activity, whereby 1 mL of the wet biomineral samples are suspended in 25 mL of a 7.3 pH 200 mM buffered 2.5% m/m urea solution.

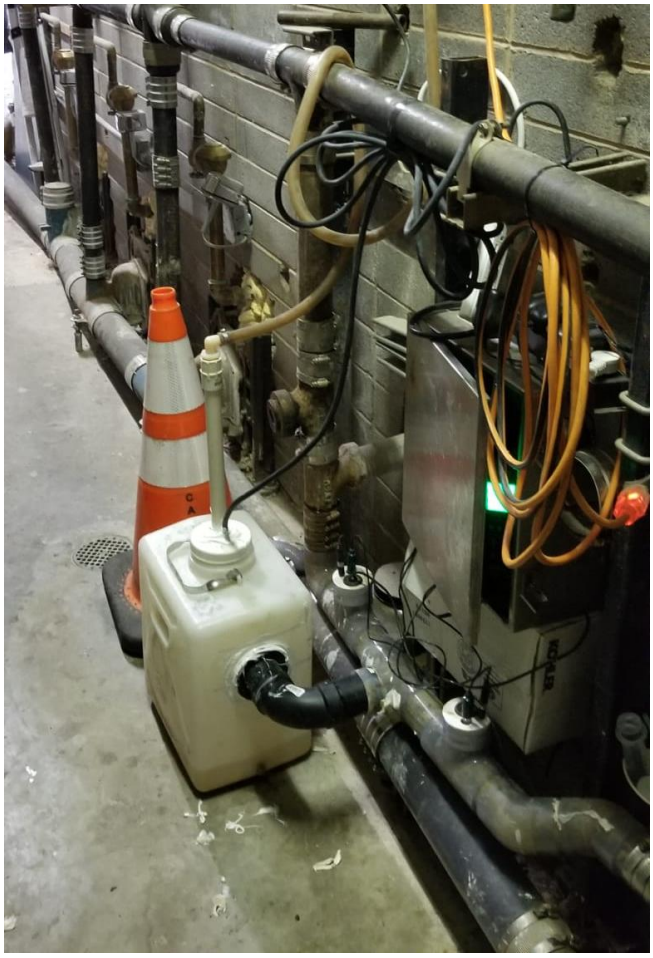

Figure 12: Sedimentation tank in the urine drain line gallery at the Dunnigan northbound rest area.

Figure 13: Biomineral production is quantified using a graduated cylinder, whereby the settled volume represents the biomineral formed in the sedimentation tank in a week's time.

4.1 Results and Discussion

Biomineral urease activity of samples obtained from the sedimentation tank does not appear to be affected by the presence of NBPT as shown in Figure 14. Unexpectedly, the maximum urease activity observed occurred during the treatment period. The average EC formation rate during the control study was 57 μS/cm-min while the EC formation rate during treatment was 57.5 μS/cm-min. The lack of difference between the enzymatic activities may be

due to degradation of the NBPT in the sedimentation tank or active ureolytic cultures producing enough urease to overcome the inhibitory effects over time.

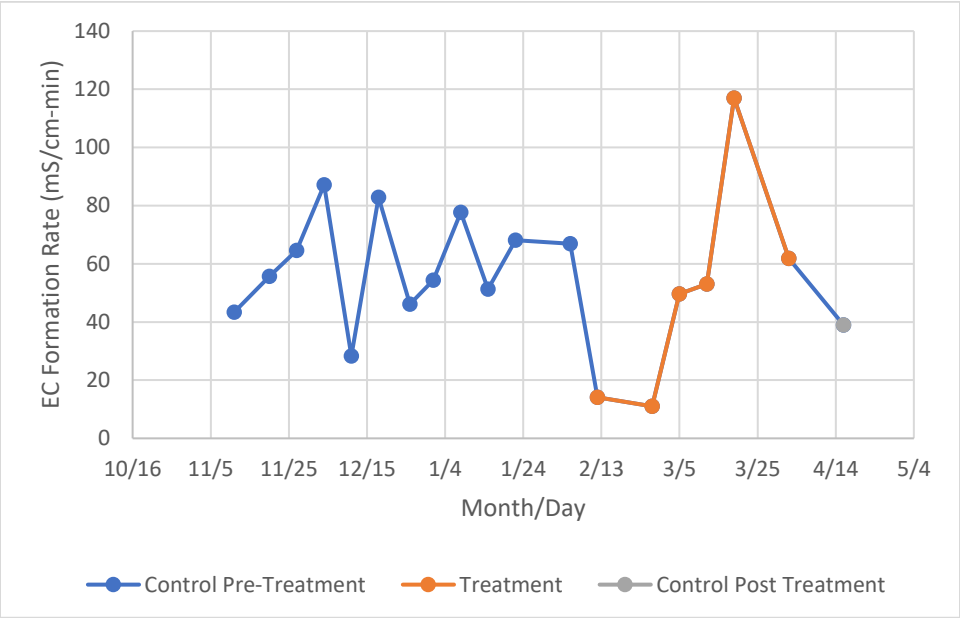

Figure 14: Biomineral urease activity associated with the precipitates in the sedimentation tank were measured weekly. Missing data is attributed to rest area closures or researcher data loss.

While biomineral urease activity was not substantially affected by the presence of NBPT, the settled volumes of biomineral produced each week witnessed a marked decrease as shown in Figure 15. During the treatment period between 02/12/2021 and 03/26/2021, weekly biomineral volumes were on average reduced 55% from that observed for control studies pre and post treatment. The decrease in biomineral volumes produced over several weeks is evidence that the NBPT treatment had an effect of minimizing biomineral fouling within the urine drain lines and sedimentation tank. More so, the weeks after concluding the urinal cake treatment yielded settled biomineral volumes that were similar to that observed before the treatment period began.

One limitation of the custom NBPT urinal cake used in this study is the high erosion rates of the cake. A total of 800 g of NBPT cakes were used in the 6-week treatment period. This is likely due to a formulation issue, as it takes time to optimize a blend that has the appropriate dose, erosion rate, and consistency. Variations in urinal cake formulation were not explored in this study. Future studies should improve the formulation to minimize the erosion rate and to maximize NBPT dosage.

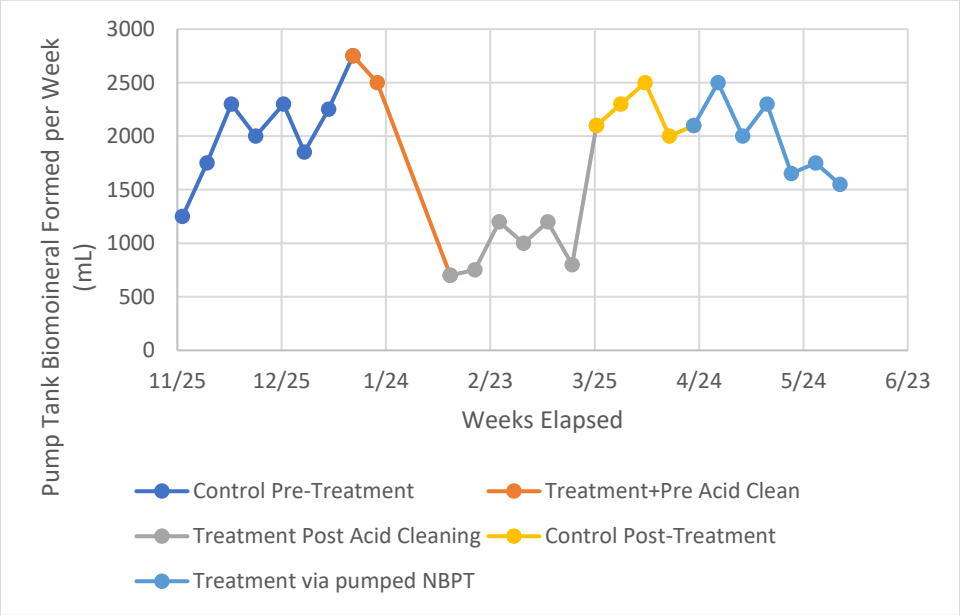

Figure 15: Biomineral production is quantified using a graduated cylinder, whereby the settled volume represents the biomineral formed in the sedimentation tank in a week's time.

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APPENDIX

Attachment 1: Supplementary Information for Microbial Ecology Study

Attachment 2: Supplementary Information for Multiple Regression Study

Attachment 1: Supplemental Information

S1 File

A biogeographic 16S rRNA survey of bacterial communities of ureolytic biomineralization from California Public Restrooms

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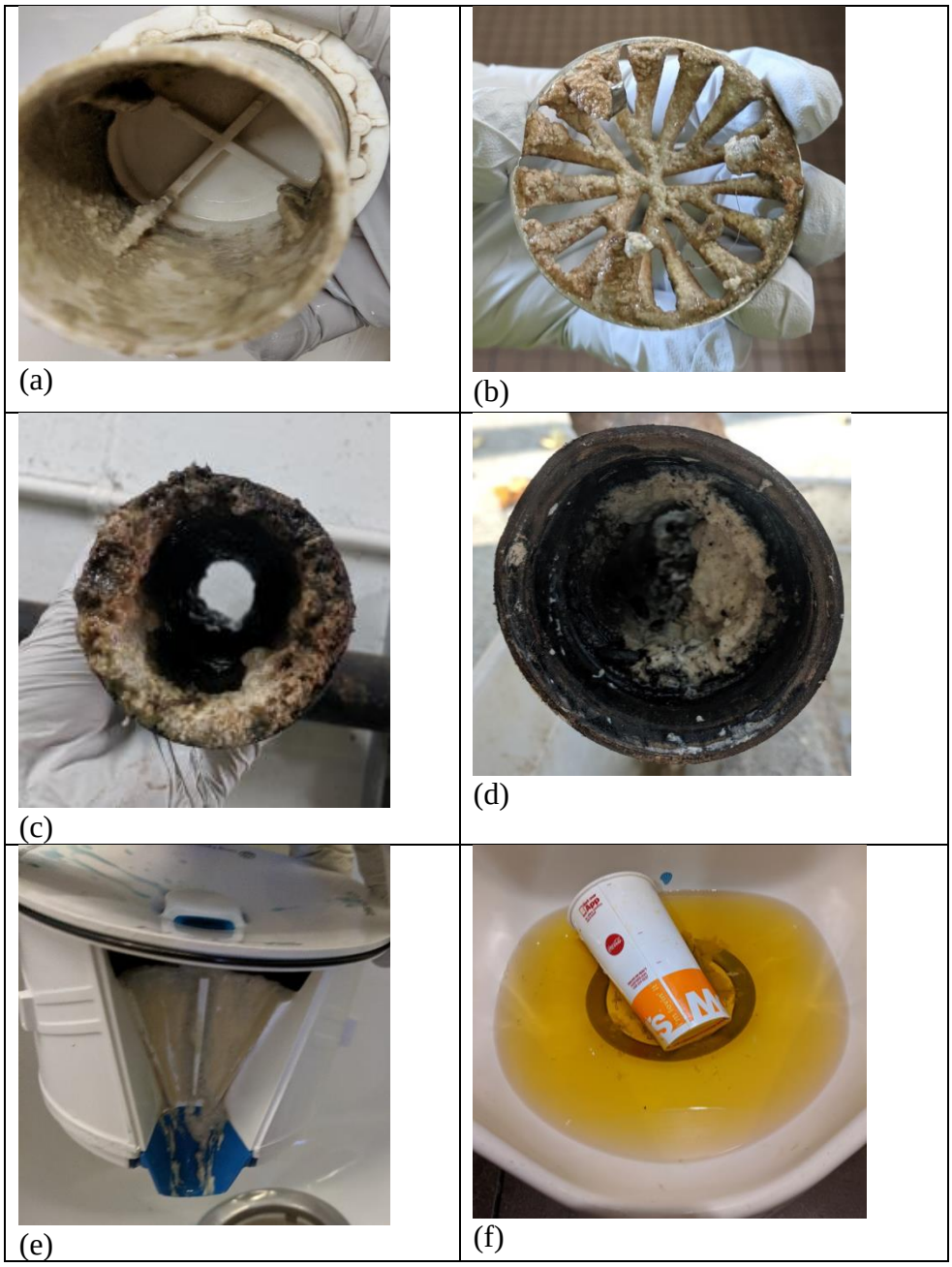
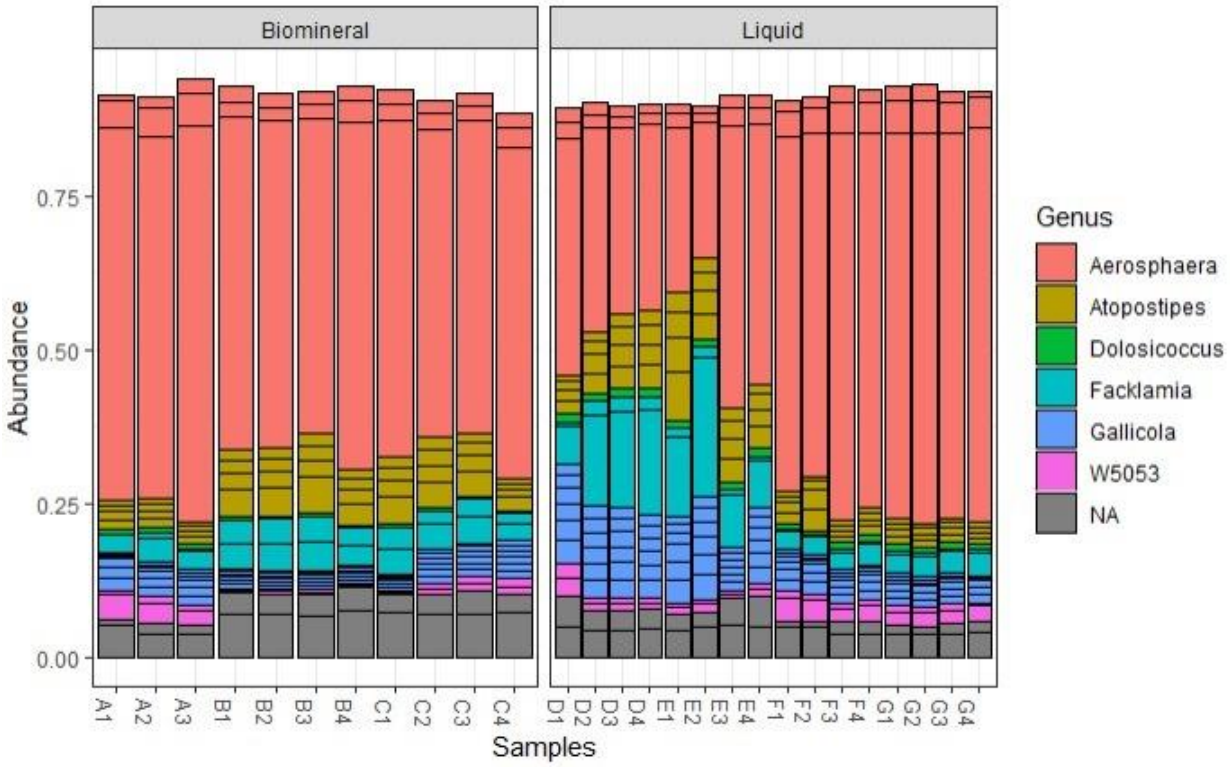
For Submission To: PLOS ONE

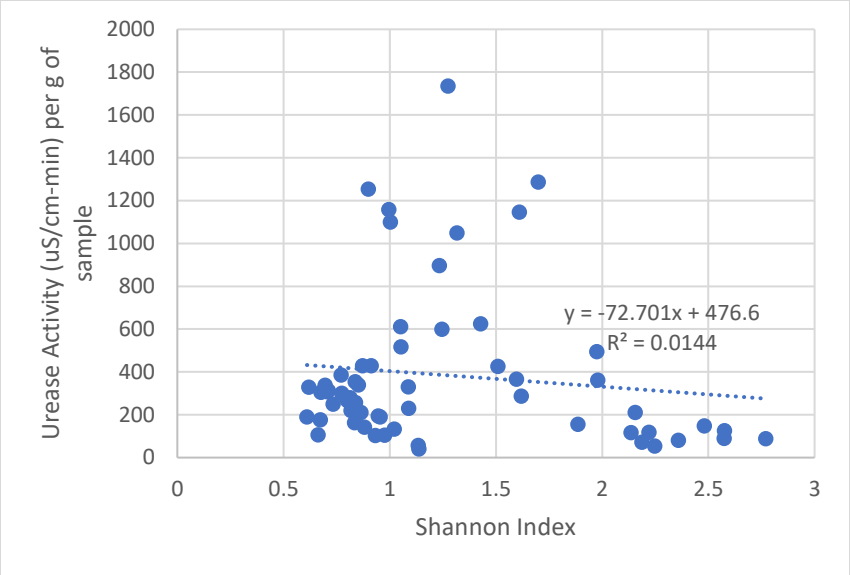


S1 Fig. Panels a-e demonstrate the extent of biomineral formation in conventional, low-flow, and waterless urinals. Any surface that urine touches is susceptible to biomineralization, including cartridges, screens, drain traps, and drainpipes. The pictures are a) biomineral formation on a cartridgeless trap design at Grass Lake on 27 Aug 2019, b) Biomineralization on a metal screen inside a urinal at RE Collier south men's restroom on 28 Aug 2019, c) view of typical combined biomineral formation and corrosion of 2" iron pipe, d) Another view of reduced internal pipe diameter by biomineralization in urine drainage pipe at Dunnigan northbound SRRA on 12 Dec 2019, e) biomineralization formation on waterless urinal cartridge at Erreca on 16 Sep 2019, f) Example of vandalism that leads to clogging observed at a waterless urinal in a gas station along I-5 on 12 Mar 2020.



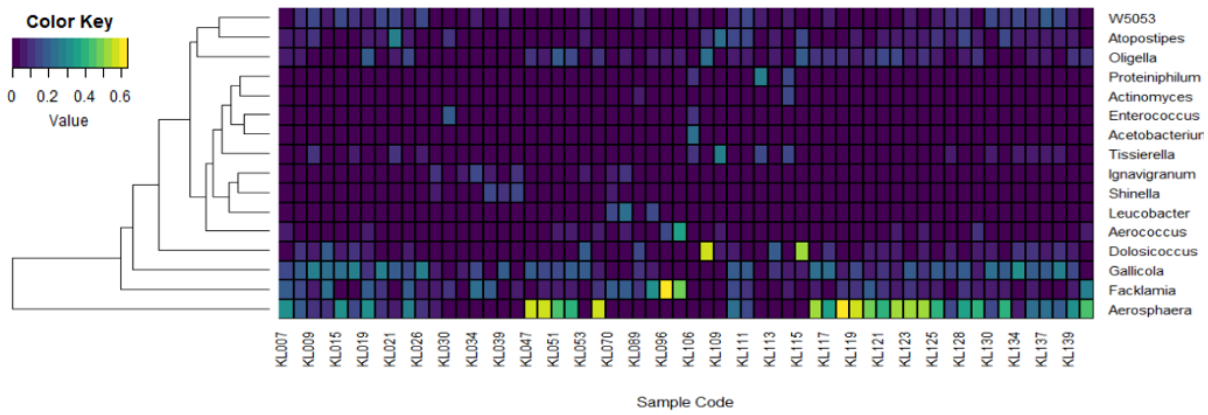
S2 Fig. An exploratory bar plot for comparing the effects of sample storage time and temperature on the outcome of high throughput sequencing is shown. Biomineral samples from Dunnigan northbound SRRA sampled from a single day were subject to various storage conditions to provide evidence that samples taken during the study were adequately stored during transportation.

Sample Group A1-3: 4^{o}C storage at 0, 2, 5 days prior to freezing
Sample Group B1-4: 4^{o}C storage at 0, 2, 5, 35 days prior to freezing
Sample Group C1-4: 21^{o}C storage at 0, 2, 5, 35 days prior to freezing
Sample Group D1-4: 4^{o}C storage at 0, 2, 5, 35 days prior to freezing
Sample Group E1-4: 21^{o}C storage at 0, 2, 5, 35 days prior to freezing
Sample Group F1-4: 4^{o}C storage at 0, 2, 5, 35 days prior to freezing
Sample Group G1-4: 21^{o}C storage at 0, 2, 5, 35 days prior to freezing



S3 Fig. Simple linear regression relating ureolytic activity with Shannon indices of biomineral samples. No correlation is observed between within-sample diversity scores and ureolytic activity indicated by the negligible coefficient of determination.

(a)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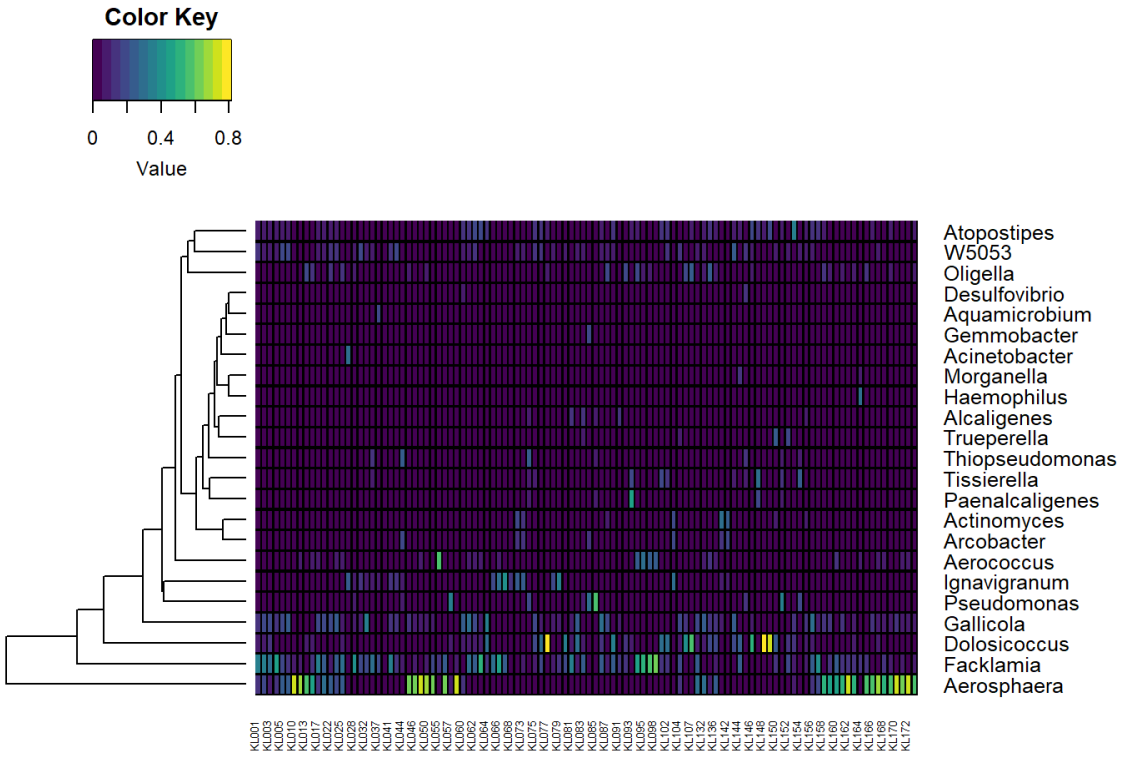
Value
W5053
Atopostipes
Oligella
Proteiniphilum
Actinomyces
Enterococcus
Acetobacteriur
Tissierella
Ignavigranum
Shinella
Leucobacter
Aerococcus
Dolosicoccus
Gallicola
Facklamia
Aerosphaera
KL007 KL009 KL015 KL019 KL021 KL026 KL030 KL034 KL039 KL047 KL051 KL053 KL070 KL069 KL096 KL106 KL109 KL111 KL113 KL115 KL117 KL119 KL121 KL123 KL125 KL128 KL130 KL134 KL137 KL139
Sample Code

(b)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Aquamicrobium
Gemmobacter
Acinetobacter
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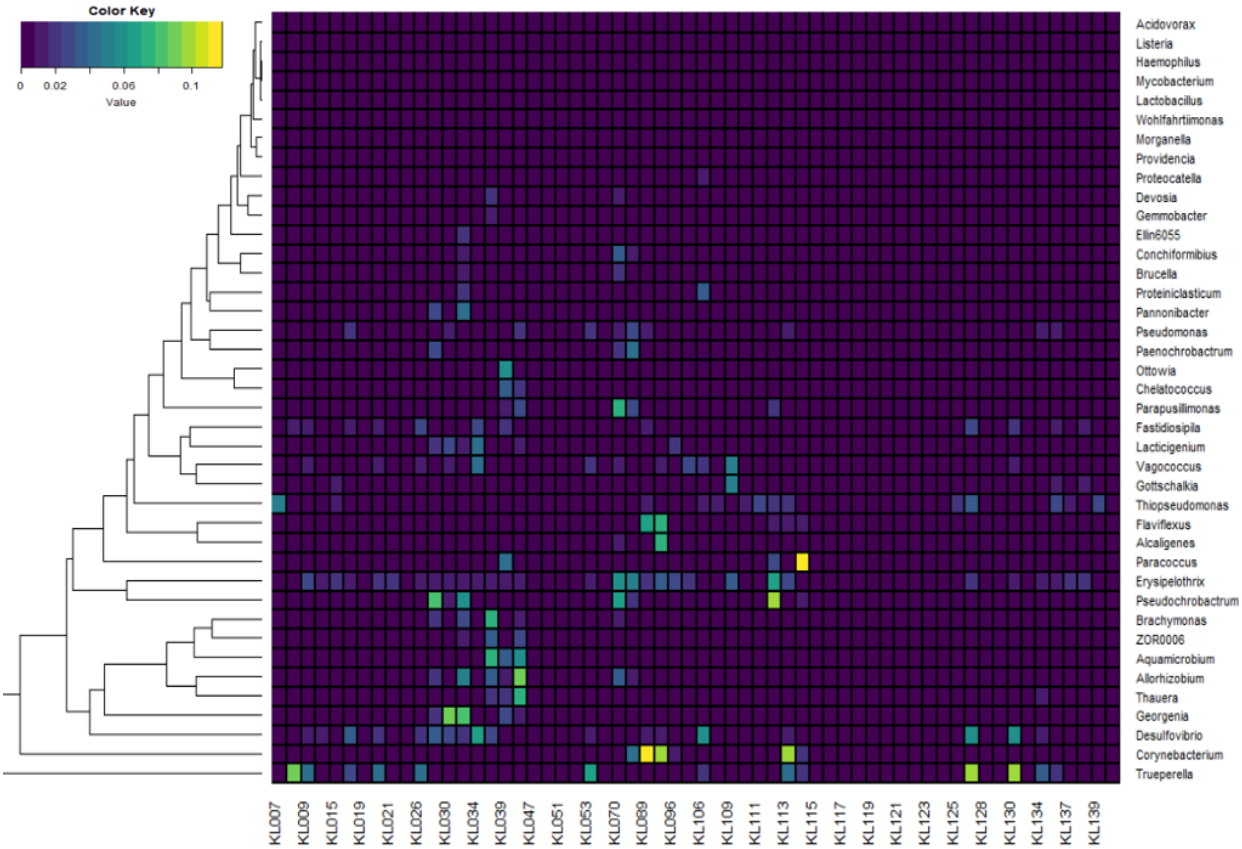
Trueperella
Thiopseudomonas
Tissierella
Paenalcaligenes
Actinomyces
Arcobacter
Aerococcus
Ignavigranum
Pseudomonas
Gallicola
Dolosicoccus
Facklamia
Aerosphaera

(c)



S4 Fig. (a) Taxonomic heatmap and phylogeny of top 15% of identifiable operational taxonomic units (OTUs) at the genus level present in biomineral samples. The color key shows the color palette pertaining to different relative abundances of the genera within each sample, (b) Taxonomic heatmap of top 15% of identifiable genera in liquid samples, (c) Taxonomic heatmap of bottom 4-15% of identifiable genera present in biomineral samples.

Attachment 2: Supplementary Information

Assessing the Biomineral Urease Activity from Urine Drainpipes of California Rest Areas

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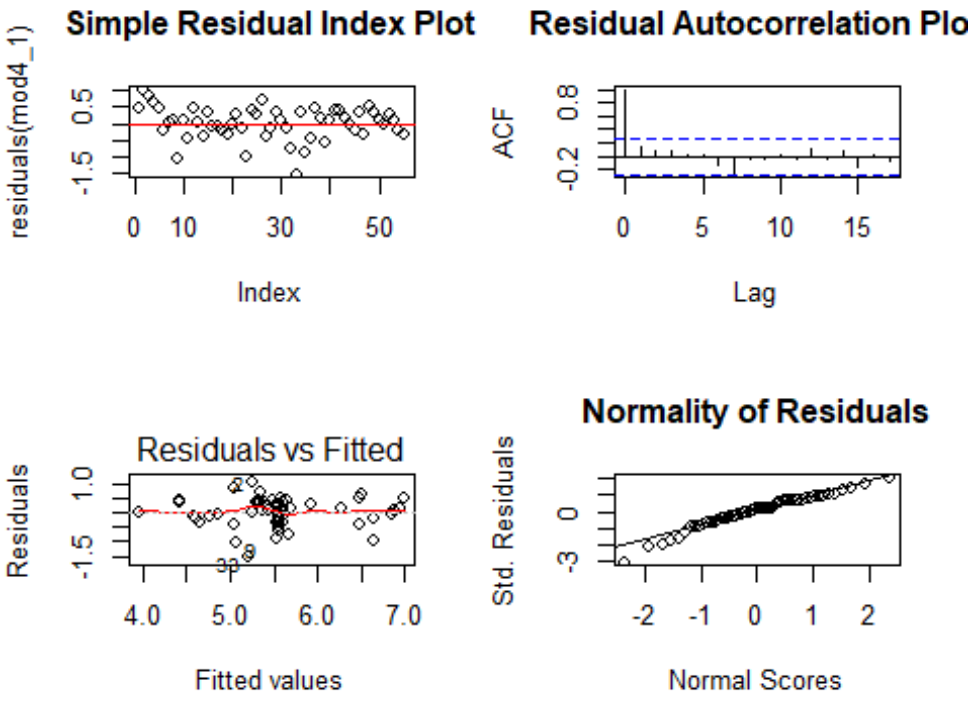
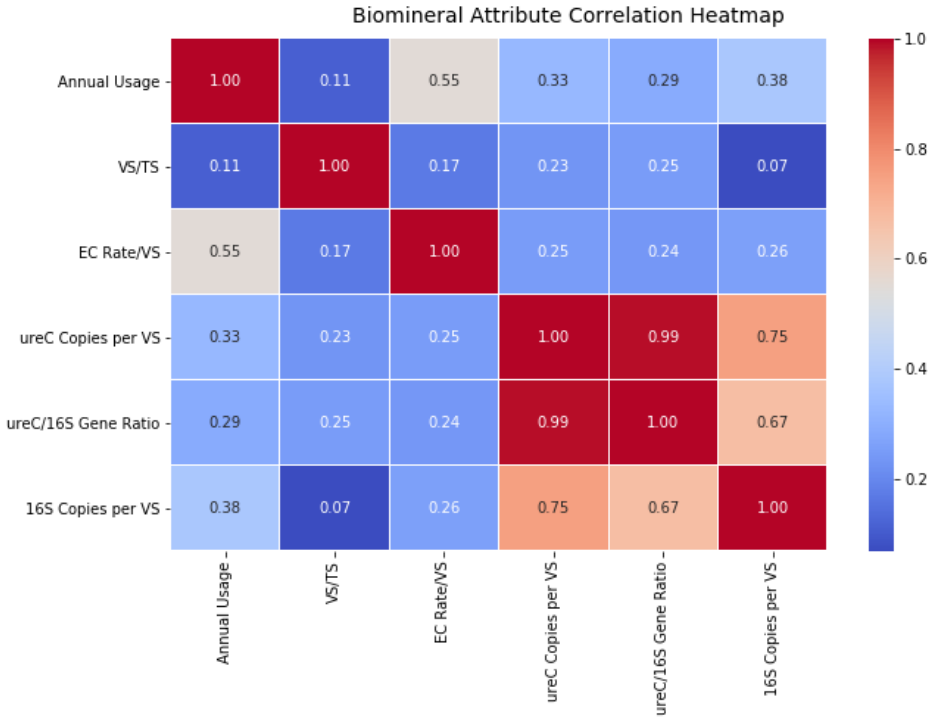
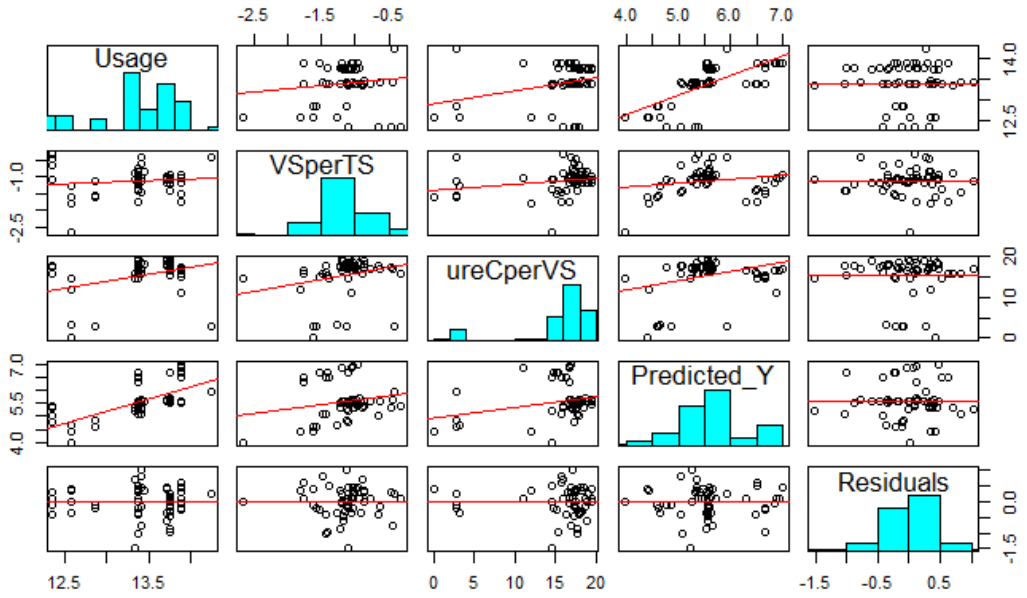


Supplemental Figure 1 The residuals of the hypothesized model including *ureC* gene concentration as a predictor (model 4) demonstrates adherence to the Gauss-Markov assumptions of the linear model described in the manuscript.

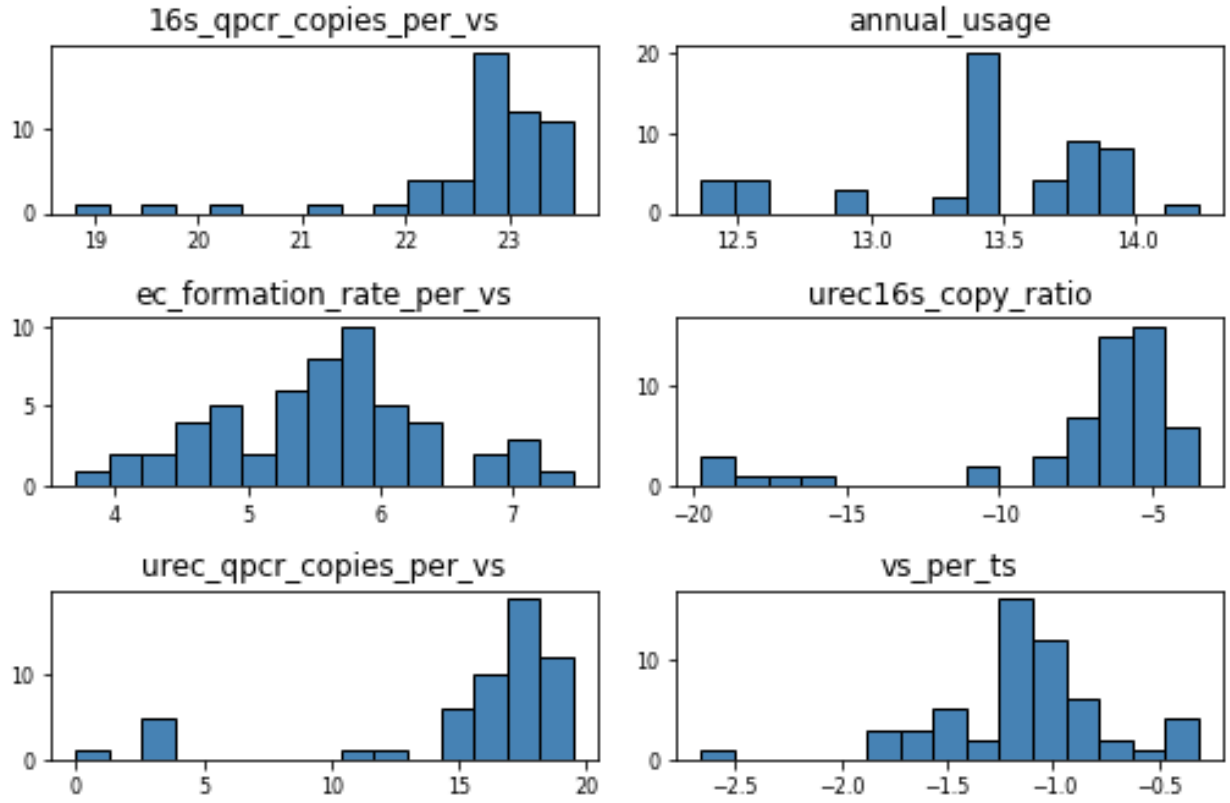
a)



b)



Supplemental Figure 2a/2b The correlation matrices suggest that the independent variables included in the multiple regression analysis is not affected by multicollinearity and that confounding factors are not observed.



Supplemental Figure 3: Histograms depicting the data distribution of natural logarithmically transformed data set.

Supplemental Table 1 Akaike Information Criterion Results for Model Selection

Model	**Delta**	**df**	**Weight**
Model 3	0	6	0.5097
Model 6	2.148	7	0.1741
Model 4	2.233	7	0.1669
Model 5	2.628	7	0.137
Model 2	7.437	5	0.01237
Model 1	32.85	3	3.751e-08

Supplemental Table 2 Summary of *in situ* trap test data

Site	Description[a]	Date	Trap volume (mL)	*In situ* activity (uS/cm-min per g VS)	Temperature (ºC)
Tejon Pass, NB	EM STD[b,c]	9/18/2019	510	115	19
	EM STD[b]		510	53	19
Sunbeam EB	EM STD[b,c]	9/17/2019	175	47	33
	EM STD[b]		175	8	33
	WM STD[c]		175	8	33
Sunbeam, WB	EM ADA[b,c]	9/17/2019	175	39	33
	EM STD[b]		175	0	33
	WM STD		175	5	33
	WM ADA[c]		175	27	33
Honey Lake	WM STD[b,c]	12/12/2019	175	93	15
	WM ADA[b]		175	35	15
	EM STD		120	15	15
	EM ADA[c]		120	19	15
RE Collier	NM ADA[b,c]	12/13/2019	130	10	12
	NM STD[b]		200	89	12
	SM ADA[c]		130	11	12
	SM STD		150	67	12
Dunnigan, NB	STD[b]	12/12/2019	400	118	25
	ADA[b,c]		400	780	25

[a] EM = eastern men's restroom, WM = western men's restroom, NM = northern men's restroom, SM = southern men's restroom, STD = standard urinal fixture height, ADA = lower urinal height to comply with American's with Disabilities Act

[b] Primary restroom facility at SRRA

[c] Urinal located nearest to door