Microbially-mediated pretreatment of aerated oil and gas produced water by addition of phosphorous or activated sludge

Alexander S. Honeyman¹, Emily R. Nicholas¹, Tzahi Y. Cath¹, John R. Spear^{1*}

¹Depart of Civil and Environmental Engineering, Colorado School of Mines, Golden, CO

*** Corresponding Author:** John R. Spear: jspear@mines.edu

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Abstract

Produced Water (PW) from oil and gas (O&G) producing wells is a unique source of water in water-stressed regions. Microbiota within O&G formations have been well-studied on site/*in-situ*, but few applied works have considered their role in the treatment of PW in engineered water treatment systems. Herein, we operated a simple aeration/mixing bench-scale bioreactor fed with produced water under three conditions: 1) PW alone (control; hereafter referred to as ‘baseline’), 2) phosphorous dosed daily as KH_2PO_4 , and 3) activated sludge (AS) dosed daily from a sequence batch membrane bioreactor (SB-MBR). Aerated and mixed PW alone (baseline) was able to attenuate PW chemistry with removal of soluble chemical oxygen demand (sCOD) and ammonia by 27.6% and 17.8%, respectively. Further KH_2PO_4 and AS additions improved water treatment efficiency markedly; in the KH_2PO_4 addition reactor, sCOD and ammonia were reduced by 50.0% and 61.5%, respectively, and in the AS addition reactor by 52.5% and 59.2%, respectively. Microbial consortia determined via 16S rRNA gene amplicons differ in composition between raw PW and all reactors; order Kiloniellales was most common in raw PW while orders Rhodobacterales, Pseudomonadales, and Caulobacterales were most abundant amongst AS, KH_2PO_4 , and baseline conditions, respectively. Several different microbial consortia are capable of treating raw PW which suggests that functional redundancy amongst microbiota in engineered treatment systems may be underappreciated. With simple addition of phosphorous and/or activated sludge to PW as part of a treatment strategy, a higher quality water can then be subjected to conventional treatment and/or local reuse.

Importance

Multiple microbiological communities are capable of treating O&G PW in a simple, applied, engineered setting. The broad possibility of PW treatment by multiple different microbial consortia elucidates the potential for easy, effective, water reuse processes in the hydraulically-stressed arid west as well as any region generating PW from O&G operations.

Keywords

Produced water; biological treatment; water reuse; microbial ecology; applied microbiology

1. Introduction

Hydraulic fracturing of oil and gas (O&G) wells may induce additional water stress in regions already experiencing water shortages and drought (1). Hydraulic fracturing of deep formations is achieved by high-pressure injection of fluids and sand into horizontal wells, after which hydrocarbons are more easily released and brought to the surface. The recovered hydrocarbons are accompanied by water consisting of both injection fluids (also known as fracturing flowback fluids—FFBs) and formation fluids with varying concentrations of cations, anions, and organics. Combined, this fluid, broadly referred to as ‘produced water’ (PW) can be an important source of clean water if methods to properly treat it are developed. PW typically contains substantive concentrations of suspended solids, total dissolved solids (TDS), organic matter, volatile compounds, metals, total radioactivity (2), and microorganisms either native to the subsurface or introduced to PW by injection fluids (or both)—all of which complicate the treatment of PW by conventional water treatment processes. Thus, the treatment of PW for beneficial use is commonly substituted by injection to deep disposal wells or, otherwise, by non-beneficial use such as elimination and volume minimization in evaporation ponds (3). Removal of total organic carbon (TOC), suspended solids, total and soluble chemical oxygen demand (COD and sCOD), and ammonia would aid subsequent downstream treatment processes such as desalination.

Publicly owned treatment works (POTW) are not designed to handle the high salinity and concentration of metals / other dissolved inorganics native to PW (3). However, Frank et al. (4) show that treatment of PW in activated sludge (AS) systems is possible at loads of 6-20% PW by volume with limited impacts on the performance of the biological process and on effluent water quality. Microbial communities have also shown remarkable resilience to disturbance of batch reactor parameters during municipal water treatment (5), suggesting there may be an untapped potential in the capabilities of microbial communities for the treatment of PW. Pre-treatment of PW by microbial communities native to PW could reduce organic and nutrient content, enabling efficient downstream water treatment by conventional municipal water treatment technologies.

While studies have investigated in detail the microbial communities inhabiting fractured subsurfaces (6-8) and shifts in their structures over time in FFBs (9-11), there exists minimal information on the stability of these communities in ambient, surface, or storage conditions over time. Foci of applied research into the microbiology of PW systems includes considerations of pipeline corrosion via microbial-induced corrosion (MIC) (12-14), biocides for the prevention of

well souring (15), and in-lab engineered microbial mats for treatment of PW (16). However, the effectiveness of simple chemical and/or microbial community additions to aerated PW for treatment improvement remains under-examined.

Herein, we have investigated the possibility of treating PW with native microbial communities through two lines of inquiry: 1) what chemical additions increase microbial treatment of produced water under aerated conditions?; and 2) what microbial communities are correlated with achieved improvements? These questions were investigated in a simplified setting whereby PW was stored at ambient temperatures in 1 m³ plastic totes for weeks at a time before addition into our experiment—mimicking the realities of PW treatment plans on-site at O&G operations. Here, we empirically determined changes in PW microbial ecology during storage and probe the subsequent behavior of those communities in an aerated treatment system. The ability for microbial communities native to PW to ‘self-treat’ water (i.e., improve water quality by the activity of native microbes alone) when mechanically aerated are not understood. Further, amendments of either KH₂PO₄ or AS test whether or not treatment of PW by native and / or introduced microbial communities can be improved. A better understanding of the biology underpinning successful treatment schemes is necessary for process upscaling and next-generation technology development—both of which are informed by this investigation. Lessons-learned about microbial ecology and the energetics thereof in the deep hydraulically fractured subsurface (6, 7) are yet to be optimized in treatment technologies. Subsurface-derived microorganisms possibly responsible for self-treatment of PW in aerated systems inherently require carbon, nitrogen, and other key elements (such as phosphorous) for assimilation into biomass. Therefore, the growth of microorganisms can remove undesirable constituents from water—suggesting that pathways to water treatment optimization by native microbial communities may exist.

Experiments were conducted to assess the viability of PW treatment with mechanical aeration as the primary mechanism to facilitate biological treatment. Microorganisms (primarily bacteria and archaea) in PW are of measurable abundance and, if aerated, may possess metabolisms capable of lowering concentrations of undesirable organic and inorganic constituents in PW—either alone or with the assistance of chemical and biological amendments. Through a controlled, long-term water treatment experiment we paired water chemistry and treatment data with 16S rRNA gene taxonomic profiling to investigate biological mechanisms of PW treatment efficacy—both under

PW alone (baseline) and amendment conditions. This study represents a pairing of biological and chemical data throughout a simple analysis of the potential for self-treatment of PW in comparison to improved treatment by two widely available amendments (either KH_2PO_4 or AS). PW is known to be phosphate-limited for microbial growth and activity (4), suggesting that simple amendments of either phosphate (as KH_2PO_4) or microbial communities and nutrients (including phosphorous) from a traditional AS source may affect microbial consortia and their resultant treatment capabilities on PW. Herein, we experimentally verify the role of microbial consortia in PW treatment; further, we expound on ecology-informed alterations to PW treatment engineered systems that may optimize PW treatment under realistic, ambient settings—either in the lab or on-site at O&G operations.

2. Materials and methods

2.1. Produced water sourcing

PW was acquired approximately every 3 weeks from the Denver-Julesburg (D-J) basin in Weld County, Colorado. The water was collected from the same well pad throughout the duration of the study. The PW was stored in the laboratory in a closed, 1 m³ plastic tote at indoor ambient temperature (20 °C); this untreated PW was used as the influent (hereafter referred to as ‘IN’) for all reactors.

2.2. Reactor experiment

Three 2-L reactor vessels filled with PW were operated constantly with a repeated one-hour cycle: mixing and aeration for 30 minutes, and then mixing without aeration for 30 minutes to simulate a sequencing batch reactor (SBR). The system was operated at ambient lab temperatures (i.e., 20 °C) over the course of 256 days. One reactor was fed with only PW and was used as a control (baseline), while two additional reactors were used to test the effect of specific additives on both microbial communities and treatment efficiency (i.e., organic and nutrients removal).

Every 24 hours, aeration and mixing were paused, solids were allowed to settle for at least 20 minutes, and then 600 mL of liquid was decanted from each vessel. 600 mL of raw PW was then added in order to maintain a 2 L volume in each reactor. At this point, one experimental reactor was dosed with 200 mg of return activated sludge (RAS) from a municipal sequence batch membrane bioreactor (SB-MBR) (5), and the second reactor was dosed with 7.5 mg/L of

phosphorous as KH_2PO_4 . Due to evaporation, deionized water was added to each jar every 3-4 days to maintain the complete 2 L volume.

The 600 mL of decanted supernatant was used for water chemistry analyses. On study days 80 and 221, 45 mL of mixed liquor was collected prior to pausing the aeration cycle, followed by a 550 mL decant after settling. The 45 mL samples of mixed liquor were frozen immediately at -20°C in 50 mL sterile polypropylene conical tubes (Corning Science Mexico, Reynosa, Tamaulipas, Mexico) until downstream microbiological processing. On study days 39, 80, and 102, 45 mL of raw produced water influent (IN) was collected, frozen, and stored with the same protocol as for reactor microbiome samples.

2.3. Chemical analysis

Anion measurements were conducted via ion chromatography (IC) on a ThermoFisher Dionex ICS-900, including a ThermoScientific DS5 conductivity detector, a Dionex ACRS 500 2 mm suppressor, a Dionex IonPac AG14A-5 μm RFIC 3x30 mm guard column, and a Dionex IonPac AS14A-5 μm RFIC 3x150 mm column. Metal analyses were conducted by inductively coupled plasma atomic emission spectroscopy (ICP-AES) on a Perkin Elmer Optima Model 5300 dual view spectrometer.

pH, conductivity, and turbidity were measured on daily calibrated benchtop instruments. sCOD, COD, ammonia, and ortho-phosphate were measured using Hach[®] TNT Plus[™] (Loveland, CO) reagent vials and Hach DR 6000[™] spectrophotometer. Total suspended solids, fixed suspended solids, and volatile suspended solids were measured following Standard Method 2540.

2.4. DNA extraction

The periodically collected 45 mL mixed liquor samples from IN and reactors were frozen in 50 mL polypropylene conical tubes that were later thawed over 30 minutes at the time of microbiological processing. 40 mL of each sample (samples normalized to 40 mL for centrifuge balancing) were then centrifuged at 7,000 G for 45 minutes. The supernatant was decanted and the solid pellet was frozen until DNA extraction kit procedures. The ZymoBIOMICS DNA MiniKit was used according to manufacturer instructions to extract DNA from each pellet. Prior to DNA extraction, each pellet was thawed over 5 minutes; the lysis solution from the Zymo kit was then

added to each sample conical. Conicals were vortexed until the pellet was completely solubilized in the lysis solution. The solution/pellet mixture was added to Zymo bead beating tubes, and the remainder of the DNA extraction protocol was conducted according to manufacturer instructions.

2.5. DNA sequencing

DNA amplicons were shipped to the Duke Center for Genomic and Computational Biology and sequenced on an Illumina MiSeq (Illumina Inc., San Diego, CA) instrument with V2 PE250 methods. 16S rRNA genes were amplified by PCR in duplicate 25 μ L reactions. Reaction concentrations: 1x QuantaBio 5PRIME HotMasterMix, 0.2 μ M 515-Y Forward Primer (5'-**GTA AAA CGA CGG CCA GT** CCG TGY CAG CMG CCG CGG TAA-3') where the M13 forward primer (bold) is ligated to the 16S RNA specific sequence with a 'CC' spacer connection, 0.2 μ M 926R (5'-CCG YCA ATT YMT TTR AGT TT-3') Reverse Primer, and sample DNA. Primers are from Parada et al. (2015) (17) with the modification of forward primers by the addition of the M13 linker sequence (18). PCR was carried out on a Techne TC-5000 thermocycler. PCR parameters: primary denaturation at 98 °C for 30 s, followed by 30 cycles of 98 °C for 10 s, then 52 °C for 20 s and 72 °C for 10 s, followed by a final sequence extension of 72 °C for 5 min and a 10 °C hold. Six additional cycles of PCR with the same parameters as above were then performed to attach barcodes (19) to the M13 linker region of the forward primer (18). 16S rRNA amplicon cleanup was performed with KAPA Pure Beads (Kapa Biosystems, Indianapolis, IN) at 0.8x concentration according to the manufacturer's instructions.

2.6. Bioinformatics

DADA2 (20) was used to—in order—remove forward and reverse primers from sequences; determine true sequence variants; merge paired-end reads; build a sequence table; and detect / eliminate chimeras. Untreated influent PW (IN) samples were rarefied to a sequencing depth of 12,874 sequences; i.e., all IN samples were forced to have the same sequencing depth via random subsampling. All IN samples had a sequencing depth greater than or equal to 12,874 and could be rarefied without rejection due to shallow sequencing depth. All experimental and control reactors were rarefied to 6,018 sequences and all samples from these vessels, similarly, were retained for post-rarefaction downstream analyses. Both rarefaction levels were based on an analysis of rarefaction curves, and values were chosen that allowed inclusion of all samples without

substantial sacrifice of alpha diversity. For reproducibility of our code, a specific random start seed was defined for sequence rarefaction. Taxonomy assignments were called with a greengenes training file (version 13.8) (21) through the DADA2 taxonomy assignment script. Chloroplast and mitochondrial sequences were removed such that only bacterial and archaeal sequences remained (the greengenes database does not include eukaryotes). Operational taxonomic unit (OTU) tables, taxonomy tables, and sample meta-data were compiled within the R package ‘phyloseq’ (22).

3. Results

3.1. Raw PW and experimental reactor chemistry

Water quality analysis by mean variables summarized in Table 1 was conducted on untreated influent PW (IN) and reactors daily or weekly (sampling frequency is summarized in a table in our raw chemistry data submission), just prior to refilling of all 2 L vessels (after decanting/sampling). Throughout the study, samples from IN and all reactors were taken for IC (major anion) and ICP-AES (metal) analyses; mean values over the course of the study and the number of measurements (‘n’) are summarized in Table 2. Both AS and KH_2PO_4 additions to reactors improved ammonia, sCOD, and COD removal relative to the no-addition baseline throughout the duration of the study (Fig. 1, Table 1).

Table 1. Mean values of chemistry measurements throughout the study (plus or minus one standard deviation), and % removal (by mean) of same variables from IN levels by reactor addition type. Data are adapted from Nicholas, E.R. 2017 (23, 24). ‘n’—the number of measurements for each variable—are reported in a table in our raw chemistry data submission. ‘n.m.’ indicates not measured.

	Raw PW (IN)	Baseline	Activated Sludge (AS)	KH_2PO_4	Mean % removal by Baseline	Mean % removal by AS	Mean % removal by KH_2PO_4
pH	6.84±0.32	8.08±0.14	8.08±0.24	8.05±0.29	n.a.	n.a.	n.a.
Turbidity (NTU)	89.9±73.6	82.3±63.4	832±624	348±289	8.45	-826	-287
TSS (mg/L)	43.8±46.2	138±257	1193±1233	292±326	-215	-2621	-566
VSS (mg/L)	20.7±21.5	66±182	847±503	146±143	-220	-3992	-604
sCOD (mg/L)	1509±859	1093±169	717±195	754±215	27.6	52.5	50.0
COD (mg/L)	1564±967	1102±300	1067±355	861±188	29.5	31.8	45.0
Ammonia (mg/L)	24.9±2.5	20.5±5.3	10.2±3.3	9.6±2.9	17.8	59.2	61.5
Phosphate (mg/L)	n.m.	0.07±0.13	0.85±2.46	16.8±28.2	n.m.	n.m.	n.m.
Conductivity (mS/cm)	n.m.	28.9±4.6	28.3±3.9	29.2±4.0	n.m.	n.m.	n.m.

Table 2. Mean concentrations of metals (mg/L) (for IN, n = 3; for reactors, n = 4) and anions (mg/L, except for IN which is in ppm) (for IN, n = 4; for reactors, n = 5), throughout the study. Metals and anions were determined via ICP-AES and IC, respectively. ‘n.a.’ indicates below instrument detection limit and ‘n.m.’ indicates not measured.

Metal / Anion	Raw PW (IN)	Baseline	Activated Sludge (AS)	KH ₂ PO ₄
Al	n.a.	n.a.	n.a.	n.a.
As	0.12	0.41	0.24	n.a.
B	16.7	20.1	16.5	17.6
Ba	9.07	10.5	8.47	7.55
Be	n.a.	n.a.	n.a.	n.a.
Ca	308.8	341.9	298.2	306.4
Cd	0.00	0.02	0.01	n.a.
Co	n.a.	n.a.	0.02	0.02
Cr	0.01	0.03	n.a.	0.03
Cu	0.04	0.22	0.12	0.22
Fe	1.32	4.37	1.69	0.75
K	33.9	41.7	38.7	88.8
Li	5.02	5.46	4.71	5.26
Mg	35.0	40.7	37.9	37.5
Mg	34.8	29.2	28.9	27.8
Mn	0.45	n.m.	n.m.	n.m.
Na	5157	5511	5121	5506
Ni	n.a.	2.17	3.61	7.90
P	n.m.	1.13	3.11	10.8
Pb	0.11	9.27	7.67	5.33
S	n.m.	5.42	5.82	6.60
Se	n.a.	13.8	10.3	31.3
Si	22.0	34.3	31.1	30.6
Sr	38.4	39.9	36.2	37.7
Tl	0.05	0.10	n.a.	n.a.
V	n.a.	0.47	0.31	0.23
Zn	n.m.	0.37	0.32	0.32
Sn	n.a.	n.a.	n.a.	n.a.
Mo	n.a.	n.a.	n.a.	n.a.
Sb	n.a.	n.a.	n.a.	n.a.
Ti	n.a.	n.a.	n.a.	n.a.
Fluoride	n.a.	n.a.	n.a.	n.a.
Chloride	8807	9703	8404	9296.1
Nitrite	6.06	12.2	9.88	12.7
Bromide	98.6	106.4	125.8	104.6
Nitrate	n.a.	11.1	n.a.	16.6
Phosphate	n.a.	n.a.	n.a.	99.4
Sulfate	5.9	24.21	14	37.6

Fig. 1. Concentrations of ammonia-N (A) and sCOD (B) in IN, and reactors (baseline, AS and KH₂PO₄). Vertical dotted lines indicate the time points of biological sampling of IN; the vertical dashed line indicates a time point of biological sampling of reactors; the vertical solid line indicates biological sampling of both IN and reactors.

3.2. Raw PW influent (IN) microbial community

IN fed into the reactor experiments was analyzed for order taxonomy microbial abundance over time (Fig. 2). During storage times of roughly 3 weeks in 1 m³ plastic totes, IN (measured at time of feed into reactors) coalesced into just a handful of taxonomic orders with a relative abundance greater than 1%. Similarly, the number of observed operational taxonomic units (OTUs) in IN decreased with time (Table 4).

Genus *Thalassospira* ranked as both the most prevalent and persistent genera of bacteria over time in IN; its relative abundance within classified genera increased from 11.1% to 22.4% and then to 28.4% over time. In contrast, genus *Marinobacterium* (the second most common genera in IN) decreased in abundance over time, beginning at 34.8% before decreasing to 8.3% and then slightly increasing to 15.9%.

Figure 2. IN order taxonomy relative abundances vs. day in study. The top 20 most abundant orders (A) are contrasted with a modified chart (B) where bacteria that are not one of the top 10 most abundant orders are grouped as ‘Other Bacteria’; i.e., all orders of bacteria that are not one of the 10 most common—but have known order taxonomy—are compiled into a single, shared, category denoted ‘Other Bacteria’. ‘Other Bacteria’, when treated as a composite group, become the second most common taxonomic classification (panel B). Bacteria that could not be classified are plotted separately as ‘Unclassified Bacteria’.

Table 4. Operational taxonomic unit (OTU) alpha diversities and cumulative rate of uncommon taxonomic classifications throughout the study.

Untreated Influent or Reactor Type	Study day	# Observed OTUs	% Bacteria not in top 10 orders ('Other Bacteria')
IN	39	135	17.8
	80	75	18.2
	102	75	15.5
Baseline	80	80	30.1
	221	55	7.2
AS addition	80	113	39.5
	221	329	43.9
KH ₂ PO ₄ addition	80	28	2.2
	221	38	7.0

3.3. Experimental/baseline reactor microbial community response

Both the baseline- and KH_2PO_4 - amended reactor vessels consisted almost exclusively of phylum *Proteobacteria*, while the AS vessel had greater diversity at the phylum level across all time points—the AS reactor contained dominant phyla *Proteobacteria* (62.1 - 62.2%), *Bacteroidetes* (11.5 - 28.7%), and *Chloroflexi* (1.6 - 9.7%). To more closely assess biotic differences between vessels, the order taxonomic rank was chosen to show the relative abundances of taxa in each reactor (Fig. 3). Order taxonomy was chosen because the vast majority of sequences from both IN and reactor samples were identifiable at this level. Furthermore, order taxonomy provided a greater resolution of differences between samples than the phylum level. At both reactor biological sampling time points (day 80 and day 221), all experimental vessels were distinct from one another as well as from the baseline reactor (Fig. 3). The baseline reactor had similar compositions on day 80 and day 221, and the relative abundances of present taxa varied only slightly, indicating dominant-taxa stability (Fig. 3). Specifically in the baseline vessel, orders *Kiloniellales* and *Caulobacterales* were most common and persisted over time between days 80 and 221; this is in contrast to IN in which *Kiloniellales* and *Oceanospirillales* were most abundant. Reactors dosed with KH_2PO_4 appear laden with completely different taxonomic orders relative to both the baseline and AS reactors, the most common of which were *Rhodobacterales*, *Pseudomonadales*, and *Oceanospirillales* (Fig. 3). While some orders have a noticeable presence in both the baseline vessel and the vessel dosed with KH_2PO_4 , the relative abundances of these shared taxa were remarkably different. Stark differences in community structure between the AS and KH_2PO_4 addition reactors are orthogonal to their similar removal efficacy (Fig. 1).

Figure 3. Order taxonomy relative abundances vs. day in study for AS and KH₂PO₄ amendment reactors compared to baseline (A). Panel A is modified in panel B by combining orders of bacteria that are not one of 10 most common into a new group called ‘Other Bacteria’ (B); i.e., all orders of bacteria that are not one of the 10 most common—but have known order taxonomy—are compiled into a single, shared, category denoted ‘Other Bacteria’. ‘Other Bacteria’, when treated as a composite group, become the second most common taxonomic classification (panel B). Bacteria that could not be classified are plotted separately as ‘Unclassified Bacteria’.

Notably, at the genus taxonomic rank, *Thalassospira* existed at no more than 7.7% relative abundance in either the AS or KH₂PO₄ reactors at both time points, marking a substantial reduction in abundance from IN. However, genus *Marinobacterium* (the second most common genera in IN) completely disappeared from the AS vessel but remained highly abundant in the KH₂PO₄ vessel. In the baseline reactor, the most common genera were largely unchanged from IN: *Thalassospira*, *Marinobacterium*, and *Mycoplana* remained the most abundant.

3.4. Microbial Diversity Response to Experiments

As time progressed microbial community diversity increased within both the AS and KH₂PO₄ test groups while the diversity in the baseline reactor decreased with time (Table 4). ‘Other Bacteria’, existing at nominally low relative abundances as individuals—but have known order taxonomy, collectively represented a substantial component of the total community in all of baseline, AS, and KH₂PO₄ conditions (Fig. 3B, Table 4). The baseline, AS, and KH₂PO₄ reactors

showed different trends in the percent of identified bacteria not in the top 10 most abundant orders (i.e., ‘Other Bacteria’). The AS condition had an abundant coalition of less common microbes (i.e., microbial community members in low abundance representative of the so-called rare biosphere); though less pronounced, these lesser-appreciated constituents of a canonical ‘abundant’ microbiome were still a substantial component of both the baseline and KH_2PO_4 reactors (Table 4).

4. Discussion

Results demonstrate that additions of AS or KH_2PO_4 were simple and effective means to enhance the pretreatment of PW in an intermittent aeration/mixing system. Pretreatment of PW in a microbial system was viable over the extended study duration, even when PW was stored for weeks at indoor ambient lab conditions. Periodic peaks in PW feed water quality (e.g., sCOD (Fig. 1B)) were well tolerated by the system, suggesting that pretreatment of PW can be achieved on-site at O&G operations with little intervention. Here, we report that ammonia, sCOD, and COD removal was modest with intermittent aeration cycles in the baseline reactor while removal of the same chemical constituents was substantially enhanced by the addition of either AS or KH_2PO_4 . Given that the raw PW (IN) fed into the experimental and baseline reactors had little-to-no detectable phosphate (Tables 1 and 2), and the subsequent measurable concentrations of phosphate during treatment (Tables 1 and 2), it is likely that the microbial consortia contained within our treatment scheme were nutrient limited with respect to phosphorous prior to AS or KH_2PO_4 addition.

The KH_2PO_4 reactor had lower microbial diversity relative to the baseline reactor, but had better treatment performance; however, we noticed that the reactor with added AS had a similar increase in treatment performance over baseline, but with a complete opposite microbial diversity response—i.e., the number of observed OTUs was 41% greater relative to the baseline reactor on day 80, and is 498% greater on day 221 (Table 4). The changes in relative abundances of the most common order-classified taxa are deceptively slight in comparison to shifts in the composite abundance of less common microbiota—all with known order taxonomy; in both the raw PW feed and the experimental reactors, greater changes in ‘Other Bacteria’ and the number of observed OTUs are seen (Table 4) relative to what may be perceived were one to look only at the top-most abundant orders. Collectively, comparisons of the most abundant taxa, the number of observed

OTUs, and the contribution of uncommon microbiota to the total population suggest that a broad interpretation of ‘who’s there’ is essential for a thorough assessment of biological PW treatment systems.

As observed in our experimental setups, it may be well true that microbial community composition of the rarer members of the community, those in least abundance and possibly present as mere singletons, likely play a substantial role in both community support to abundant members and functional redundancy of metabolic response (25, 26). Microbiota native to PW and those that live within AS are not identical. Nonetheless, we empirically show functional redundancy between these two distinct communities; i.e., similar PW treatment efficacy is conducted by both. Louca et al. (25) describe community functional capacity as decoupled from the abundance of taxonomic clades; further, this decoupling is thought to systemically arise from the need for microbial communities to possess a base set of critical metabolic functions. In our experiment, it is possible that stochastic processes are not responsible for the similar treatment efficacy of KH_2PO_4 - and AS-additions but, rather, these critical metabolic functions are actively selected for from distinctly different microbial communities; i.e., an inherent property of microbial communities native to PW in engineered systems may be to adapt to the task at hand independent of the overwhelming diversity of microorganisms within ‘The deep, hot biosphere’, as hypothesized by Thomas Gold in 1992 (27, 28).

Genus *Thalassospira* was most common in the raw PW feed and is a known chemotactic bacterium that pursues inorganic phosphate (29), further suggesting that raw PWs are initially phosphorous-limited. In addition to genus *Thalassospira*, genus *Marinobacterium* (a known carbon opportunist in O&G fracture systems (7)) was found to be persistent over time in the raw PW feed, despite a reduction in alpha diversity as time progressed (Table 4); the persistence of microbiota native to subsurface systems in PW suggests that the functional capacity to treat PW may prevail under ambient, surface conditions. Persistence of subsurface metabolisms in surface, engineered treatment systems may enable more techno-economic flexibility in the treatment of PW for beneficial reuse. Genus *Marinobacterium* was also common in the baseline and KH_2PO_4 -amended reactors; however, despite similar chemistry removal performance of the AS-amended reactor, *Marinobacterium* in those samples was neither present nor enriched with time. Disparate fates of *Marinobacterium* in the experimental reactors suggest that there exist multiple

microbiological consortia capable of treating PW, and that some of those communities—such as those inoculated by AS—may not necessarily derive exclusively from the subsurface or PW.

In light of Frank, et al. (4), it is likely that the treatment of PW in POTWs—seen optimally between 6-20% by volume—is a synthesis of performance by both AS and native PW microbial communities. Nonetheless, microbial communities existing within AS may have a limit on their ability to treat PW—suggesting that simple additions of phosphorous—optimized within a range centered at daily doses of 7.5 mg P/L—in an aerated/mixed system could be an excellent mechanism to enhance pre-treatment of PW. Further, entirely different microbial consortia appear to have similar chemistry removal efficacy; this is promising for the treatment of PW because an exact replication of a particular treatment-capable microbial consortia may not be necessary, indeed likely impossible given the variance of PW microbial communities, to reach treatment goals.

At the order taxonomy level, *Rhodobacterales* are the most greatly enriched taxa in both AS- and KH_2PO_4 -amended reactors across all time points (Fig. 3). This increase in relative abundance may indicate a role in PW treatment by *Rhodobacterales*. *Rhodobacterales* are known surface colonizers in saline coastal marine waters (30); their exceptional adaptation for early surface colonization may position them well for establishment as a biofilm in PW treatment reactors. Shortly after exposure to crucial nutrient additions (i.e., phosphorous), it is possible that biofilm colonization by *Rhodobacterales* is potentiated—which would make sense in light of *Rhodobacterales*'s enrichment in AS- and KH_2PO_4 -amended reactors. Future work should consider the role of planktonic versus biofilm communities in the treatment of PW, revealing more optimal physical designs of treatment reactors better tuned to the needs of surface colonizers.

5. Conclusion

Herein, we have described a simple, minimal-intervention bench scale PW treatment scheme. Microbial consortia native to PW are capable of attenuating concentrations of sCOD, COD, and ammonia. Further, simple additions of either AS or phosphorous as KH_2PO_4 are sufficient to further improve water treatment. Microbiome analyses of this simple, O&G field-site compatible engineered system suggest that several microbial consortia can treat PW across a range of both chemical and environmental conditions—a promising finding for practical considerations of PW treatment system implementation in the field at O&G sites where advanced systems are less

tractable and it is essential to consider techno-economic viability. Via simple additions of AS or KH_2PO_4 , PW (a phosphorous-limited system) water quality can be enhanced by microbial communities jumpstarted by these additions. In light of this study, future work on the treatment of PW should consider PW a ‘living system’. Both microbiota native to the subsurface and introduced to PW by AS apparently tolerate changes in environmental conditions while consistently enhancing the water quality of PW. Future work should focus on greater understanding of the mechanisms of ecological functional redundancy in engineered PW treatment systems; improved knowledge about basic ecological phenomena during applied interventions will likely lead to better optimized PW treatment schemes for beneficial water reuse.

Data Availability

All raw data, R code, files, and R objects used in the production of this manuscript are available for review and enable immediate reproduction of results (link to DropBox below).

<https://www.dropbox.com/sh/7fpfkc0lu3d28sa/AACsyRAeWTXx773BK1SPSIEma?dl=0>

If the manuscript is accepted, all raw DNA sequencing reads will be uploaded to NCBI SRA with accession numbers listed here; additionally, the same raw data folder shared for review on DropBox will be shared publicly on figshare with a DOI listed here.

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References

1. **Fry M, Hoeninghaus DJ, Ponette-González AG, Thompson R, La Point TW.** 2012. Fracking vs Faucets: Balancing Energy Needs and Water Sustainability at Urban Frontiers. *Environmental Science & Technology* **46**:7444–7445.

- 435 2. **Murali Mohan A, Hartsock A, Bibby KJ, Hammack RW, Vidic RD, Gregory**
436 **KB.** 2013. Microbial Community Changes in Hydraulic Fracturing Fluids and
437 Produced Water from Shale Gas Extraction. *Environmental Science & Technology*
438 **47**:13141–13150.
- 439 3. **United States Environmental Protection Agency.** 2015. Technical Development
440 Document for Proposed Effluent Limitations Guidelines and Standards for Oil and
441 Gas Extraction 1–190.
- 442 4. **Frank VB, Regnery J, Chan KE, Ramey DF, Spear JR, Cath TY.** 2017. Co-
443 treatment of residential and oil and gas production wastewater with a hybrid
444 sequencing batch reactor-membrane bioreactor process. *Journal of Water Process*
445 *Engineering* **17**:82–94.
- 446 5. **Vuono DC, Benecke J, Henkel J, Navidi WC, Cath TY, Munakata-Marr J,**
447 **Spear JR, Drewes JE.** 2014. Disturbance and temporal partitioning of the activated
448 sludge metacommunity. *The ISME Journal* **9**:425–435.
- 449 6. **Borton MA, Daly RA, O'Banion B, Hoyt DW, Marcus DN, Welch S, Hastings**
450 **SS, Meulia T, Wolfe RA, Booker AE, Sharma S, Cole DR, Wunch K, Moore**
451 **JD, Darrah TH, Wilkins MJ, Wrighton KC.** 2018. Comparative genomics and
452 physiology of the genus *Methanohalophilus*, a prevalent methanogen in
453 hydraulically fractured shale. *Environmental Microbiology* **20**:4596–4611.
- 454 7. **Evans MV, Panescu J, Hanson AJ, Welch SA, Sheets JM, Nastasi N, Daly RA,**
455 **Cole DR, Darrah TH, Wilkins MJ, Wrighton KC, Mouser PJ.** 2018. Members of
456 *Marinobacter* and *Arcobacter* Influence System Biogeochemistry During Early
457 Production of Hydraulically Fractured Natural Gas Wells in the Appalachian Basin.
458 *Frontiers in microbiology* **9**:337–17.
- 459 8. **Borton MA, Hoyt DW, Roux S, Daly RA, Welch SA, Nicora CD, Purvine S,**
460 **Eder EK, Hanson AJ, Sheets JM, Morgan DM, Wolfe RA, Sharma S, Carr TR,**
461 **Cole DR, Mouser PJ, Lipton MS, Wilkins MJ, Wrighton KC.** 2018. Coupled
462 laboratory and field investigations resolve microbial interactions that underpin
463 persistence in hydraulically fractured shales. *Proc Natl Acad Sci USA* **115**:E6585–
464 E6594.
- 465 9. **Cluff MA, Hartsock A, MacRae JD, Carter K, Mouser PJ.** 2014. Temporal
466 Changes in Microbial Ecology and Geochemistry in Produced Water from
467 Hydraulically Fractured Marcellus Shale Gas Wells. *Environmental Science &*
468 *Technology* **48**:6508–6517.
- 469 10. **Lipus D, Roy D, Khan E, Ross D, Vikram A, Gulliver D, Hammack R, Bibby K.**
470 2018. Microbial communities in Bakken region produced water. *FEMS*
471 *Microbiology Letters*, 21st ed. **365**:2069.

- 472 11. **Struchtemeyer CG, Elshahed MS.** 2011. Bacterial communities associated with
473 hydraulic fracturing fluids in thermogenic natural gas wells in North Central Texas,
474 USA. *FEMS Microbiology Ecology* **81**:13–25.
- 475 12. **Liang R, Davidova IA, Marks CR, Stamps BW, Harriman BH, Stevenson BS,**
476 **Duncan KE, Suflita JM.** 2016. Metabolic Capability of a Predominant
477 *Halanaerobium* sp. in Hydraulically Fractured Gas Wells and Its Implication in
478 Pipeline Corrosion. *Frontiers in microbiology* **7**:116.
- 479 13. **Booker AE, Borton MA, Daly RA, Welch SA, Nicora CD, Hoyt DW, Wilson T,**
480 **Purvine SO, Wolfe RA, Sharma S, Mouser PJ, Cole DR, Lipton MS, Wrighton**
481 **KC, Wilkins MJ.** 2017. Sulfide Generation by Dominant *Halanaerobium*
482 Microorganisms in Hydraulically Fractured Shales. *mSphere* **2**:2278–17.
- 483 14. **Sowards JW, Williamson CHD, Weeks TS, McColskey JD, Spear JR.** 2014. The
484 Effect of *Acetobacter* sp. and a Sulfate-Reducing Bacterial Consortium from
485 Ethanol Fuel Environments on Fatigue Crack Propagation in Pipeline and Storage
486 Tank Steels. *Corrosion Science* **79**:128-138.
- 487 15. **Gaspar J, Mathieu J, Yang Y, Tomson R, Leyris JD, Gregory KB, Alvarez PJJ.**
488 2014. Microbial Dynamics and Control in Shale Gas Production. *Environmental*
489 *Science & Technology* **1**:465–473.
- 490 16. **Akyon B, Stachler E, Wei N, Bibby K.** 2015. Microbial Mats as a Biological
491 Treatment Approach for Saline Wastewaters: The Case of Produced Water from
492 Hydraulic Fracturing. *Environmental Science & Technology* **49**:6172–6180.
- 493 17. **Parada AE, Needham DM, Fuhrman JA.** 2016. Every base matters: assessing
494 small subunit rRNA primers for marine microbiomes with mock communities, time
495 series and global field samples. *Environmental Microbiology* **18**:1403–1414.
- 496 18. **Stamps BW, Lyles CN, Suflita JM, Masoner JR, Cozzarelli IM, Kolpin DW,**
497 **Stevenson BS.** 2016. Municipal Solid Waste Landfills Harbor Distinct
498 Microbiomes. *Frontiers in microbiology* **7**:507.
- 499 19. **Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N,**
500 **Owens SM, Betley J, Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G,**
501 **Knight R.** 2012. Ultra-high-throughput microbial community analysis on the
502 Illumina HiSeq and MiSeq platforms. *The ISME Journal* **6**:1621–1624.
- 503 20. **Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP.**
504 2016. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat*
505 *Meth* **13**:581–583.
- 506 21. **DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T,**
507 **Dalevi D, Hu P, Andersen GL.** 2006. Greengenes, a Chimera-Checked 16S rRNA
508 Gene Database and Workbench Compatible with ARB. *Applied and Environmental*
509 *Microbiology* **72**:5069–5072.

510 22. **McMurdie PJ, Holmes S.** 2011. PHYLOSEQ: A Bioconductor Package For
511 Handling And Analysis Of High-Throughput Phylogenetic Sequence Data.
512 Proceedings of the Pacific Symposium 235–246.

513 23. **Nicholas ER.** 2017. Evaluation of a sequencing batch reactor followed by media
514 filtration for organic and nutrient removal from produced water. Masters Degree
515 Thesis, Dept of Civil and Environmental Engineering, Colorado School of Mines,
516 Golden, CO.

517 24. **Nicholas ER, Cath TY.** 2020. Evaluation of sequencing batch bioreactor followed
518 by media filtration for organic carbon and nitrogen removal in produced water.
519 Under Review in Journal of Water Process Engineering.

520 25. **Louca S, Polz MF, Mazel F, Albright MBN, Huber JA, O'Connor MI,**
521 **Ackermann M, Hahn AS, Srivastava DS, Crowe SA, Doebeli M, Parfrey LW.**
522 2018. Function and functional redundancy in microbial systems. *Nat Ecol Evol*
523 **2**:936–943.

524 26. **Trivedi CB, Stamps BW, Lau GE, Grasby SE, Templeton AS, Spear JR.** 2020.
525 Microbial Metabolic Redundancy Is a Key Mechanism in a Sulfur-Rich Glacial
526 Ecosystem. *mSystems* **5**:e00504–20.

527 27. **Gold T.** 1992. The deep, hot biosphere. *Proc Natl Acad Sci USA* **89**:6045–6049.

528 28. **Colman DR, Poudel S, Stamps BW, Boyd ES, Spear JR.** 2017. The deep, hot
529 biosphere: Twenty-five years of retrospection. *Proc Natl Acad Sci USA* **114**:6895–
530 6903.

531 29. **Hütz A, Schubert K, Overmann J.** 2011. *Thalassospira* sp. Isolated from the
532 Oligotrophic Eastern Mediterranean Sea Exhibits Chemotaxis toward Inorganic
533 Phosphate during Starvation. *Applied and Environmental Microbiology*, 2nd ed.
534 **77**:4412–4421.

535 30. **Dang H, Li T, Chen M, Huang G.** 2007. Cross-Ocean Distribution of
536 Rhodobacterales Bacteria as Primary Surface Colonizers in Temperate Coastal
537 Marine Waters. *Applied and Environmental Microbiology* **74**:52–60.

538