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Growth dynamics and pollutant removal efficiency of the cyanobacterium *Komvophoron* sp. in flowback and produced water: a pilot study

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Abstract

This study explores the use of a native hypersaline microalgae strain from Oklahoma, *Komvophoron* sp., for dual purposes: treating Flowback (FW) and Produced Water (PW), and generating algal biomass. The wastewaters were analyzed before and after treatment, and the resulting biomass was characterized for moisture, volatile matter, fixed carbon, and ash content. *Komvophoron* sp. thrived in both FW and PW, achieving higher biomass concentrations when cultivated in PWs compared to FWs despite nutrient limitations. It also showed high specific growth rates in both water types. The biomass had an energy content of 16–17 MJ kg^{-1}, suitable for biofuel feedstock, although salinity slightly reduced this value. Algal cultivation fully removed ammonia and significantly reduced nitrate, phosphate, boron, and metals such as zinc, manganese, and iron. This approach shows strong potential for reducing the environmental impact of hydraulic fracturing while producing biomass for biofuels and other industrial uses.

Introduction

Hydraulic fracturing, widely known as fracking, is a well-established drilling method used to extract oil and natural gas from deep underground formations. Particularly prevalent in North America, this technique has proven effective for unlocking hydrocarbon reserves in low-

permeability and unconventional formations such as shale, coal beds, and tight sands.[1] Fracking involves injecting a high-pressure fluid mixture (up to 100 MPa at the well inlet) consisting of water, chemical additives, and solid proppants to generate and expand fractures within and beneath the reservoir.[2] These proppants help keep the fractures open, enabling the hydrocarbons to flow freely back to the surface.

This large-scale technique took off in the early 1990s in the United States and later in Canada around 2005, making shale oil and gas production economically viable.[3] By 2020, there were approximately 90,000 active fracking wells in North America, with 73,000 in the USA and 18,000 in Canada.[4] Driven by increasing energy demands across various sectors, the fracking market in North America is projected to keep growing through 2030.[5] Depending on the target resource, the fracking market can be segmented into crude oil, shale gas, tight gas, coal bed methane, and others. In 2020, USA recoverable reserves included 45.62 trillion m^3 of shale gas and 174 billion barrels of tight/shale oil.[6] Fracking requires substantial volumes of water, ranging from 10,000 to 236,620 m^3 per well depending on the well design, and generates significant Wastewater (WW), including Flowback Water (FW) and Produced Water (PW). On average, a single fracking operation can use between 1.5 million and 9.7 million gallons of water.[7] Environmental challenges linked to fracking include freshwater depletion, WW contamination, aquifer pollution, induced seismicity, and unsustainable WW disposal. Notably, the fate of the large volumes of water used and generated in fracking remains poorly understood, raising public concern.

Fracking fluids typically contain chemicals for pH control, microbial inhibition, gelling, and corrosion resistance, adding to the environmental uncertainty. According to well operator data, FW represents about 22% of the water returning from the well, composed of drilling and fracking fluids as well as formation brines.[8] Once the initial flowback period concludes, all produced water is classified as PW. In USA operations, FW and PW together can constitute between 10% and 80% of the injected fracking fluid volume.[9] Recent reviews highlight that PWs are generated in greater volumes than FWs and pose heightened environmental concerns. PW composition varies widely, containing minerals like sodium, potassium, chloride, bromide, calcium, barium, and radioactive elements such as strontium, radium, and uranium.[10] Organic pollutants including polycyclic aromatic hydrocarbons, Benzene, Toluene, Ethylbenzene, and Xylenes (BTEX) compounds, long-chain fatty acids, and more, also feature prominently.[11] Total Dissolved Solids (TDS)

concentrations in PW can vary from 100 to 400,000 mg L^{-1} depending on well age, geological setting, and water sources.[12] State and federal onshore operations are responsible for most PW production in the USA (over 20 billion gallons), with offshore federal and tribal land activities accounting for roughly 3%. In 2017, Texas led PW generation (nearly 10 billion gallons, or 41% of the national total), followed by California, Oklahoma, Wyoming, and Kansas.[13] The environmental impact of these WW streams depends on factors like well numbers, local geology and hydrology, proximity to freshwater sources, available treatment and disposal options, and PW chemical characteristics.[14] Effective, eco-friendly disposal remains a key sustainability challenge for the fracking sector. Current disposal practices include deep-well injection, reuse in agriculture and industry, onsite and offsite treatment, and surface discharge.[13] In states like Pennsylvania and Oklahoma, deep-well injection is the dominant practice, but concerns over induced seismicity in Oklahoma have prompted regulatory measures to mitigate these risks.[15]

While treatment and reuse of PW have been explored using various techniques, there's a pressing need to cut costs and environmental impacts while meeting national discharge standards (*i.e.* oil and grease $\leq$ 29 mg $>L^{-1}$ monthly average under strict limits in offshore discharges).[16] This has spurred interest in alternative treatment approaches like microalgae-based bioremediation. Studies demonstrate that microalgae can survive in these challenging WW environments and contribute to improving water quality, though their growth is often limited by low nutrient availability.[17-19] Interestingly, adding nutrient-rich effluents (*i.e.*, animal WW) can boost both contaminant removal and algal biomass yields.[20] Microalgae adapted to high salinity can thrive in hypersaline fracking waters, making algal treatment an attractive option to tackle the large volumes and high salinity of FWs and PWs.

Komvophoron sp., a genus of filamentous motile cyanobacteria belonging to the class of Cyanophyceae, has been reported to grow both in FWs and PWs.[18] The filaments (trichomes) occur alone or grouped in clusters or delicate, mucilaginous mats, appearing straight, slightly wavy, or curved. Trichomes, usually short and rarely extending up to 650 μm, are deeply constricted and do not taper at the ends, showing slight motility (trembling). Cells are generally spherical or barrel-shaped, up to 10 μm in width, lacking gas vesicles but may contain large, irregularly scattered granules. Reproduction occurs by splitting trichomes in the middle without necrotic cells, producing isopolar hormocytes that germinate from both ends. These are mainly benthic forms

found alone or in delicate mats on sandy or muddy bottoms of clean lakes, pools, small reservoirs, and streams, as well as in metaphyton on plants and rarely in plankton.[21]

In this study, *Komvophoron* sp. was cultured in PW and FW samples from Oklahoma wells, and its growth and biomass chemical profiles were characterized. We also analyzed inorganic contaminant concentrations before and after algal treatment.

Materials and Methods

Inoculums and culture medium preparation

The strain *Komvophoron* sp. examined in this study was obtained from the culture collection of the University of Texas (UTEX) at Austin. This strain, identified as SP33, was originally collected from Great Salt Plains (GPS), OK, USA and maintained in the A+ culture medium at room temperature. It was maintained at room temperature in 50 mL glass tubes and grown in the medium that was recommended by UTEX, that is A+. This medium contained 18 g L^{-1} NaCl (Fisher S271-500; 0.308 M) and 5 g L^{-1} $MgSO_4 \cdot 7H_2O$ (Sigma 230391; 0.02 M) as the primary salts. Metal chelation was ensured by adding $Na_2EDTA \cdot 2H_2O$ (Sigma ED255; 0.08 mM). Additional macronutrients were supplied through KCl (Fisher P217; 8.05 mM), $CaCl_2 \cdot 2H_2O$ (Sigma C-3881; 2.52 mM), $NaNO_3$ (Fisher BP360-500; 11.8 mM) as the nitrogen source, and KH_2PO_4 (Sigma P0662; 0.37 mM) as the phosphorus source. Buffering capacity was provided by Trizma Base (pH 8.2; 8.26 mM). A sterile A+ trace-element solution was added at 1% (v v^{-1}). All chemicals used in this study were reagent-grade unless otherwise stated. The culture tubes, gently covered with a plastic cap to allow for air diffusion and shaken regularly by hand, were illuminated with a Photosynthetic Photon Flux (PPF) of 40 µmol m^{-2} s^{-1} provided by two 32 W white fluorescent tubes (F32T8/SP65/ECO, General Electric Company, Fairfield, CT, USA) and exposed to a light/dark photoperiod of 12/12 h.

Wastewater collection and pre-treatment

PWs and FWs samples were collected from active wells located in Kingfisher county, Oklahoma, USA. The produced water samples, identified as PW-A, PW-B, and PW-C, were collected from

JR Burton (1H-28X), Alig (1H-35), and Santana (1H-29X) wells, while the flowback water samples, identified as FW-A and FW-B, were collected from Carol (1H-18) and Judy (1H-17) wells. Upon collection, the samples were stored in clean plastic buckets at ambient temperature and processed within three months.

To separate the oil phase, the samples underwent a 3-hour treatment with an oil skimmer (Anabaki Oil Skimmer, Chagrin Falls, OH, USA). Next, an overnight separation in a separatory funnel allowed complete phase segregation, and the lower aqueous phase was retrieved. This water was heated to 40° C for 1 h with continuous stirring using a Model Arex 6 hot plate (Velp Scientifica Inc, Deer Park, NY, USA) at 40° C for 1 h with continuous stirring to volatilize any residual organic compounds (Volatile Organic Compunds, VOCs). Following filtration through paper disks (#1, Whatman, Cambridge, UK), the samples were sterilized at 121°C for 20 min in an autoclave (Hirayama, HVE-50, Ramsey, MN, USA) before use in cultivation experiments.

Algae cultivation

Komvophoron sp. was grown in 2 L borosilicate glass Photobioreactors (PBRs) (Kimble Chase Life Science and Research Products LLC, Vineland, NJ, USA), sealed with GL45 3-port caps (CPLabSafety, Novato, CA, USA). Gas exchange was enabled via sterile syringe filters (Argos Technologies, Elgin, IL, USA) and controlled with a polypropylene check univalve (VWR Science, Bristol, CT, USA). The PBRs were placed in a controlled growth chamber (118.8 cm length × 58.4 cm height × 76.9 cm width) at 23 ± 4 °C. Twelve 23 W cool white fluorescent bulbs (Osram Sylvania, Wilmington, MA, USA) delivered a PPF of 85 ± 4 μmol m^{-2} s^{-1}, measured by a quantum meter (QMSW-SS, Apogee Instruments Inc., Logan, UT, USA), following a 12 h light/12 h dark photoperiod. During the light phase, aeration was on, while in the dark phase, it was switched off. The aeration rate was 20 mL min^{-1}, and the concentration of CO_2 (industrial carbon dioxide, Airgas, Stillwater, OK, USA) in the air (Grade D breathing air, Airgas, Stillwater, OK, USA) was 5% v v^{-1}. Cultures were inoculated at an initial biomass concentration of 0.1 g L^{-1} and maintained at a working volume of 1.2 L. All cultivation equipment was thoroughly cleaned, autoclaved (121°C, 20 min), and UV-sterilized in a laminar flow cabinet (Air Science Ltd. Purair 24-PCR, Lydiate, UK). Strict aseptic techniques, including the use of sterile disposable pipettes and loops, were employed throughout.

Characterization of microalgae growth pattern

Algal growth was monitored daily by measuring absorbance (ABS) at 680 nm using a spectrophotometer (DU 520, Beckman Coulter, Brea, CA, USA), with distilled water as the blank over a period of one month. A calibration curve was established to relate absorbance to dry biomass concentration. For gravimetric biomass quantification, 5 mL culture samples (V) were filtered through pre-weighted (W_1) glass microfiber filters (GF/C^{TM} 55 mm diameter, Whatman, Cambridge, UK), dried in a forced-air oven (model F Air 2.3 CF, VWR Science, Radnor, PA, USA) at 105°C overnight to a constant weight (W_2), and re-weighed using an analytical scale (model A-160, Denver Instrument, Bohemia, NY, USA).

The dry biomass concentration (X_{dw}, g L^{-1}) was calculated using the following equation:

$$X_{dw} = W / V \quad (1)$$

where W is the weight of dried biomass (g) and V is the culture volume (L).

Maximum biomass productivity (ΔX, g $L^{-1}d^{-1}$) was determined as:

$$\Delta X = \max X_{dw} / t_{max} \quad (2)$$

where max X_{max} is the maximum biomass concentration (g L^{-1}) obtained at t_{max}, which is the time point at which the X_{max} is reached during the cultivation

Specific growth rate (μ, d^{-1}) was calculated according to the following equation:

$$\mu = (\ln X_2 - \ln X_1) / (t_2 - t_1) \quad (3)$$

where X_2 and X_1 are the dry biomass concentration (g L^{-1}) at time t_2 and t_1, respectively.

and doubling time (t_d, days) was calculated as:

$$t_d = 1 / (\mu / \ln (2)) \quad (4)$$

Culture pH was measured with a pH meter (AR20, Fisher Scientific, Waltham, MA, USA).

Algal biomass characterization

Thermogravimetric Analysis (TGA) was used to determine the proximate composition of algal biomass.[22,23] In summary, samples were heated from 25 °C to 110 °C at 20 °C min^{-1} under nitrogen atmosphere (flow rate 50 mL min^{-1}) and held at 110 °C for 6 min to measure Moisture (M) content. Subsequently, the temperature was increased to 575 °C at 80 °C min^{-1} and held for 10 min to measure Volatile Matter (VM). Then, the atmosphere was switched from nitrogen to air (50 mL min^{-1}), and the temperature was increased to 800 °C at 80 °C min^{-1} to quantify Fixed Carbon (FC) and residual Ash (A).

The higher heating value (HHV, MJ kg^{-1}), was estimated as:[24]

$$HHV = 0.3536 \times FC + 0.1559 \times VM - 0.0078 \times A \quad (5)$$

Wastewater quality

Chemical analyses of PW and FW samples (pre- and post-algae treatment) were conducted by the Soil, Water and Forage Analytical Laboratory (SWFAL) at the Oklahoma State University employing standard analytical methods.[25] At the end of the cultivation, samples were centrifuged at 8,000 rpm for 10 min (Sorval RC-5C Plus centrifuge, Ramsey, MN, USA). The supernatant was filtered (Whatman GF/C™, UK), and Chemical Oxygen Demand (COD) was determined with USEPA Method using a DBR200 vial heater reactor (HACH, Loveland, CO, USA).[22] A calibration curve generated using standard solutions was used for COD determination.

Data analysis

All experiments were performed in duplicate or more, and average values with standard deviations were reported. Statistical analyses of data were carried out using SAS 9.2 and SAS 9.3 (SAS Institute Inc., Cary, NC, USA). Regression equations and data visualization were prepared in Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA, USA).

Results

The chemical composition of the PWs and FWs before and after the cultivation of *Komvophoron* sp. is summarized in Table 1.

Sodium and chloride dominated the ionic profile of all water samples, except for FW-B. This ion pattern is typical of water from Oklahoma's Anadarko Basin.[23] Additionally, bicarbonate was present in notable quantities across samples. Calcium emerged as the second most abundant cation, followed by potassium, which is an essential nutrient for algae cultivation. High calcium levels in PW are known to contribute to scaling and the precipitation of minerals and A. Generally, PW is characterized by elevated concentration of sodium, chloride, calcium, magnesium, potassium, sulfate, bromide, bicarbonate, and iodide. However, the specific ionic ratios can differ from those in seawater, potentially influencing PW's aquatic toxicity. The pH of WW samples was generally alkaline, ranging from 7.6 to 9, except for PW-C, which exhibited slightly acidic characteristics. As expected for WWs generated during hydraulic fracturing operations, the concentration of key nutrients, such as Nitrogen (N) and Phosphorous (P), were relatively low. Essential micronutrients for algae growth, including iron, copper, manganese, and zinc, were also present in very small quantities, with the notable exception of higher iron concentrations detected in PW-C and FW-B.

To better understand the physiological response of *Komvophoron* sp. in the various WW media, the evolution of both Optical Density (OD) and pH during cultivation was monitored in Figure 1a and 1b, respectively. Except for PW-B, all cultures exhibited an initial lag phase of approximately four days, after which exponential growth occurred and eventually plateaued into a stationary phase lasting around 20 days. This initial delay is likely a reflection of the organism's need to adapt to the extreme conditions presented by some of the tested waters, particularly the elevated salinity and heavy metal loads characteristic of certain fracking WWs. A comparison between Electrical Conductivity (EC) in PW-C (EC = 76,400 μmhos cm^{-1}) and FW-B (EC = 9,830 μmhos cm^{-1}) supports the idea that higher salinity is associated with longer lag phases. However, confirming this relationship will require more in-depth analysis.

When normalized to the initial OD values, cultures grown in PW-A and PW-C exhibited the most significant increase in OD over time, consistent with the high X_{max} and specific growth rate values reported in Table 2. This suggests that, despite the elevated salinity and ionic loads in these PWs (Table 1), the strain was able to maintain active growth, possibly due to its halotolerant origin from the GSP in northern Oklahoma.

In contrast, FW-B showed only a marginal increase in OD, aligning with its low X_{max} (0.40 g L^{-1}) and biomass productivity (19 mg L^{-1} day^{-1}). This confirms that the specific ionic composition,

particularly lower levels of sodium and chloride, and nutrient scarcity likely constrained growth performance.

Beyond salinity, other growth-inhibiting factors in fracking waters may include elevated concentrations of metals like Zn, Cu, and Mn, as well as persistent organic contaminants potentially toxic to cyanobacteria. Despite these harsh environmental stressors, *Komvophoron* sp. demonstrated the ability to grow in both PWs and FWs, highlighting its resilience.

As for pH dynamics (Figure 1b), all media experienced an initial drop in pH following the introduction of 5% CO_2 (v v^{-1}), as expected from CO_2 dissolution in aqueous media. Over time, however, the pH gradually rose, driven by the biological uptake of Total Inorganic Carbon (TIC) through photosynthesis. Cultures in FW-A and PW-B showed moderate pH increases during cultivation, likely due to CO_2 uptake and nitrate assimilation, common in photoautotrophic growth. Since pH can strongly influence algal growth and metabolic efficiency, maintaining it within an optimal range is crucial.

The specific growth rates (μ) observed in this study ranged from 0.03 to 0.12 day^{-1} across all tested PWs and FWs (Table 2). These values were notably lower than the 0.21 day^{-1} previously reported for other PWs,[26] but consistent with the value of 0.03 day^{-1} observed in other FWs for the same strain.[17]

The maximum biomass concentration (X_{max}) observed ranged from 0.40 g L^{-1} to 1.78 g L^{-1} across PWs and FWs (Table 2). Overall, PWs consistently supported higher X_{max} values compared to FWs. Remarkably, cultures grown in PW-A and PW-C reached X_{max} nearly four times greater than those in FW-B.

Selecting the appropriate strain that can reliably yield biomass with a specific chemical composition is a critical factor for its commercial viability, particularly when it comes to energetic production. To achieve this goal, it is essential to characterize the biomass in terms of M, VM, FC, A contents, and HHV. These parameters for *Komvophoron* sp. grown both in PWs and FWs are provided in Table 3.

The VM content of the algal biomass generated in all the waters tested was high (ranging from 56% to 77%), except for the culture grown in PW-B, which showed an anomalously low VM (16%). When cultivated in PW-A and PW-C, the VM values were higher than the VM value of 49% obtained for the same strain in previous studies using different tested PWs.[26]

A general trend was also observed comparing the growth of *Komvophoron* sp. across all the PWs: the higher was the TDS content in the PW (as shown in Table 1), the lower the VM value (as indicated in Table 3). The FC content ranged from 7.6% to 40% for all the PWs and FWs tested. These values were someway not aligned with FC content of 16% exhibited by the same strain when cultivated in another PW.[26] It is worth noting that the FC values in PW-C and FW-A fell within the range reported for other cyanobacteria like *Spirulina platensis*.[27] High VM and FC contents are desirable attributes when it comes to producing pyrolysis oil from biomass. This is because high VM and FC content make the algal biomass easy to ignite and subsequently gasify or oxidize, which is crucial for energy production.

As for M content, the cyanobacterium exhibited values around 4% in all the PWs and FWs investigated except in PW-B (8%) (Table 3), still within acceptable ranges for thermochemical processing. Low M content is essential for the thermal conversion of biomass to alcohol, gas, or oil, with typical requirements being below 50%. Therefore, microalgae biomass with low M content is considered the most efficient source for thermal conversion to liquid fuels, such as biodiesel.

The A content of the algal biomass depended on the specific WWs where they were cultivated, as indicated in Table 3. The exceptionally high A content exhibited by *Komvophoron* sp. when grown in PW-B (35%), compared to the lowest one (9%) in FW-A, suggests possible inorganic salt accumulation associated with the elevated salinity if this WW. While this interpretation is plausible, it remains hypothetical, as no direct analytical evidence, e.g., Scanning Electron Microscopy – Energy-Dispersive X-ray spectroscopy (SEM-EDS) analysis of surface salt deposition, was obtained in this study. Additional work would be required to confirm whether external salt precipitation or intracellular mineralization mechanisms underlie this deviation.

It is important to recognize that the overall composition of algal biomass, including VM, FC, M, and A content, is highly species-dependent and influenced by the developmental stage of harvest. Notably, the HHV values of biomass produced in PW-based systems typically ranged between 17 and 21 MJ Kg^{-1}, aligning with the energy standards required for biofuel feedstocks.[28]

To evaluate the effect of *Komvophoron* sp. on WW quality, the chemical comparison of PW and FW were analyzed before and after algal treatment (Table 1). The efficiency of contaminant removal is illustrated in terms of anions and cations in Figures 2a and 2b for PWs and FWs, respectively. This investigation aimed to determine whether algal cultivation could not only purify

WW for potential use in irrigation or industrial processes but also generate valuable biomass for bio-based products like biofuels.

It is important to note that Figures 2a and 2b illustrate the qualitative trends rather than statistically supported differences in removal efficiency. Each wastewater (PW-A, PW-B, PW-C, FW-A, and FW-B) was analyzed as a single culture due to the limited sample volumes available, and no biological or analytical replicates were performed. As a result, standard deviations and confidence intervals could not be calculated, and error bars are not reported. The comparisons among waters should therefore be interpreted as indicative of possible patterns rather than as statistically significant variations. The terms used to describe what is depicted in these figures (such as "remarkable or significant reduction" or "complete removal") are merely descriptive, denoting observable tendencies under the experimental conditions rather than outcomes confirmed by statistical testing.

A key observation was the complete removal of nitrate across all WW samples following *Komvophoron* sp. growth likely via assimilation, although adsorption or volatilization contributions cannot be excluded under current data resolution. Ammonium levels also showed substantial reductions (generally >80% in both PWs and FWs), particularly in PW-B, which displayed the highest decrease, reaching approximately 96%. However, no such reduction was detected in PW-C. Phosphorous removal mirrored this trend, with PW-B again showing the most effective uptake (76%). A remarkable reduction in Chemical Oxygen Demand (COD) and five-day Biological Oxygen Demand (BOD5) was also reported when cultivating *Komvophoron* sp. in PW-B and FW-A.

Together, these data reinforce the conclusion that *Komvophoron* sp. is a promising strain for bioremediation of fracking WW. However, its performance is highly matrix-specific, influenced by both the chemical complexity of the water and the balance of nutrients available. This underscores the need for targeted pre-treatment or co-cultivation strategies (e.g., nutrient supplementation or sequential polishing steps) to enhance treatment efficiency in large-scale applications.

Discussion

All WW samples analyzed in this study were sourced from actively producing oil wells, as confirmed by the Frac Focus Chemical Disclosure Registry.[29] The hydraulic fracturing fluid used

in the well where PW-A was collected included proppants (notably silica and quartz, marketed as 40/70 White), a friction reducer (FRW-200), biocides (AQUACAR GA 50 and B-445-50), a breaker (ammonium persulfate, LTB-1), a polymer breaker (CS POLYBREAK 210W), along with other unspecified additives not listed in the Material Safety Data Sheet (MSDS). Similarly, PW-B and PW-C samples originated from wells employing 40/70 White silica and quartz as proppants, biocides (AQUCAR GA 50, B-445-50 and ICI-340 DP), the same friction reducer (FRW-200), breaker (LTB-1), polymer breaker (CS POLYBREAK 210W), and additional undisclosed compounds. The composition of FW-A and FW-B was comparable to that of PW-B and PW-C, except for their use of 100 Mesh Sand in addition to 40/70 White proppants.

Notably, as shown in Table 1, magnesium and potassium concentrations in PW-A and PW-C were significantly higher compared to the other samples. Interestingly, the Mg^{2+} levels in these samples were lower than those reported for other USA sites, while K^{+} levels were consistent with previous studies.[30] TDS levels in PW-A and PW-C were substantially higher, three to five times, than those in other WW samples (Table 1). These TDS concentrations align with the National Alliance for Advanced Biofuels and Bioproducts (NAABB) report for Southwestern USA wells, which documented TDS levels between 7.744 mg L^{-1} and 38.000 mg L^{-1}.[31] The TDS in FW-A and FW-B exceeded those found in fracturing water used for algae cultivation in previous studies[32] but remained lower than those measured in wells from the Denver–Julesburg Basin in Colorado[33] and in Texas.[34] Cyanobacteria typically thrive in neutral to alkaline pH environments, conditions mirrored in synthetic culture media (pH from 7.5 to 10). Previous research by Fecteau *et al*.[35] indicated that, while green algae prefer slightly acidic to neutral pH (5-7), they can still grow at pH levels as low as 4 or as high as 9. Therefore, the pH values observed in these samples fall within the optimal range for growth of both cyanobacteria and green algae.

Interestingly, *Komvophoron* sp. achieved its highest final biomass concentration (1.61 g L^{-1}) in PW-C, the most acidic medium tested, outperforming other WWs with pH values above 7.5. The pH in PW-C remained relatively stable despite a significant increase in OD, possibly indicating internal buffering effects or reduced CO_2 uptake due to stress from its extremely high salinity (TDS > 51 g L^{-1}). These findings support the hypothesis that *Komvophoron* sp. possesses metabolic flexibility to adapt to distinct chemical environments but may face physiological constraints in media with both high TDS and low bioavailable nutrient content. Notably, cultures grown in PW-A, PW-C, and FW-A displayed a fluctuating growth trend, likely tied to the light regime employed

during the experiments. Under a 12-hour light/12-hour dark photoperiod, biomass tends to accumulate during the illuminated phase due to active photosynthesis. In contrast, the dark phase sees a reduction in biomass as cellular respiration, cell death, and degradation processes dominate. This rhythmic alternation between light and darkness explains the observed oscillations in growth patterns during cultivation.

The reduced growth performance in terms of μ (0.03–0.12 d^{-1}) can be primarily attributed to extrinsic environmental limitations rather than intrinsic physiological constraints of *Komvophoron* sp. The low inoculum density (0.1 g L^{-1}) likely delayed the onset of exponential growth due to insufficient self-shading and low cell-to-cell metabolic coupling typical of filamentous cyanobacteria. Limited nutrient availability reported for PW and FW matrices, with N and P concentrations one to two orders of magnitude lower than in conventional synthetic media such as A^+, is not optimized to sustain microalgal growth, since it does not provide balanced and sufficient levels of essential nutrients. In addition, N/P ratios < 1 are far below the optimal 10–20 range that supports balanced anabolic activity. Under these conditions, nutrient limitation (particularly N) was the dominant factor constraining μ. High salinity (TDS > 30 g L^{-1} in some samples) imposed osmotic stress that further reduced photosynthetic efficiency and protein synthesis, compounding the effect of nutrient scarcity. Consequently, it is expected that strains grown in enriched media achieve higher specific growth rates compared to those cultivated in WWs where nutrients may be deficient.[36]

Conversely, intrinsic physiological limitations of the strain were minor. *Komvophoron* sp., which has been isolated from hypersaline soils such as those of the GPS, exhibits inherent halotolerance and robust photophysiology, suggesting that its potential μ under non-limiting conditions is considerably higher. The 12 h light/12 h dark photoperiod explains the observed biomass oscillations between illumination and darkness but cannot alone justify the low average μ values relative to those reported (up to 1.1 d^{-1}) for other strains in nutrient-replete media. Thus, the modest growth rates measured here mainly reflect extrinsic environmental stress with nutrient limitation and salinity, while the light–dark cycling only modulated temporal biomass fluctuations without fundamentally restricting the organism's intrinsic capacity for growth. Nutrient requirements differ between algal strains, and growth rate is often positively correlated with both absolute concentration of N and P and their N/P ratio in the medium. In the present study, A+ medium provided N and P as nitrate (NO_3^-) and phosphate (PO_4^{-3}), with an N/P ratio of 20. Notably, a

previous study reported a μ of 0.61 day^{-1} for the cyanobacterium *Pseudoanabaena* sp. cultivated in A+ medium.[37] Liu and Vyverman[38] found that *Pseudoanabaena* sp. achieved its highest growth rate (μ of 1.10 day^{-1}) when cultivated in a standard medium with NO_3^- and PO_4^{-3} at an N/P ratio of 12, outperforming other benthonic filamentous strains evaluated. Optimal N/P ratio of 7 was also identified for the filamentous green alga *Klebsormidium* sp., corresponding to a maximum μ value of 0.6 day^{-1}. Similarly, Erratt *et al*.[39] reported a μ of 1 day^{-1} for the cyanobacterium *Pseudoanabaena* sp. cultivated in Bold Basal Medium with an N/P ratio of 7. In contrast, the present study cultivated *Komvophoron* sp. in PWs and FWs characterized by N/P ratios < 1, significantly below the optimum values (10-20) (Table 1). These findings underscore the importance of strain-specific nutrient requirements, particularly regarding the N/P balance, in achieving optimal growth in various cultivation environments.

Komvophoron sp. topped the chart with an X_{max} of 1.78 g L^{-1} in PW-A, comparable to the biomass levels reported for *Picochlorum oklahomenis*, *Pseudoanabaena* sp. and *Geitlerinema carotinosum* under similar conditions in the same PW.[19] When cultivated in FW-A, *Komvophoron* sp. reached an X_{max} of 0.96 g L^{-1}, approximately double the value recorded in FW-B (0.40 g L^{-1}). Interestingly, even though FA-A and FW-B were collected from two distinct wells just a few miles apart within Kingfisher County, Oklahoma, their chemical profiles differed markedly especially in sodium, chloride, bicarbonate, and overall TDS. Additionally, since all water samples, both PWs and FWs, originated from oil producing wells, it's likely that the presence of organic compounds, including petroleum hydrocarbons, played a role in enhancing microalgal growth. This aligns with recent insights by Miazek *et al.*,[40] highlighting the supportive role of such compounds in microalgal cultivation.

Many *Komvophoron* species acclimate to changing light conditions by modulating their phycobiliprotein content. In high-light environments, they often downregulate phycobiliprotein synthesis to prevent photodamage, while under low-light conditions they boost phycobiliprotein levels to maximize light harvesting.[41] Algae cells can adhere to PBR walls and form biofilms, reducing light penetration to underestimation of biomass when samples are drawn only from the liquid phase. To avoid this bias, it is common practice to exclude wall-attached cells from concentration measurements. In our experiments, however, the filamentous strain did not develop any visible biofilm on the glass PBR wall. This phenomenon can be attributed to the constant gas bubbling, which kept the culture well-mixed and prevented cell settlement. It is worth to note that

adhesion propensity varies by species and surface materials, in this case, smooth glass likely further discouraged attachment.

Oklahoma State University, the University of Tulsa, and Wichita State University teamed up under the Salt Plains Microbial Observatory (SPMO) program to survey life in the GSP, an 840-acre hypersaline expanse in northern Oklahoma near Kansas.[42] There, soils and brine pools yielded 105 distinct aerobic bacterial isolates across 46 phylotypes, along with more than 200 halotolerant cyanobacteria, diatoms, and green algae. The strain used in our work came from GSP's surface soils and brine pools, an "extreme" habitat where salinity routinely exceeds 5% and daily temperature swings can reach 15 °C. With no plant cover to shield against intense UV radiation, conditions are harsh for most organisms.[43] Yet these very stresses select for microalgae with unusual resilience, traits that make them promising candidates for large-scale WW treatment and bioproduct cultivation. Because treating the massive volumes of FW and PW in closed PBRs can be prohibitively expensive, open evaporation ponds are often preferred. Employing locally sourced algae in these ponds minimizes the ecological and public-health risks tied to non-native or genetically modified strains, whose escape and potential invasiveness remain a concern despite limited experimental data. By focusing on native Oklahoma isolates, whose capabilities for WW remediation are still underexplored,[36] we both reduce introduction risks and tap into organisms already adapted to regional extremes.

Spherical green algae like *Picochlorum oklahomensis* tend to exhibit low A content when cultivated in both PWs and FWs environments, as reported by Lutzu *et al.*[19] In contrast, the filamentous cyanobacterium *Komvophoron* sp, which is non-motile and form extensive colonies or filaments, has a higher tendency to trap salts on its surface. This is particular relevant given that markedly TDS concentration were observed in PW-A (34.225 mg L^{-1}) and PW-C (51.334 mg L^{-1}), compared to the relatively lower TDS level in FW-A (11.240 mg L^{-1}). Because of this, it is likely that much of the A in *Komvophoron* sp. biomass resides externally, rather than being absorbed into the cells. This surface accumulation likely contributes to the elevated A content measured in PW-A and PW-C cultures, relative to FW-A. This trend aligns with previous findings that filamentous cyanobacteria often show higher A content than green algae when both are grown in standard media with lower TDS levels. For instance, Zhou and Dunford[37] analyzed the biomass composition of various green algae (*Dunaliella* sp., *Tetraselmis striata*) and cyanobacteria (*Phormidium keutzingianum*, *Pseudoanabaena* sp., *Tyconema bornetii*) cultured in A+, BG11 and

f/2 media, reporting A contents ranging from only 4.3% to 9.7%. Elevated A levels are generally undesirable for downstream processing, as they tend to increase operational costs, reduce combustion efficiency, and complicate disposal.

Globally, Figures 2a and 2b collectively underscore the species-dependent nature of contaminant uptake. The interaction between WW composition and algal biomass concentration plays a central role in treatment efficiency. Moreover, different metabolic pathways between green algae and cyanobacteria like *Komvophoron* sp. suggest that further studies are essential to unravel the precise biological mechanisms driving contaminant absorption. It is important to note that nutrient removal efficiency is closely linked to the N:P ratio in WW and varies between algal species. For example, decreasing the N:P ratio, while keeping nitrate levels constant, enhanced nitrogen removal for the filamentous green algae *Klebsormidium* sp. and the filamentous cyanobacterium *Pseudoanabaena* sp., yet reduced it for *Cladophora* sp.[38]

Broadly speaking, although several solutes show higher concentrations after algal growth (Table 1), this pattern is largely explained by concentration and speciation effects rather than net solute release from biomass. In particular, conservative tracers (e.g., chloride) indicate measurable concentration factors in some matrices (notably FW-B ≈×2.35, PW-C ≈×1.04), consistent with evaporation/aeration-driven volume loss during culturing. Concurrent pH elevation under photosynthesis shifts the carbonate system toward HCO_3^-/CO_3^{2-}, which appears as higher alkalinity and, where measurable, carbonate. Increases in Ca/Mg in select freshwaters likely reflect dissolution of carbonate scale or particle-bound phases under changing pH/ionic strength, while the Cu increase in PW-C is more consistent with material leaching/corrosion in a high-chloride matrix than with biological release. In contrast, reactive nutrients show the expected decreases (NO_3^- to <DL - under detection limit; NH_4^+ declines in most cases), and BOD/COD generally decrease, indicating assimilation and oxidation of organics.

Despite these promising nutrient reductions, TDS levels remained largely unchanged. In some cases, no significant reduction was observed. Elevated salinity, especially concentrations approaching 10,000 mg L^{-1}, can inhibit biological treatment processes due to osmotic stress, including plasmolysis and reduced metabolic activity. As highlighted by Guo *et al.*,[44] lowering salinity diminishes both the salt content and the electrical conductivity of WW, since salts facilitate ionic movement. High TDS levels also contributed to COD reduction. PW-C, for example, had the

highest salinity (51.3 g L^{-1}) and showed only a marginal decrease in COD (data not shown), underscoring the challenges posed by extreme salt concentrations.

Another critical parameter, Boron (B), is especially relevant for irrigation water due to its narrow optimal range for plant health. *Komvophoron* sp. showed minimal or negligible effectiveness in B removal across all samples, except in FW-A with only an 8% reduction (data not shown). Prior research by Saveedra *et al.*[45] found that green algae such as *Scenedesmus almeriensis* removed up to 38% of B among various microalgae tested. Their study also highlighted the pH-dependency and strain-specific nature of B uptake, and pointed to gasps in our understanding of B metabolism in both green algae and cyanobacteria.

Conclusions

The hydraulic fracturing industry generates substantial volumes of PW and FW as byproducts of oil and gas recovery. Effective treatment or disposal of these saline and chemically complex waters is critical, yet often costly. Biological treatment using microalgae presents a promising, cost-effective alternative, particularly when employing strains adapted to hypersaline environments. These resilient species are well-suited for growth in the challenging conditions typical of fracking WW.

In this context, the current study demonstrates that the cyanobacterial strain *Komvophoron* sp. is capable of growing in both PWs and FWs. However, while the organism can tolerate these harsh media, biomass production remains limited due to a scarcity of essential nutrients, a common characteristic of such WWs.

The composition of the resulting algal biomass was found to vary depending on both the species and the specific chemical profile of the growth medium. Importantly, cultivation of *Komvophoron* sp. led to the effective removal of several key contaminants including ammonia, nitrate, phosphate, and trace elements such as zinc, manganese, iron, and copper. Newly acquired data also demonstrate the species' capacity to partially reduce sulfate, calcium, magnesium, and COD, though pH shifts and conductivity changes remained limited. These insights further highlight the matrix-specific performance of *Komvophoron* sp. and suggest its suitability for site-tailored polishing strategies. This study positions PW not only as a waste stream to be managed but as a potential resource for sustainable algal biomass production. Utilizing native or locally adapted microalgae for the treatment of hydraulic fracturing WW offers a viable path toward lowering

treatment costs, generating valuable bio-based materials, and enhancing the environmental sustainability of unconventional fossil fuel extraction practices.

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Table 1. Chemical composition of Produced Waters (PWs) and Flowback Waters (FWs) before and after *Komvophoron* sp. growth

	PW-A before	**PW-A after**	**PW-B before**	**PW-B after**	**PW-C before**	**PW-C after**	**FW-A before**	**FW-A after**	**FW-B before**	**FW-B after**
Cations (mg L^{-1})										
Sodium	11.72	11.73	3.73	3.9	17.88	18.33	3.66	3.48	2.35	2.59
Calcium	1.01	1.02	41	43	2.334	2.317	69	99	50	27
Magnesium	140	150	11.5	12.3	328	359	13	14	5.6	4.8
Potassium	161	175	71	73	422	403	49	46	56	60
Anions (mg L^{-1})										
Nitrate-N	0.1	< DL	0.1	0.01	0.1	< DL	0.2	< DL	0.1	< DL
Chloride	19.9	19.35	3.94	4.13	29.4	30.7	4.35	4	758	1.78
Sulfate	683.5	740	906	961	559	583	328	308	1.06	1.17
Boron	53	56	55	59	56	56	56	51	67	72
Bicarbonate	587	623	1.35	1.48	402	514	1.19	1.34	1.5	1.71
Carbonate	na	na	na	83	na	na	24	18	70	112
pH	7.8	7.8	7.7	8.4	5.4	5.9	8.5	8.4	8.7	8.7

EC (µmhos cm^{-1})	50.8	51.5	15.62	15.68	76.4	86	17.03	15.76	9.83	9.28
Trace elements (mg L^{-1})										
Zinc	0.2	0.06	0.04	< DL	< DL	< DL	0.08	0.08	0.08	0.02
Copper	0.2	0.06	0.05	0.06	0.12	0.58	0.11	0.11	0.21	0.02
Manganese	0	0	0	< DL	1	0	< DL	< DL	0	0
Iron	1.89	0.14	1.63	0.21	10.12	0.91	1.12	1.08	5.2	2.1
Ammonium	15.2	1.81	3.4	0.13	58.2	55.3	0.7	0.1	1.3	0.13
ICAP_P	0.27	0.21	0.29	0.07	0.27	0.29	0.15	0.08	0.62	0.26
Derived values										
TDS (mg L^{-1})	34.22	34.11	10.3	10.74	51.33	56.76	11.24	10.4	6.48	7.47
SAR (%)	91	88	133	130	92	93	106	87	84	121
PAR (%)	0.7	0.8	1.5	1.4	1.3	1.2	0.8	0.7	1.2	1.6
Residual carbonates (meq L^{-1})	na	na	19	23	na	na	16	16	24	30
Sodium percentage (%)	89	88	98	98	84	84	97	96	97	98
Hardness (mg L^{-1})	3.116	3.208	149	159	7.176	7.258	226	305	148	87
Alkalinity (mg L^{-1} as $CaCO_3$)	481.5	510.5	1.11	1.32	329	421	1.01	1.13	1.35	1.59
COD (mg O_2 L^{-1})	2.64	1.85	1.08	931	2.02	2.03	2.77	1.56	1.96	1.92
BOD5 (mg O_2 L^{-1})	21	11	14	12.45	13.15	7.83	14.5	9.75	12.86	11.32

TDS, Total Dissolved Solids; PAR, Potassium Absorption Ratio;EC, Electrical Conductivity; ICAP_P, phosphorus measured by inductively coupled argon plasma emission spectrophotometer; SAR, Sodium Absorption Ratio; COD, Chemical Oxygen Demand; BOD5, five-day Biological Oxygen Demand; nd, not detected; na, not available, because carbonate was measured by titration method which is good for the pH higher than 8.3; < DL, under detection limit

Table 2. Effect of produced waters (PWs) and flowback waters (FWs) media on the growth characteristics of *Komvophoron* sp.

Medium	**µ (day^{-1})**	**t_d (day)**	**X_{max} (g L^{-1})**	**ΔX (mg L^{-1} day^{-1})**

PW-A	0.05 ± 0.01	14.7 ± 3.04	1.78 ± 0.67	67 ± 0.01
PW-B	0.12 ± 0.002	5.6 ± 0.09	0.95 ± 0.32	31 ± 0.005
PW-C	0.08 ± 0.03	8.8 ± 3.58	1.61 ± 0.22	87 ± 0.03
FW-A	0.03 ± 0.01	13.8 ± 1.17	0.96 ± 0.18	43 ± 0.02
FW-B	0.05 ± 0.02	10.6 ± 0.00	0.40 ± 0.07	19 ± 0.01

Note: μ, specific growth rate; $t_{d,}$ doubling time; $X_{max,}$ maximum biomass concentration, ΔX; average biomass productivity; PW-A, PW-B, and PW-C, produced waters; FW-A and FW-B, flowback waters.

Table 3. Chemical composition of biomass obtained when cultivating *Komvophoron* sp. in produced waters (PWs) and flowback waters (FWs) determined by thermogravimetric analysis (TGA).

Medium	**M**	**VM**	**FC**	**A**	**HHV**
PW-A	4.2	56.3	23.7	15.8	17.8
PW-B	8.7	16.3	40.0	35.0	18.0
PW-C	3.7	69.1	7.6	19.6	13.8
FW-A	3.4	77.0	10.5	9.1	16.2

Note: M, Moisture (%), VM, Volatile matter (%), FC, Fixed carbon (%), A, Ash (%) and HHV, Higher Heating Value (MJ kg^{-1}); PW-A, PW-B, and PW-C, Produced Waters; FW-A and FW-B, Flowback Waters.

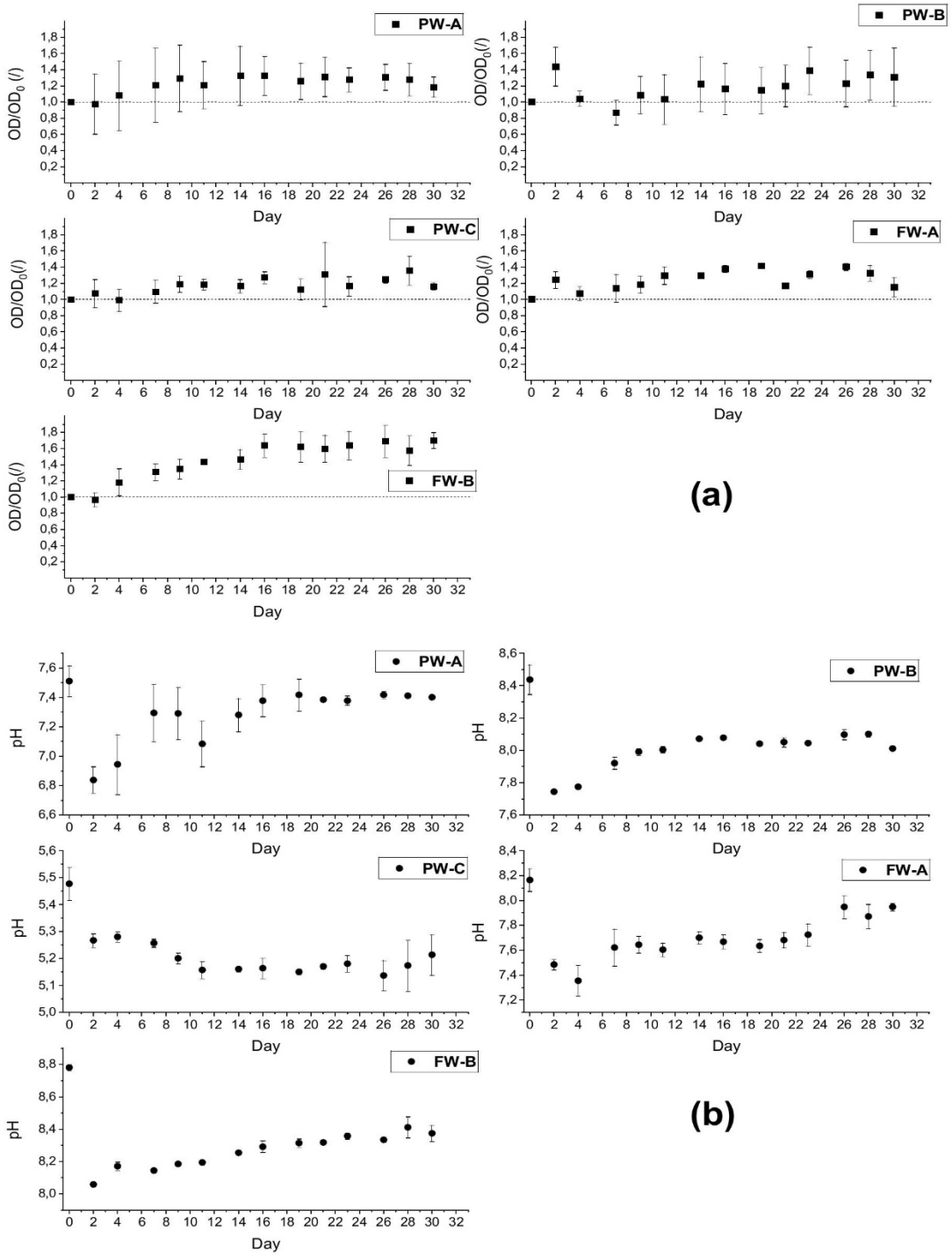

Figure 1. Time evolution of cultures' Optical Density (OD) (a) and pH (b) referred to their initial values, obtained by growing *Komvophoron* sp. in different Produced Water (PWs) and Flowback Water (FWs).

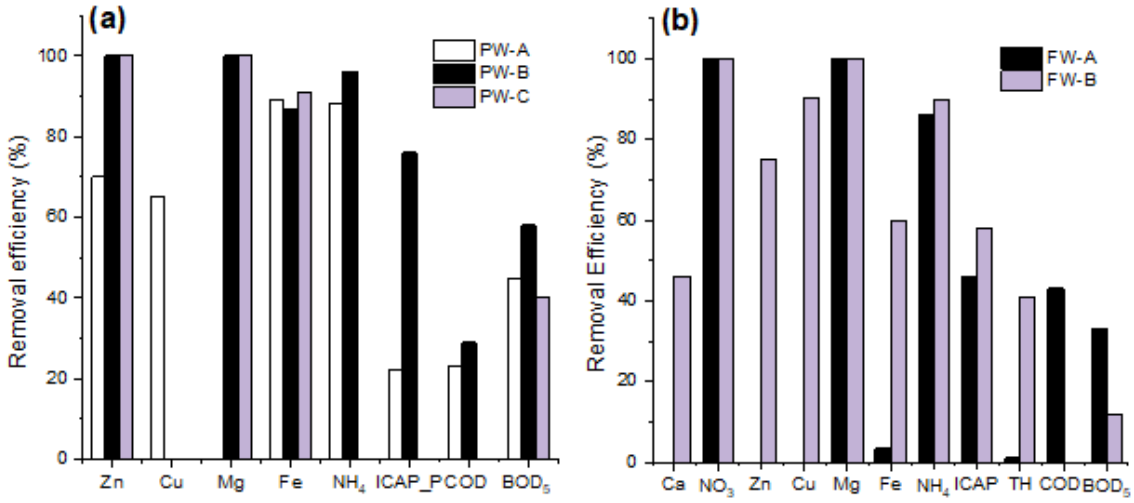

Figure 2. Removal efficiencies achieved by *Komvophoron* sp. with the three investigated produced waters PW-A, PW-B, and PW-C (a) and two investigated flowback waters FW-A and FW-B (b). ICAP_P, phosphorus measured by inductively coupled argon plasma emission spectrophotometer; COD, Chemical Oxygen Demand; BOD5, five-day Biological Oxygen Demand.