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Quorum sensing autoinducers enhance biofilm formation and power production in a hypersaline microbial fuel cell

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ABSTRACT

Intensive hydraulic fracturing of shale oil and gas reservoirs and other industrial activities can produce large volumes of high-organic-strength and high-salinity wastewaters that are difficult to treat by conventional technologies. Microbial fuel cells (MFC) can treat high-strength wastewater while generating electricity. We enhanced anodic biofilm formation by the extremophile *Halanaerobium praevalens*, a prevalent species in saline oil and gas reservoirs, in a hypersaline MFC (100 g/L NaCl), by promoting quorum sensing (QS) through addition of exogenous QS signals (i.e., *Pseudomonas aeruginosa* QS signal (PQS) and 4-hydroxy-1-methyl-2-quinolone (quinolone)). A 95% increase in biofilm mass was observed with the addition of 100 nM quinolone, yielding a sustained 30% increase in generated power density. Enhanced bacterial attachment to the anode's surface was corroborated by confocal microscopy. Overall, QS stimulation through the addition of trace levels of such autoinducers might be a feasible approach to enhance MFC performance under high-salinity conditions.

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1. Introduction

The production of highly saline and high-strength wastewaters by unconventional oil and gas production and other industrial processes poses a significant treatment and disposal challenge at many locations where deep well injection is not feasible [33]. In addition, produced and flow-back waters disposal legislation is becoming more stringent, underscoring the need for novel treatment approaches. Microbial fuel cells (MFC) offer the potential to treat high-strength produced and flow-back waters while generating electricity [21]. In MFCs, bacteria oxidize organic pollutants under anaerobic conditions using the anode as electron acceptor [25]. This allows the generation of electrical power that can be harvested [27]. Although some salinity can be beneficial, allowing better proton transfer and higher conductivity in the system [14], high salinity levels can exert osmotic stress and pose a major challenge to MFC or any treatment process with a significant microbial component [2]. Therefore, the success of MFCs for such applications hinges upon finding an effective and resilient microbial community that can degrade hydrocarbons and other organics under saline conditions.

Halanaerobium species belonging to the phylum *Firmicutes* are anaerobic and extreme halophilic bacteria able to ferment and degrade complex organic matter such as aromatic compounds [26]. Interest in this genus is growing in the oil and gas industry since such bacteria are commonly found in oil reservoirs and shale formations [20] as well as in produced waters from hydraulic fracturing [5,19]. Because *Halanaerobium* spp. adapt to high hydrocarbon concentrations under extremely saline conditions, such extremophile bacteria might be relevant for biological treatment of produced and flow-back waters.

We recently reported that *Halanaerobium* spp. (including *H. saccharolyticum*, *H. kushneri*, *H. praevalens* and *H. congolense*) became dominant in a MFC seeded with ocean sediments from the Gulf of Mexico and operating at 100 g/L NaCl [21]. The power production under such extreme saline conditions was steady, yet low at 71 mW/m^2. This suggests that MFCs could steadily generate electricity while treating saline wastewater from unconventional oil and gas production. *Halanaerobium* spp. were found to form microcolonies and small biofilm patches on the surface of the carbon brush anode, and full biofilm coverage was not achieved.

Biofilm coverage of the anode is critical for MFC power generation. Halophiles such as *Halanaerobium* spp. exhibit optimum growth at 70–130 g/L NaCl [13], and would not be stressed by hypersaline wastewaters. Hence, it is important to understand the biofilm formation and exoelectrogenic capabilities of these

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extreme halophiles to enhance the performance of hypersaline MFCs.

It is generally accepted that all bacterial genera employ cell to cell communication strategies to coordinate collective behavior [32]. In one approach, diffusible signals (autoinducers) are synthesized by bacteria and secreted out of the cell. The extracellular concentration of these signals increases with bacteria density, leading to their diffusion back into the cells. This phenomenon, known as quorum sensing (QS), serves to coordinate gene expression and regulate various traits, from virulence to biofilm formation [6,15,22].

There are very few microorganisms for which QS mechanisms (signal molecules and genes) are well understood (e.g., *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*). For *P. aeruginosa,* promotion of biofilm formation by QS has been demonstrated [8,32], and this process can be induced even by exogenous autoinducers [7]. QS autoinducers in Gram(−) bacteria generally belong to the family of *N*-acylhomoserine lactones (AHL) but also include butyrolactones, alkyl-quinolones and furanones [34]. In contrast, little is known about QS by *Halanaerobium* spp. and to our knowledge, no prior publications have studied QS or biofilm formation by *Halanaerobium* spp. Thus, it is unknown what are the *Halanaerobium* QS signals and whether QS could be stimulated by autoinducers from other bacteria. However, the genus *Halanaerobium* is known to produce quinolone type signals (LuxR family proteins) such as 4-hydroxy-methyl-2-quinolones coded by the *hmqF* gene [1]. Furthermore, *Halanaerobium* species are considered as Gram (−) bacteria, although strictly speaking they are low G + C Gram(+) bacteria that lack a cell wall. Genes involved in QS by Gram(−) bacteria generally belong to the *luxI* and *luxR* families, which code for quinolone-like transcriptional regulator and synthesis proteins respectively [10,30]. Thus, it is plausible that some quinolones might be involved in QS by *Halanaerobium* spp.

In this work, we assess the potential to enhance anodic bacterial attachment and MFC power generation under high salinity conditions by the addition of exogenous QS signals such as PQS and quinolone. *Halanaerobium praevalens* was chosen as a model extreme halophilic bacterium because its entire genome has been sequenced [13], which facilitates transcriptomic analysis.

2. Experimental methods

2.1. Biofilm growth

A *Halanaerobium* spp. enrichment from sea sediments, with 70% predominance of *H. praevalens,* was used in biofilm growth tests to screen potential QS signal molecules. Two different *N*-acylhomoserine lactones (AHLs) typically found in Gram(−) bacteria were considered: *N*-(3-oxo-dodecanoyl)-homoserine lactone (3-oxo-C12-HSL, Sigma–Aldrich, MO, USA), and *N*-(butanoyl)-homoserine lactone (C4-HSL, Cayman Chemicals, MI, USA). A signal belonging to the family of 2-alkyl-4-quinolones (AQs), the PQS, better known as the *Pseudomonas* quinolone signal, was also tested (2-heptyl-3-hydroxy-4-quinolone, Sigma–Aldrich, MO, USA).

Subsequent experiments were conducted with a pure culture of *H. praevalens* (strain 2228, purchased from DSMZ, Germany) to avoid potential confounding effects associated with microbial population shifts and discern the transcriptomic response to potential QS autoinducers. Based on preliminary results with the sea sediments enrichment, two AQs were used: the PQS and 4-hydroxy-1-methyl-2-quinolone (quinolone) (Sigma–Aldrich, MO, USA). Chemical structures of the quinolone type signals are illustrated in Fig. 1.

The effect of potential QS molecules (autoinducers) on biofilm formation was assessed by growing bacterial cultures under anaerobic conditions in 24-well plates. QS molecules were added separately, at 0.1 μM or 100 nM. The growth medium (pH 7.5) contained glucose (2.5 g/L), $MgSO_4$.(5 g/L), KCl (7 g/L), yeast extract (8.5 g/L), peptone (1.7 g/L), casamino acids (1.7 g/L) and 100 g/L NaCl. The 24-well plates (triplicates) were cultivated at 37 °C with shaking at 50 rpm for 5 days. Crystal violet stain was used to quantify the attached biomass in Costar® cell culture 24-well plates from Corning Inc. (Corning, NY). After discarding the medium, the biofilm was heat-fixed at 60 °C for 1 h. Then 250 μL of crystal violet (0.1%) was added in each well to stain the biofilm. After 15 min in dark crystal violet was removed and the plates were washed with 0.1 M phosphate buffer (PBS, pH 7.4) to remove the excess dye. The dye absorbed in the biofilm was then extracted with 70% ethanol and was quantified by measuring its absorbance at 590 nm in a microtiter plate reader (Spectramax 384, Molecular Devices, CA, USA).

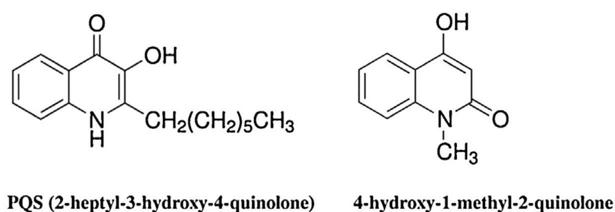

Fig. 1. Chemical structure of the quorum sensing signals used in this study.

2.2. Effect of QS signals on gene expression

H. praevalens was grown in Costar® cell culture 24-well plates (Corning, NY) to OD_{600} = 0.05 at 37 °C and 50–60 rpm shaking in the presence of exogenous QS signals (added at 100 nM). The plates were placed in a GasPak™ EZ Standard Incubation Container (BD, Franklin Lakes, NJ) to ensure anaerobic conditions. Controls, receiving no QS signals, were treated in the same way. After 45-h (exponential growth phase), the biofilm was rinsed with PBS to remove unbound cells and subsequently removed from the plate with a cell scraper. Biofilm RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, and concentrations were determined using a Nanodrop ND-1000 instrument (Nanodrop products Inc., Wilmington, NE). cDNA was synthesized overnight at 42 °C by reverse transcription polymerase chain reaction (PCR) using random primers RNase-OUT, dNTPs and Superscript II reverse transcriptase. Quantitative real-time polymerase chain reaction (q-rt-PCR) was performed in 15 μL of reaction mixture composed of 2 ng cDNA, SYBR Green Master Mix (7.5 μL), 0.3 μM of each primer and water, to quantify the expression of target chromosomal genes involved in QS (i.e., carbon storage regulator gene *csrA*), lipopolysaccharide (LPS) biosynthesis (i.e., lipopolysaccharide biosynthesis gene *Hp_2077* and polysaccharide biosynthesis gene *capD*) and multidrug and metal resistance (efflux-pump) gene *Hp_1819*. The sequence of quantitative PCR primers (Table S-1) and standard curves are included in the supplementary information section (Fig. S-1). These target genes were chosen according to the genome annotation of *H. praevalens* [13]. The $2^{-\Delta CT}$ method was used to quantify differential gene expression with DNA gyrase subunit A *gyrA* as the reference gene, and the results were analyzed with SDS 1.3.1 [17]. All treatments and q-rt-PCR analyses for each sample were run in triplicate.

2.3. MFC reactor

A single chamber reactor with an air cathode (250 mL volume) was used to study the effect of the addition of quinolone molecule

on the electrical output of hypersaline MFCs (a schematic of an air-cathode MFC is shown in Fig. S-2). Carbon brush (PANEX 35, Gordon Brush, Commerce, CA) and carbon cloth containing 0.5 mg/cm^2 Pt (Fuel Cell Store, College Station, TX) were used as anode and cathode, respectively, as previously described [21,35]. The experimental setup was described previously [21]. MFCs were seeded with a *H. praevalens* pure culture (OD_{600} = 0.05) and the hypersaline growth medium (100 g/L NaCl) was the same as used in the biofilm growth assay. To study the effect of QS signal, 100 nM of quinolone (4-hydroxy-1-methyl-2-quinolone) was included in the growth medium, which was the best performing concentration for both biofilm formation and transcriptional response.

2.4. Confocal scanning microscopy

Sample preparation for confocal imaging of the anode's carbon fibers was made as previously described [21]. Confocal imaging was performed using a Nikon A1-Rsi inverted confocal microscope with 60× water immersion objective (NA 1.27, Nikon Instruments, Melville, NY) in a unidirectional Single-track mode without averaging. A 405-nm Argon laser was used for DAPI imaging with a 425–475 nm emission filter. Laser intensities were set at 5% of the maximum output power (25.7 mW for 405 nm). The pinhole was set at 1 AU. Images were all captured at the same setting and analyzed using Nikon NIS Element (Nikon Instruments, Melville, NY).

3. Results and discussion

3.1. Biofilm induction by QS signals

AHLs (i.e., 3-oxo-C12-HSL and C4-HSL) had no discernible effect on biofilm formation by the *H. praevalens* enrichment (Fig. S-3). In contrast, considerable stimulation of biofilm formation was achieved by PQS (2-heptyl-3-hydroxy-4-quinolone) (Fig. S-4). Thus, subsequent experiments and transcriptomic analysis were conducted with a pure culture of *H. praevalens* exposed to quinolone-type signals (AQs).

The addition of both AQs at concentrations as low as 100 nM significantly promoted biofilm formation by *H. praevalens* on a polystyrene surface (Fig. 2 and Fig. S-5). A minimum threshold signal concentration has to be reached to achieve QS, although the concentration of exogenous QS signal needed to stimulate biofilm growth may be system-specific and vary by microorganism and signal type. The promotion of quorum sensing biological factors has been shown to occur over a broad range of exogenous autoinducer concentrations [24]. Common QS signal threshold values are in the nanomolar range [32], although higher threshold concentrations have been reported. A study conducted with *P. aeruginosa* reported that homoserine lactone (HSL) concentrations

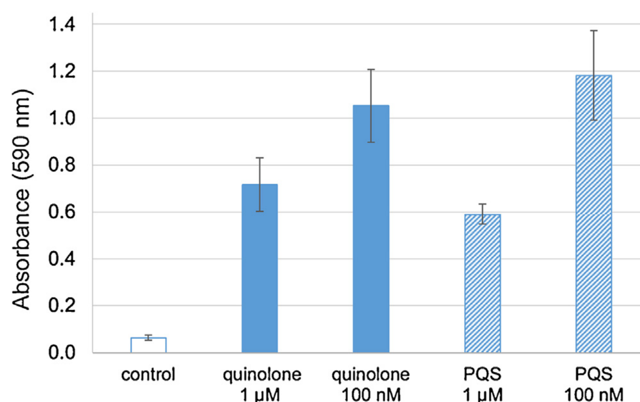

Fig. 2. Enhanced biofilm development by *H. praevalens* exposed to quinolone or PQS signals. Absorbance values from the crystal violet staining assay were used to assess biofilm formation.

reached up to 1.5 mM [4], and another study [12] reported higher concentrations of 3.36 mM in a 6-day-old biofilm. Overall, signal concentrations reported in the literature are around 500 nM. That is why concentrations of 1 μM and 100 nM were chosen for our study.

A similar behavior was reported for *P. aeruginosa*, where PQS enhanced biofilm development [6]. Our results further substantiate the positive effect of QS enhancement on biofilm formation by other bacteria, including *Pseudomonas putida* [32], *B. cenocepacia*, *Escherichia coli* [3,15] and *Halomonas pacifica* [16].

Results from Fig. 2 suggest that alkyl-quinolones may be involved in inter-species signaling to enhance biofilm formation by *H. praevalens*. For PQS, previous studies found that this signal can also enhance QS in other bacterial species (e.g., *Burkholdeira* spp.) when the latter possess homologous genes [28]. Such potential interspecies communication could be of great relevance to biofilm formation in a wide variety of systems. The fact that two different quinolone-based signals induced QS in the same bacterium is noteworthy. Some *Pseudomonas*, *Alteromonas*, and *Burkholderia* spp. produce multiple quinolone molecules [9]. In *P. aeruginosa*, in addition to the well-known PQS, the quinolone (4-hydroxy-2-quinolone) used in our study has been also detected [11], although its specific function in the cell remains unknown. Our results show that this quinolone signal has a stimulatory effect on biofilm formation by *H. praevalens* (Fig. 2).

To further understand biofilm promotion mechanisms, transcription of biofilm-related genes was assessed following exposure to QS signals at 100 nM. Note that these target genes are putative; whereas the databases relate some genes to biofilm formation and QS sensing [23], there are no published studies demonstrating

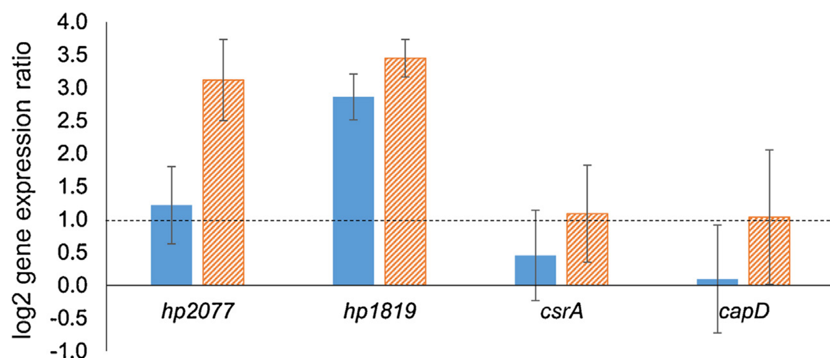

Fig. 3. *H. praevalens* gene expression results. *Hp_2077* and *cap D* represent biofilm formation genes, *csrA* is a carbon storage gene related to QS mechanisms and *Hp_1819* a multidrug resistance gene. Values greater than one indicate significant up-regulation (Michels et al., 2015).

their role. The influence of QS signals on the expression of a metal resistance (efflux pump) gene was also considered because heavy metals such as cadmium, silver and zinc are commonly found in oil and gas reservoirs [2]. Such metals could be carried over, and upregulation of metal resistance could mitigate their impact on MFC performance.

We found up-regulation of four target genes after the addition of 4-hydroxy-1-methyl-2-quinolone (quinolone), while only *Hp_2077* (1.5–3.5 fold increase) and *Hp_1819* (6–9 fold increase) were stimulated with PQS (Fig. 3). The up-regulation of *Hp_2077* and *capD* is consistent with the enhanced biofilm formation observed in crystal violet assay, as polysaccharides are the main components of the biofilm matrix that hold bacterial cells together, providing architecture, adhesion to the surface, and resistance [31].

Hp_1819, the metal resistance gene, was highly overexpressed in the presence of both signals, with up to 9-fold increase with PQS and 13-fold with quinolone. Hp_1819 codes for the multidrug efflux pump homologue of AcrB that belongs to Resistance-Nodulation-Division (RND) family. This efflux pump plays an important role in providing heavy metal resistance to gram-negative bacteria including cobalt, zinc and cadmium. The addition of QS signal increase the expression of genes coding for AcrB pump in a single step, which would make *H. praevalens* more resistant to residual heavy metals that may reach an MFC.

Gene expression results corroborate the data from biofilm growth plates demonstrating the induction of biofilm formation by QS signals. Overall, higher up-regulation was achieved with quinolone than PQS, so in the analysis of the MFC performance we only studied the influence of quinolone, which is also more economical and accessible.

3.2. Effect of QS signal on the pure-culture MFC performance

Two MFCs seeded with *H. praevalens* culture and the same hypersaline medium (100 g/L NaCl) were run simultaneously. One MFC was amended with 100 nM quinolone signal and the other one served as a control. Fig. 4 shows two consecutive cycles, with the arrow indicating replacement of the medium. For the second cycle no signal was added to evaluate the persistence of the QS signal effect. Voltage production (Fig. 4) was significantly stimulated by quinolone addition, reaching up to 0.3 V compared to the control (mean value ≈ 0.1 V) at R_{ext} = 510 Ω. The first cycle experienced fluctuations in voltage output that are common prior to cell stabilization in the system [18], but the produced voltage was clearly higher in the MFC amended with QS signal throughout the experiment. This improvement cannot be attributed to an increase in electron donor concentration in the medium, since the addition of 100 nM quinolone (only 30 μg/L COD) represents less than 0.0002% of the total COD added to the MFC.

After replacement of the medium (without signal), the same pattern was observed with more stabilized voltage production. A stable value of 0.185 ± 0.009 V (accounting for 3 subsequent cycles) was reached in the MFC previously amended with quinolone, compared to 0.101 ± 0.004 V (mean value for 3 cycles) in the unexposed control. Therefore the beneficial effect of the QS signal persisted, indicating that there is no need for continuous addition. Power density curves also show a significant benefit on the maximum power production capacity (Fig. 5)., with an increase of up to 75% right after the addition of quinolone and a long-term stable increase of 30% (based on an external resistance of 510 Ω).

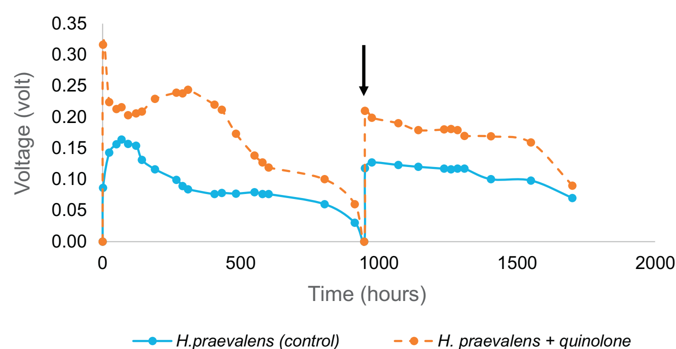

Fig. 4. Enhanced voltage production in MFC seeded with *H. praevalens* by addition of 100 nM quinolone. Arrow indicates replacement of the medium.

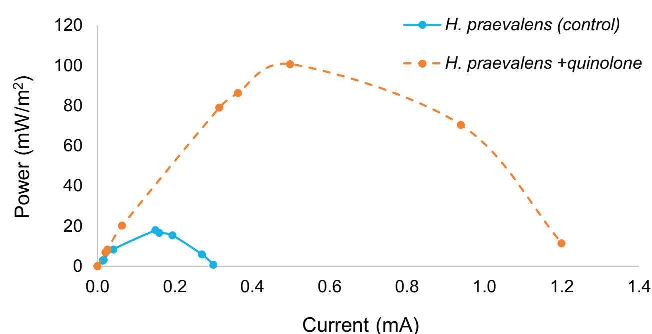

Fig. 5. Enhanced power density in MFC seeded with *H. praevalens* by the addition of 100 nM quinolone.

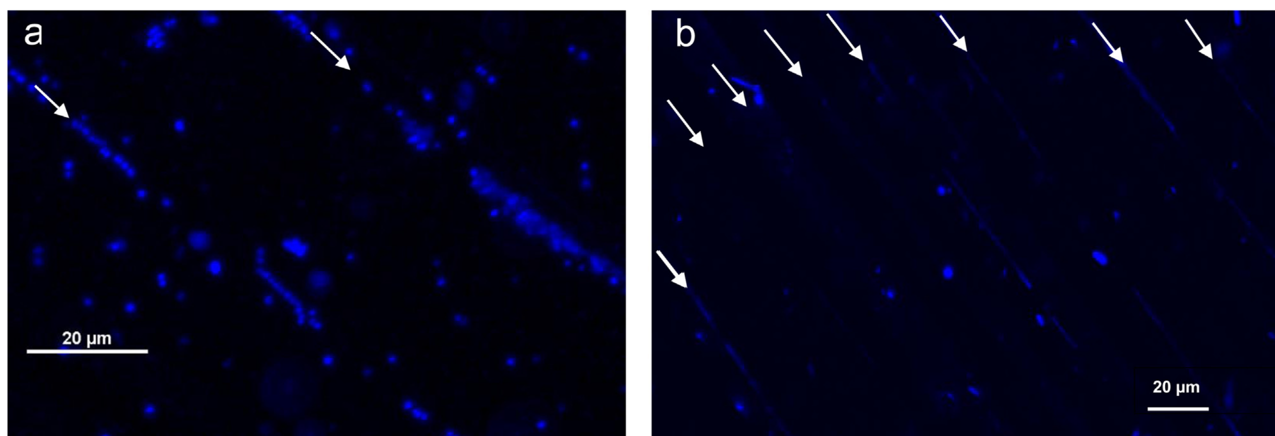

Fig. 6. Confocal scanning laser images of enhanced biofilm colonization of anodic strands in an MFC amended with 100 nM quinolone. Two strands highly covered by bacteria can be clearly identified (a) versus control, where the image shows multiple strands that are sparsely covered (b) (strands indicated by arrows).

Confocal microscopy images (Fig. 6) confirm that bacterial attachment to the anode was stimulated by exogenous quinolone molecules. In the absence of QS signal (Fig. 6b.) the anode carbon strands were sparsely covered with isolated microcolonies, whereas the quinolone substantially enhanced bacterial attachment (Fig. 6a). More detailed enhanced attachment to a single strand is shown in the Supplemental information section (Fig. S-6).

The positive effect on energy output can be largely attributed to the increase in the anodic biofilm formation, although we cannot rule out the possibility that the autoinducers also stimulated the exoelectrogenic capabilities of the bacteria as has been previously reported for *P. aeruginosa* [29]. *P. aeruginosa* generates extracellular virulence factors (i.e., phenanzines) that act as electron shuttles facilitating the transfer of the electrons to the MFC anode. Phenanzine production is upregulated by QS, and was reported to increase current generation by 28-fold in the MFC [29]. In another study, [36] assessed the effect of the overexpression of *rhl* (QS related gene) in *P. aeruginosa* on MFC performance. The overexpression of this gene led to the production of various electron shuttles with lower redox potential, resulting in a 1.6-fold increase in current. Assessing the potential for such exoelectrogenic enhancement mechanism represents a much greater challenge due to the limited information about QS by *H. praevalens*, as well as the prevailing extreme salinity that interferes with the analysis of trace chemicals and electron shuttles in the medium.

4. Conclusions

We demonstrated that MFCs could be used to treat hypersaline high-strength wastewaters (e.g., produced and flow-back waters) while producing electricity, which would alleviate a major economic and environmental challenge for the oil and gas industry. Power production can be significantly enhanced by adding trace levels of exogenous QS signals such as 4-hydroxy-1-methyl-2-quinolone and PQS. This enhances quorum sensing, which promotes anodic biofilm formation. Transcriptomic analysis corroborated enhanced biofilm formation through increased expression of genes associated with polysaccharides biosynthesis. Overall, these results suggest a promising approach to expand the limits of MFC applications and enable biological treatment of hyper saline wastewaters.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bej.2016.01.023.

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