Investigation of the response mechanisms of halophilic and mixed culture aerobic granular sludge under hypersaline conditions

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Abstract

The high salinity of brine wastewater generated by industrial processes could limit options for biological treatment and reuse of these wastewaters. In the present study, several bioresponse mechanisms to cope with high osmotic pressure created in hypersaline wastewater were investigated. Production of extracellular polymeric substances (EPS), microbial community shifting, and intracellular accumulated cations were compared between aerobic granular reactors seeded with activated-sludge and an enriched halophilic culture to investigate different adaption strategies. The selected cultures exhibited different responses toward the gradually increased salt content (1 – 85 g. L^{-1} as NaCl). EPS production was consistently higher in enriched halophilic culture under hypersaline conditions ($\geq$ 40 g. L^{-1} NaCl), which corresponded with better granule size and integrity in this same region. Microorganisms in the activated sludge culture relied on higher concentrations of intracellular cations to cope with high osmotic extracellular pressure. Genomic analysis showed that salinity had a significant influence on microbial community composition, resulting in both communities becoming dominated by halophilic organisms, with the richness of the two cultures converging at hypersaline conditions. Despite these broad similarities in the microbial communities, the use of halophilic inoculum resulted in measurable differences in microbial response and better maintained granule structure under hypersaline conditions.

Introduction

Saline wastewaters are byproducts of many industrial processes, including oil and gas production, sea food canning, tanning, and vegetable pickling (Ou et al., 2018; Sharghi et al., 2014; Wang et al., 2017a). The use of aerobic granular sludge (AGS) for biological treatment of these wastewaters has potential advantages, including the capability to withstand shocks due to changes in wastewater composition while maintaining high constituent removal efficiency (Ramos et al., 2015; Wang et al., 2017a). Previous studies have examined the use of AGS to treat saline wastewater, but have usually experienced difficulty maintaining granule structure above 30-50 g. L^{-1} NaCl (Corsino et al., 2017; Li et al., 2017; Ramos et al., 2015; Wang et al., 2017b). Expanding this range would allow for broader treatment of concentrated brine wastewaters, but may require seeding the system with organisms adapted to these high-salt conditions (halophiles).

Microbial communities respond to harsh environments by developing external and internal salt tolerance mechanisms (Wang et al., 2017a). Production of extracellular polymeric substances (EPS) is one response mechanism used by organisms to regulate high external osmotic pressure (Deng et al., 2016; Wang et al., 2017a). According to divalent cation bridging theory (DCB), the presence of divalent cations induces bioflocculation through bridging of EPS to negatively charged sites on bio cells surface or to by binding EPS with each other (Fig. 1). However, monovalent cations have a detrimental impact on AGS aggregation through replacement of divalent cations in the EPS matrix (Kara et al., 2008; Li et al., 2017), which may make this approach sensitive to high NaCl concentrations. A second strategy used by microorganisms to adapt to high salinity is the salt-in strategy (Fig. 2), in which microorganisms

regulate higher external osmotic pressure by accumulation of intracellular cations, such as Ca^{+2}, Mg^{+2}, Na^{+}, and K^{+} (Hänelt and Müller, 2013; Plemenitaš et al., 2014; Wan et al., 2014). Alternatively, microorganisms may accumulate or produce compatible solutes, primarily amino acids, as an alternative to cation accumulation (Hänelt and Müller, 2013).

The objective of this study is to reveal adaption strategies used by two initially distinct microbial cultures to cope with a hypersaline environment. Correlation of microbial adaption strategies with the investigated response mechanisms, such as EPS production and granule size, could lead to a better understanding of AGS formation under hypersaline conditions and improve AGS activity in this harsh environment. The microbial community of both systems was also monitored to assess changes that may occur as a response to the change in surrounding environment salinity (Ou et al., 2018; Wan et al., 2014).

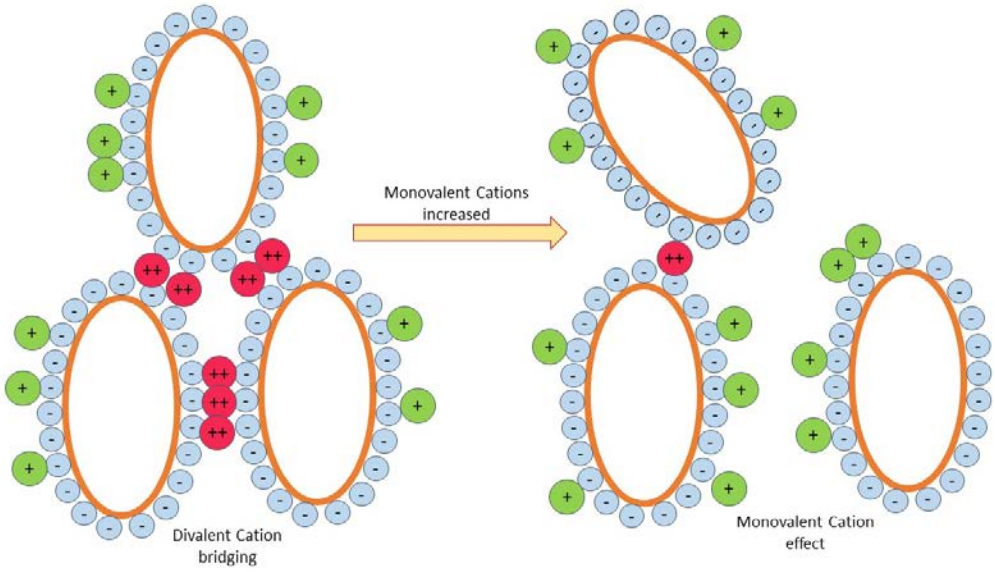

Figure 1: Conceptual model of divalent cation bridging theory.

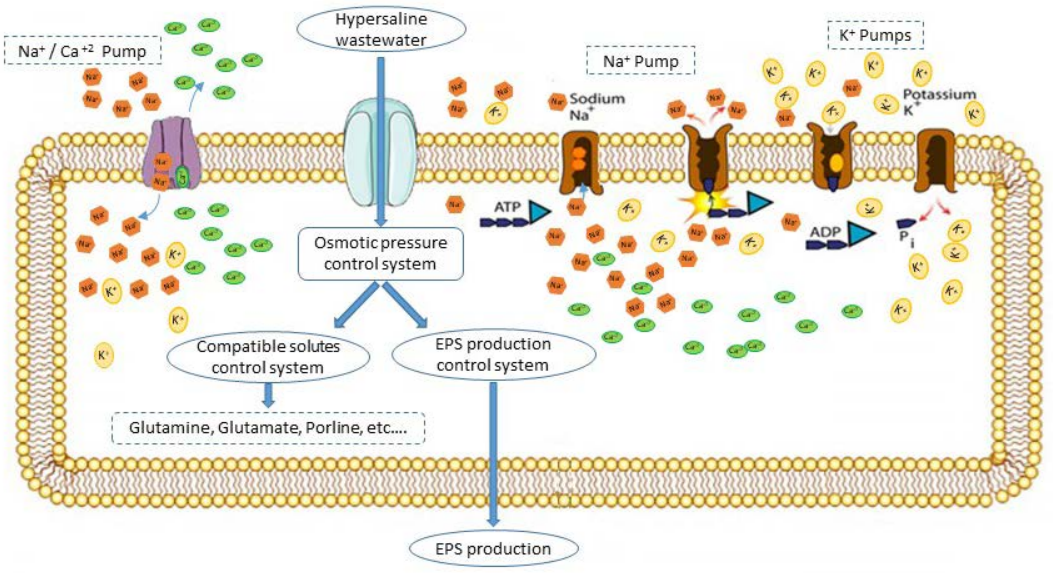

Figure 2: Model of the key osmoadaption strategy.

Materials and Methods

Two identical bench scale sequencing batch reactor reactors (SBR), operated with a working volume of 3.0 L, were utilized in the current study. The first SBR was inoculated with an enriched halophilic culture, and the second with a mixed culture of activated sludge from a local wastewater treatment plant. Both reactors were fed a synthetic brine solution with a composition modeled after that reported in previous literature (Pendashteh et al., 2012; Sharghi et al., 2014). The C/N/P ratio was adjusted to approximately 100/10/1 by adding NH_4Cl and KH_2PO_4. Sodium acetate was the only carbon source, and NaCl concentrations ranged from 0 to 85 $g.L^{-1}$.

Samples and analytical methods

EPS extraction was carried out using the heat method described by Li and Yang (2007). A temperature controlled microwave aided digestion procedure based on Özsoy (2006) was used to extract intracellular cations. Image analysis using Fiji imaging processing software was

implemented to characterize granule properties (diameter and aspect ratio) and to observe the changes in AGS morphology.

Duplicate mixed liquor samples for DNA extraction were collected at inoculation and at every stepwise increase in saline concentration from 40 g/ L^{-1} on in both reactors. DNA extraction was performed using the Qiagen DNeasy PowerSoil Kit (Germantown, MD), according to the manufacturer's protocol.

16S rRNA gene amplification and Illumina targeted gene sequencing

PCR was performed to amplify the V4 hypervariable region of the 16S rRNA gene using primers 515F and 806R (Caporaso et al., 2011). PCR products were purified using AmpureXP beads and dual indexed libraries were prepared using the Illumina Nextera® XT v2 indices (Illumina, San Diego, CA following Illumina's 16S Metagenomic Sequencing Library Preparation protocol. The prepared libraries were pooled and sequenced as a paired-end 300cycle sequence run on an Illumina MiSeq sequencer (Illumina, San Diego, CA at the Integrated Genomic Facility at Kansas State University, Manhattan, KS.

Sequence data analysis

Sequence processing

Illumina sequence data was analysed by the Center for Microbial Metagenomic Community Analysis at the University of Kansas, Lawrence, KS. The raw reads were processed using the Quantitative Insights into Microbial Ecology2 (QIIME2 v2017.12) (Caporaso et al., 2010). Sequence data was demultiplexed into individual samples using the QIIME2 demux plugin (https://github.com/qiime2/q2-demux). The reads were denoised, dereplicated into operational taxonomic units (OTUs) and filtered for chimeras using the QIIME2 dada2 plugin

(Callahan et al., 2016). Sequences representative of the OTUs were aligned using multiple sequence alignment (MAFFT) software version 7 (Katoh and Standley, 2013). Taxonomic identity was assigned by RDP classifier (Wang et al., 2007) using SILVA release 128 (Quast et al., 2013; Yilmaz et al., 2013), and a phylogenetic tree was generated using FastTree2 (Price et al., 2010). Biases against sequencing depth and testing were accounted for by normalizing the feature data using the cumulative-sum scaling method (CSS) implemented in the R package metagenomeSeq (Paulson et al., 2013).

Statistical Analysis

Statistical analysis was performed using Minitab 17 (Minitab 17 Statistical Software, 2010) and R version 3.4 (R Core Team, http://www.r-project.org/). The effect of high salinity on AGS formation in both reactors was assessed using one-way ANOVA analysis. The differences between group means were considered statistically significant when $p < 0.05$ (95% confidence interval). Relative abundances at different taxonomic levels were calculated for each sample using the R package phyloseq (McMurdie and Holmes, 2013). Faith's Phylogenetic Diversity (Faith, 1992) and Shannon Index for richness and evenness, respectively, were calculated using R packages phyloseq (McMurdie and Holmes, 2013) and Picante (Kembel et al., 2010).

Results and discussion

Salt content and AGS appearance

Both reactors were fed a brine solution with 7.5 g. L^{-1} total dissolved solids (TDS) until granules appeared, which occurred after 37 days of cultivation. Starting from this point, salt (as NaCl) was added to the influent wastewater at a concentration of 10 g. L^{-1}. Salt concentration was increased gradually over time to a maximum of 85 g. L^{-1}. Image analysis and showed that

AGS shape and appearance varied as salinity increased (Fig. 3). Granule size in the halophilic reactor was much lower at low salt concentrations (Fig. 3 A and D), which may be because halophilic microorganisms require higher levels of salt to thrive (Ma et al., 2010). At a salt content of 40 g. L^{-1}, granules in the halophilic reactor had a light yellow appearance and an irregular shape with a rougher surface (Fig. 3 B) while mixed culture granules had a solid and smooth surface, a dark yellow appearance, and a regular shape (Fig. 3 E). At the highest salt concentration (85 g. L^{-1}), halophilic granule size decreased slightly and their shape became more regular, with a solid appearance (Fig. 3 D). In contrast, the mixed culture granules had small decreases in granule size but significant decreases in density (Fig. 3 F).

EPS production as a response mechanism to cope with high salinity

EPS production was higher in the mixed culture reactor while the NaCL concentration remained below 40 g. L^{-1} (Fig. 4). As the solution became hypersaline ($\geq$ 40 g. L^{-1} NaCl), EPS production decreased notably in the mixed culture reactor. The halophilic culture reactor showed less change, and EPS production in this system was consistently higher from 40 - 85 g. L^{-1}.

EPS production may have initially increased in the mixed culture reactor as a response to salt stress. Corsino et al. (2017) observed a similar effect, with a reduction in granule stability above 50 g. L^{-}, slightly higher than the 40 g. L^{-1} threshold observed here. EPS contributes to cell-cell and/or cell- aggregate linking and plays a key role in bioflocculation (Deng et al., 2016). However, EPS aggregation is affected by the presence of accumulated cations. The replacement of divalent cations in the EPS matrix process could alter the balance between production and detachment (Pronk et al., 2014) affecting both EPS levels and granule structure. The harsh

surrounding environment could also affect metabolic activity of microorganisms and result in the use of produced EPS as a local energy source (Corsino et al., 2017; Deng et al., 2016). Regardless of specific mechanism, the strong correlation between granule size and EPS production (p-value < 0.05) makes explicit the crucial role of EPS in maintaining AGS structure under high salinity conditions. These results are in agreement with other studies findings, which reported the role of EPS in bioaggregation (Corsino et al., 2017; McSwain et al., 2005; Ramos et al., 2015; Wan et al., 2014; Wang et al., 2017a).

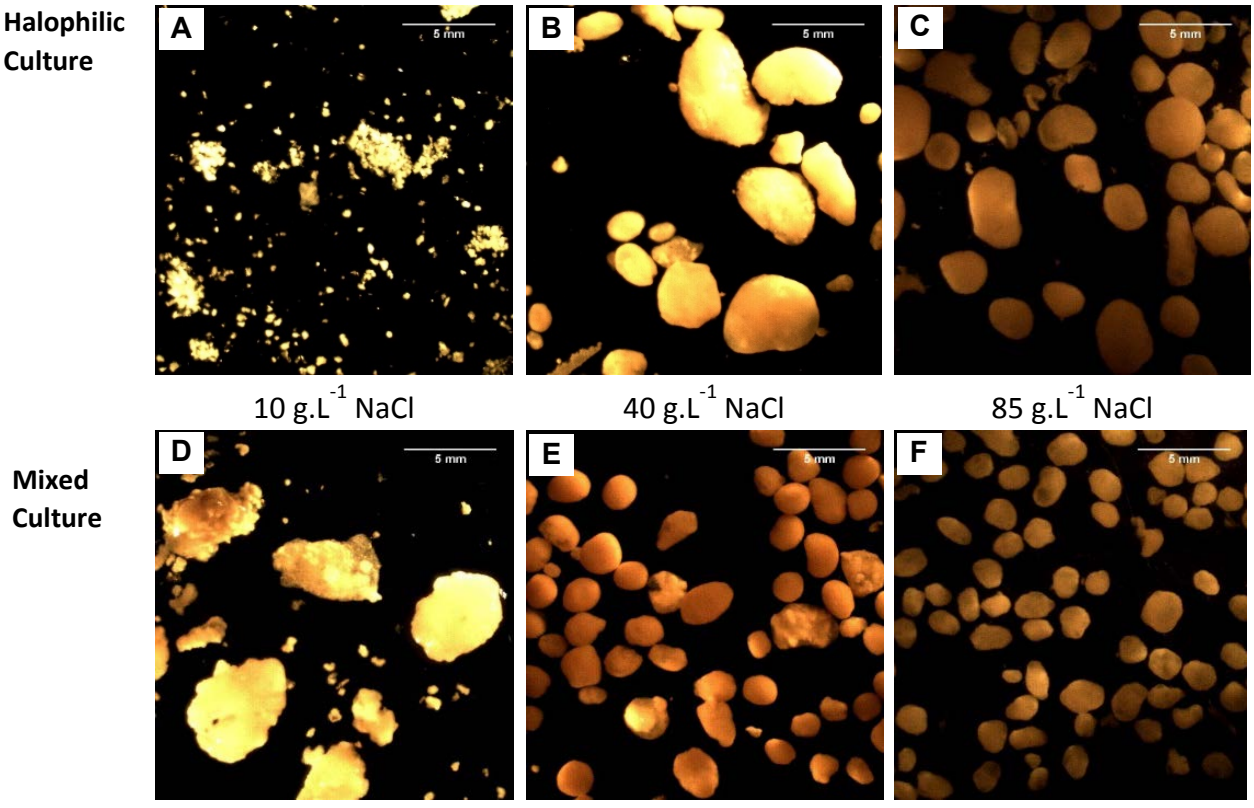

Figure 3: AGS images: (A & D) 10 g. L^{-1} NaCl, (B & E) 40 g. L^{-1} NaCl, (C & F) 85 g. L^{-1} NaCl.

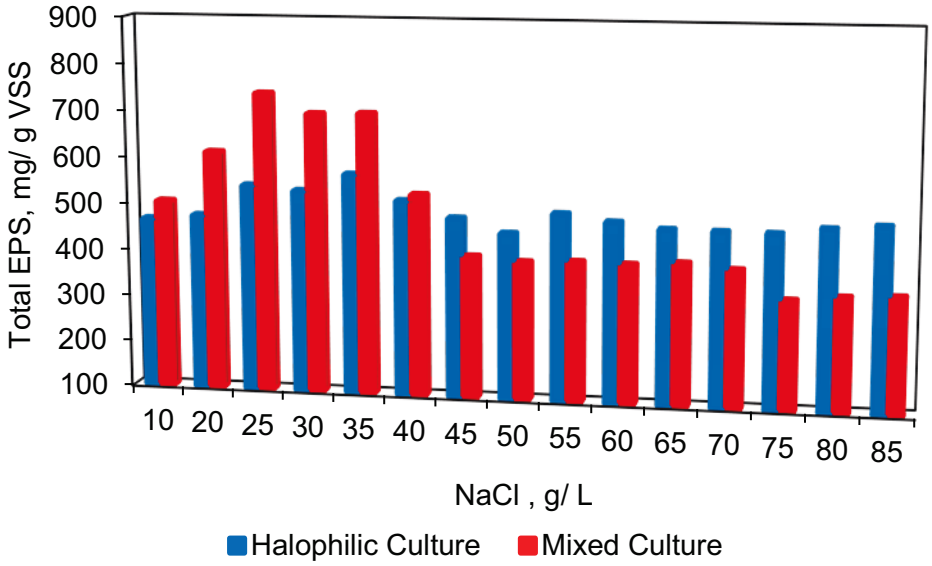

Figure 4: EPS production in halophilic and mixed culture.

Intracellular accumulated cations

Some microorganisms can utilize accumulate intracellular cations to cope with high osmotic extracellular pressure (Hänelt and Müller, 2013). Intracellular Ca^{+2} and Na^{+} were measured in both reactors to examine whether this mechanism played a role under hypersaline conditions. The mixed culture reactor had higher intracellular concentrations of both cations (Figures 5 and 6). Notably, intracellular Ca^{2+} concentrations were approximately half those of Na^{+}, despite being present in the synthetic wastewater solution at more than 1000 times lower concentration. (Ca^{2+} concentration was ~ 16 mg. L^{-1} in the feed solution.) Ca^{2+} also decreased as NaCl content increased. Previous research has noted varying intracellular Ca^{2+} and Na^{+} concentrations in aerobic granules with increased salinity, and attributed this effect to sodium-cation exchange through an electrogenic ion pump(Wan et al., 2014).

By contrast, the halophilic reactor had much lower intracellular concentrations of both ions. Intracellular concentrations of both Na^+ and Ca^{2+}were significantly different (*p* value < 0.05) between reactors. Additionally, concentrations of both ions in the halophilic reactor changed very little with increasing salinity. These differences indicate a notably different response between the two communities with respect to accumulation of intracellular cations.

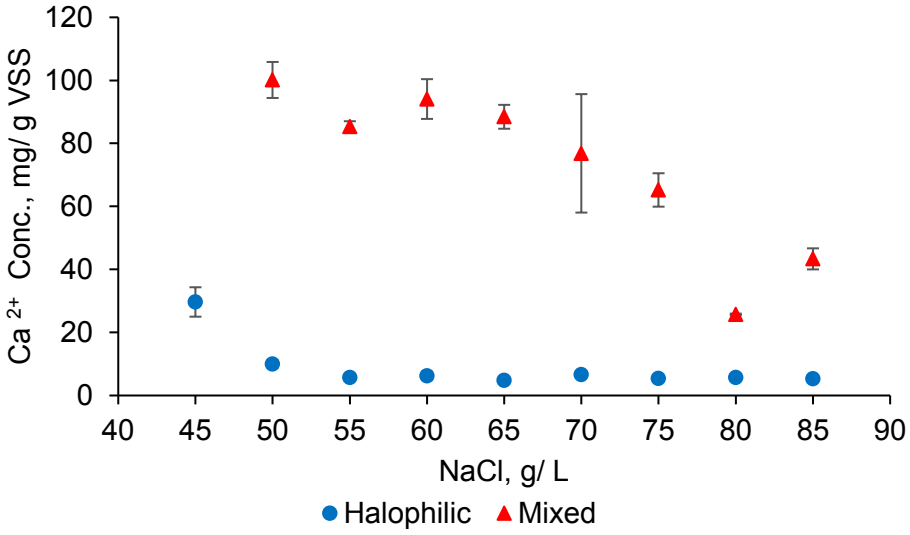

Figure 5: Intracellular calcium content.

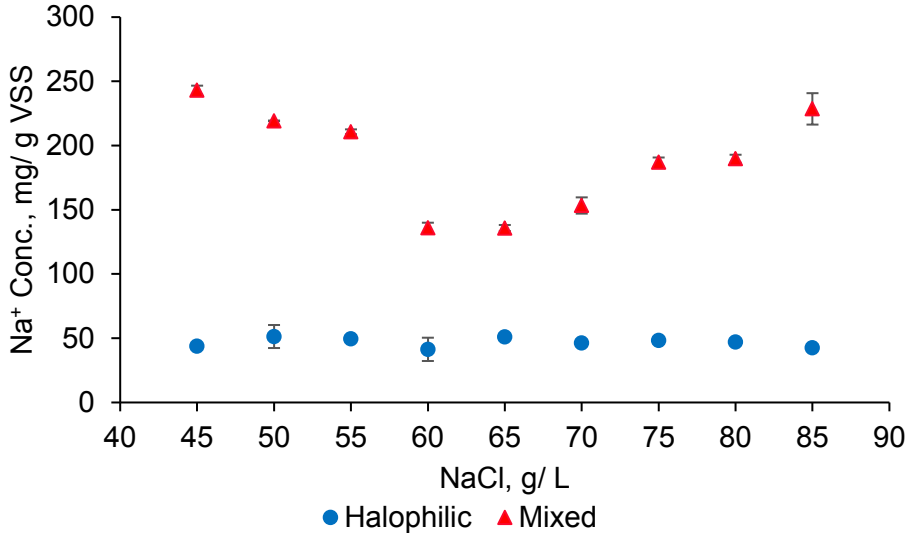

Figure 6: Intracellular sodium content.

Microbial community composition

At the genus level (Fig. 7), the top five most abundant genera in the halophilic reactor were *Halomonas* (46.21%), *Corynebacterium* (7.8%), *Nitratireductor* (3.96%), *Marinobacter* (4.43%), and *Marinicella* (3.41%), while the five most abundant genera in the mixed reactor were *Halomonas* (51.85%), *Marinobacter* (15.29%), *Marinicella* (4.34%), *Corynebacterium* (4.1%), and *Polymorphum* (3.63%). Furthermore, sequencing analysis showed that the hypersaline environment selected for halophilic bacteria, resulting in a shift in the microbial community composition from the initial inoculum for both reactors. Faith's phylogenetic diversity analysis revealed that the mixed culture inoculum diversity was significantly higher than the enriched halophilic culture inoculum (Figure 8, t-test, $p<0.05$). The richness of the two cultures converged as the salinity increased through 85 g. L^{-1}. While the Faith's diversity index in the mixed culture reactor at hypersaline conditions was much lower than in the activated sludge inoculum, the index in the enriched halophilic reactor increased from 2.6 ± 1.7 to 7.8 ± 0.2 at 40 g L^{-1} NaCl (Figure 8A). The Shannon's evenness diversity metric showed a similar trend to Faith's phylogenetic diversity, with similar species evenness in the two reactors at NaCl concentrations of 40 g L^{-1} and above.

Despite the high similarity between the two cultures, several of the identified differences in the microbial community may be relevant to granule formation. *Corynebacterium*, a genus which has been reported to be a major biofilm producers (Souza et al., 2015), is present at higher abundance in the halophilic reactor. *Bdellovibrio* genus, by contrast, is a more significant component of the mixed culture reactor. These organisms have been shown to slow biofilm formation by inhibiting biopolymer production and/ or utilizing

produced proteins as an energy source (Monnappa et al., 2014). These examples illustrate that, despite the overall similarity and presence of halophilic organisms, smaller differences in community makeup could influence granule formation and reactor performance.

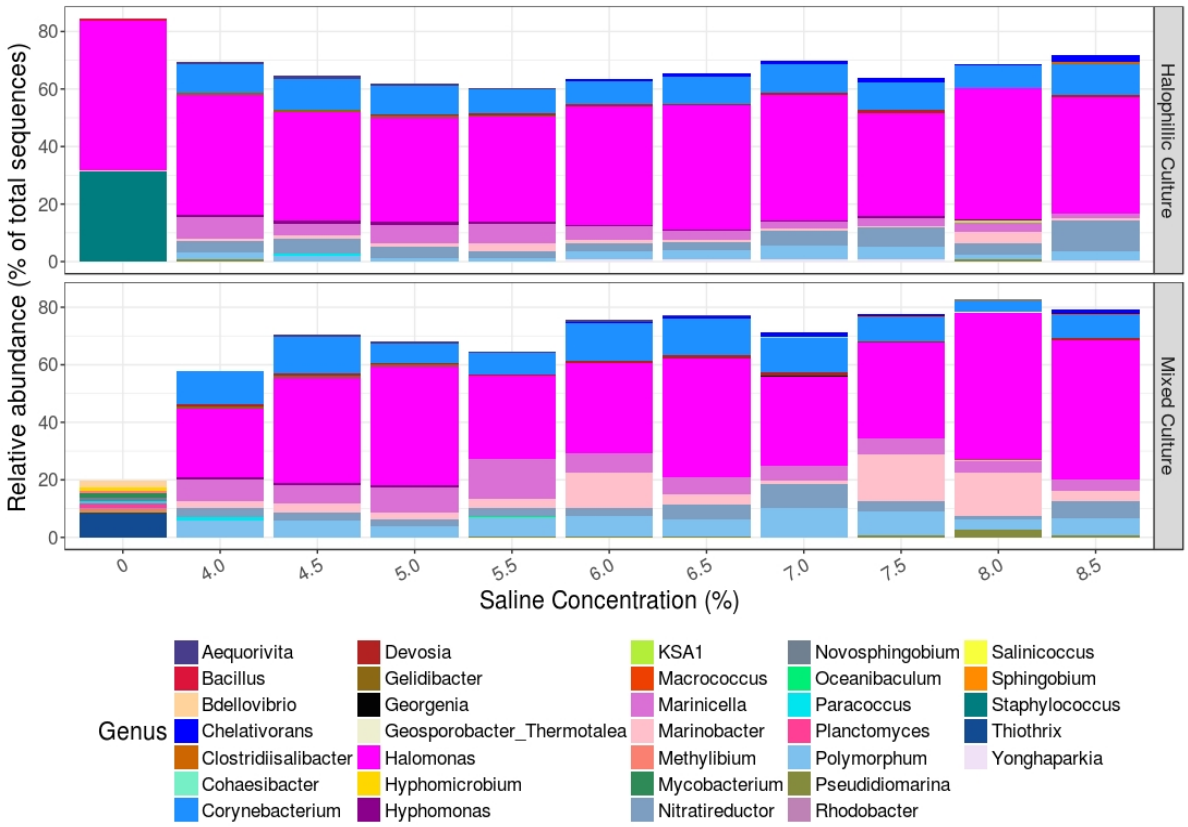

Figure 7: Relative abundance of the Top 10 genera belonging to halophilic and mixed cultures.

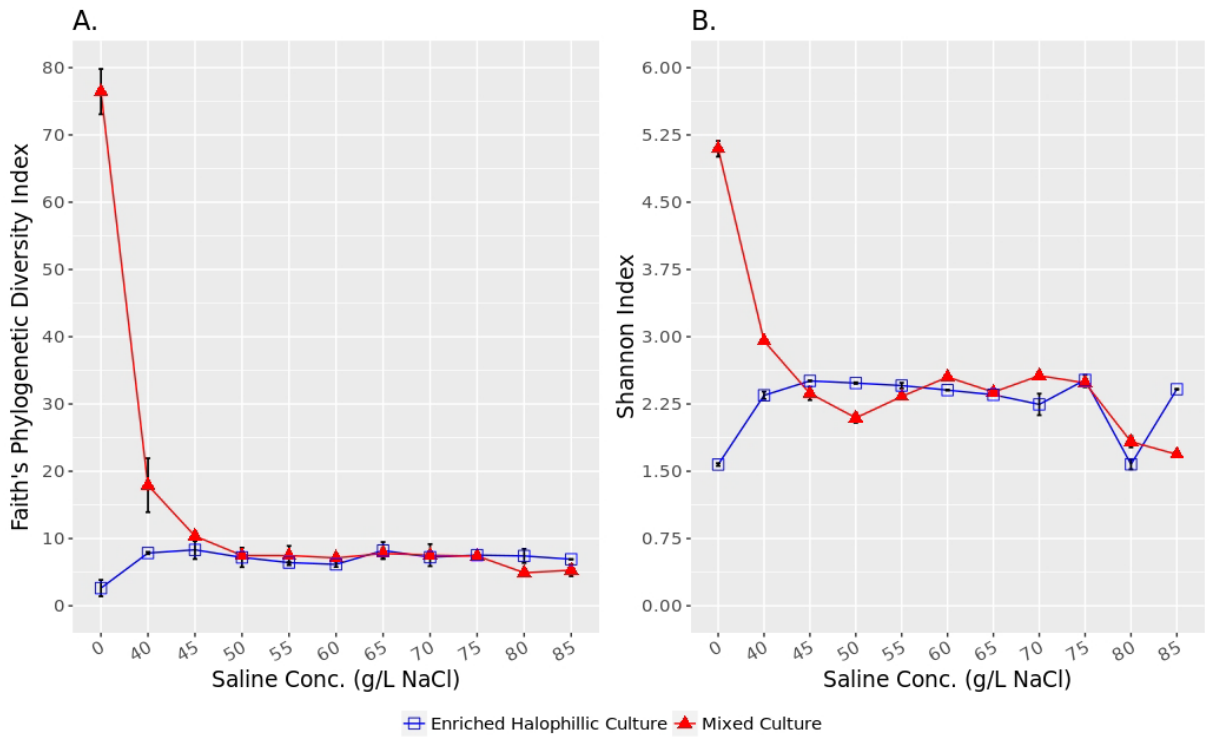

Figure 8: (A) Faith's Phylogenetic Diversity indicating richness and (B) Shannon index indicating evenness of microbial communities in the halophile-inoculated reactor (blue) and the activated sludge inoculated reactor (red) at different salt concentrations.

Conclusions

The use of a halophilic culture to inoculate an aerobic granule reactor resulted in increased granule size and stability under hypersaline conditions compared to inoculation with activated sludge. Increased EPS production at NaCl concentrations of 40 – 85 g. L^{-1} may be responsible for this behavior. Additionally, significant differences were observed in intracellular concentrations of both Na^{+} and Ca^{2+} between the two systems. At a community level, both reactors converged towards systems dominated by halophilic organisms, with similar values for species diversity. However, the differences in granule development and in community response to high salt stress indicate that the two systems were not the same. The use of a halophilic

inoculum appears to provide better granules under hypersaline conditions, and should be investigated further.

Acknowledgements

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