Biofouling Formation and Bacterial Community Structure in Hybrid Moving Bed Biofilm Reactor-Membrane Bioreactors: Influence of Salinity Concentration

Alejandro Rodriguez-Sanchez [1,2] , **Juan Carlos Leyva-Diaz** [1,3] , **Barbara Muñoz-Palazon** [1,2] , **Maria Angustias Rivadeneyra** [1,4] , **Miguel Hurtado-Martinez** [1] , **Daniel Martin-Ramos** [5] , **Alejandro Gonzalez-Martinez** [1,4,*] , **Jose Manuel Poyatos** [1,2] and **Jesus Gonzalez-Lopez** [1,4]

[1] Institute of Water Research, C/Ramon y Cajal, 4, University of Granada, 18071 Granada, Spain; arod@ugr.es (A.R.-S.); jcleyva@uniovi.es (J.C.L.-D.); bmp@ugr.es (B.M.-P.); michelhm@correo.ugr.es (M.A.R.); mrivaden@ugr.es (M.H.-M.); jpoyatos@ugr.es (J.M.P.); jgl@ugr.es (J.G.-L.)

[2] Department of Civil Engineering, Campus of Fuentenueva, s/n, University of Granada, 18017 Granada, Spain

[3] Department of Chemical and Environmental Engineering, University of Oviedo, 33006 Oviedo, Spain

[4] Faculty of Pharmacy, Campus of Cartuja, s/n, University of Granada, 18017 Granada, Spain

[5] Department of Mineralogy and Petrology, Campus of Fuentenueva, s/n, University of Granada, 18017 Granada, Spain; jdmartin@ugr.es

[*] Correspondence: agon@ugr.es

Abstract: Two pilot-scale hybrid moving bed biofilm reactor-membrane bioreactors were operated in parallel for the treatment of salinity-amended urban wastewater under 6 hours of hydraulic retention time and 2500 mg L^{-1} total solids concentration. Two salinity conditions were tested: the constant salinity of 6.5 mS cm^{-1} electric conductivity (3.6 g L^{-1} NaCl) and the tidal-like variable salinity with maximum 6.5 mS cm^{-1} electric conductivity. An investigation was developed on the biofouling produced on the ultrafiltration membrane surface evaluating its bacterial community structure and its potential function in the fouling processes. The results showed that biofouling was clearly affected by salinity scenarios in terms of α-diversity and β-diversity and bacterial community structure, which confirms lower bacterial diversity under variable salinity conditions with *Rhodanobacter* and *Dyella* as dominant phylotypes. Microorganisms identified as bio-mineral formers belonged to genera *Bacillus*, *Citrobacter*, and *Brevibacterium*. These findings will be of help for the prevention and control of biofouling in saline wastewater treatment systems.

Keywords: biofouling; bio-precipitation; calcite; MBBR-MBR; salinity wastewater; struvite

1. Introduction

Wastewater discharges to the environment are one of the main environmental problems in our world today. Several technologies have been developed for the treatment of wastewater such as the activated sludge process, which is the most commonly applied technology for urban wastewater treatment worldwide [1]. Nevertheless, more novel technologies than the activated sludge process such as the moving bed biofilm reactor-membrane bioreactor (MBBR-MBR) have been used for the treatment of urban and industrial wastewater [2–4].

Wastewater treatment bioreactors could be subjected to constant or variable salinity concentrations due to human activities such as the use of seawater as toilet flushing and the use of salt for snow-melting

operations to industrial activities such as the seafood processing industry and due to natural events such as intrusion of seawater in coastal wastewater treatment plants [5]. Salinity has been reported to affect microbial activity due to their impact in the bacterial cell mortality increase, in the increase in transmembrane osmotic pressure, and in the inhibition of different metabolic pathways [6]. The effect of salinity over the membrane bioreactor (MBR) and MBBR-MBR systems has been evaluated and is still being investigated in order to fully understand its role in the functioning of these systems and to develop tools to mitigate its impact and increase the performance of salinity wastewater treatment systems [7].

The MBBR-MBR system is a combination of the moving bed biofilm reactor (MBBR), which was followed by a membrane bioreactor (MBR) process [8,9]. Compared to the activated sludge process, the MBBR-MBR offers the development of fixed biofilm over the media placed within the bioreactor, which increases the sludge retention time of the biomass, promotes the development of more specialized microbial communities, and increases the total solids' concentration in the system without increasing the mixed liquor suspended solids. This leads to more economic treatment of wastewater [10,11]. Additionally, the placement of a downstream MBR after the MBBR process offers a very high-quality effluence due to membrane filtration, which also removes the majority of pathogens. Nevertheless, the main problem of MBBR-MBR systems is the membrane fouling, which is a process of clogging the membrane pore that leads to lower treatment efficiencies, lower wastewater flow treated per time unit, an increase of transmembrane pressure, and a destruction of the membrane.

The precipitation of minerals due to the activity of bacteria is known as biomineralization. It has been defined as a naturally occurring process comprising the formation of minerals with the mediation of metabolic activity of organisms [12,13]. Many different minerals can be formed by microorganisms in wastewater treatment systems, but close attention has been paid to calcium carbonate precipitates such as calcite, phosphate precipitates, and struvite [8,14–16]. Minerals precipitated can cause biofouling and permanent damage to membranes in wastewater treatment processes. Precipitated minerals have been identified along with biofilm as the most important components causing membrane fouling [17].

It has been observed that bacterial strains isolated from moderate and high salinity environments mediate the formation of a variety of carbonate and phosphate minerals [18]. Moreover, it has been suggested that bio-mineral formation under such environments could be of crucial relevance for the survival of the bacteria with capacity to mediate mineral formation [19]. In this sense, biofouling during the operation of membrane technologies for the treatment of saline wastewater could be endangered by the potential formation of minerals derived from saline-adapted microbial communities thriving within the system. To date, no research has been spent to shed light over this phenomenon.

The potential risk of biofouling in MBBR-MBR systems due to precipitation of biominerals including calcium carbonate and magnesium phosphate has been reported [8]. This potential risk could also be present in MBBR-MBR systems treating saline wastewater. For this reason, the aim of this research was to evaluate the effect of the influent salinity conditions over the potential risk of biofouling of an MBBR-MBR system due to bio-mineral formation. To unravel this, this technology was operated for treating saline wastewater under constant and variable saline concentrations in the influent, analyzing the bacterial community structure of the fixed biofilm (biofouling) by molecular techniques (Illumina MiSeq), and cultivating the fixed biofilm in mineral-precipitating media in order to evaluate the main bacterial strains involved in the formation of calcium and phosphate biominerals. In light of this, the potential risk of membrane biofouling of an MBBR-MBR system treating saline effluents could be established in a function of its bacterial microbiome.

2. Materials and Methods

2.1. Bioreactor Configuration and Operational Conditions

The bioreactor used in the study was configured as a four-chamber system followed by a membrane tank (Figure S1) with the potential capacity to properly remove C and N. The operational volume of each chamber was of 6 L and of 4.32 L for the membrane tank. The first, third, and fourth chambers were aerated and filled with K1 carriers in the 35% of their volume. K1 carriers (Anox-Kaldnes AS, Lund, Sweden) had a density of 0.92–0.96 g cm^{-3}, a specific surface area of 690 m^2 m^{-3}, and had a hollow cylindrical shape of 7 mm × Ø10 mm with a cross inside the cylinder with fins on the outside of the cylinder. The second chamber was not aerated (anoxic) and not filled with carriers and mixed completely by using a mechanical stirrer. The influent was introduced through the first chamber and forced to pass all the others before entering the membrane tank from where the effluent was withdrawn using an ultrafiltration membrane separation process. Additionally, a recycling flow was imposed from the membrane tank to the first chamber. This configuration has been called a hybrid MBBR-MBRn/anox [6,7,20].

The membrane tank contained an ultrafiltration membrane module continuously submerged and displaced vertically. The membrane module was made of hollow fibers of polyvinylidene fluoride with a core reinforcement of polyester that had 2.45 mm of external diameter and 1.10 mm of internal diameter with a 0.04 μm pore size. The total surface of the membrane was 0.2 m^2. The membrane was operated in a cycle of 9 min drawing and 1 min backwash in order to avoid membrane clogging. The flow rate was forced to be constant during the operation time by increasing the transmembrane pressure in the event of continuous fouling of the membrane. When the membrane pressure exceeded the value of 0.5 bar, the membrane was cleaned manually. In this sense, the flow rate was 4.72 L h^{-1} and the flux rate of the membrane was 23.6 L h^{-1} m^{-2}.

The bioreactor was operated for the treatment of salinity-amended urban wastewater with two different salinity inputs: i) constant salinity of 6.5 mS cm^{-1} electric conductivity (3.6 g L^{-1} NaCl), ii) tidal variation salinity with an upper limit of 6.5 mS cm^{-1} electric conductivity (3.6 g L^{-1} NaCl), lower limit of regular urban wastewater salinity (around 1 mS cm^{-1} electric conductivity, 0.5 g L^{-1} NaCl), and in cycles of 6 h of upper limit/6 h lower limit. The achievement of the salinity amended wastewater was accomplished differently for the two salinity conditions.

For the constant salinity conditions, NaCl was manually diluted in batches of real urban wastewater collected at the Los Vados wastewater treatment plant (Granada, Spain) until the electric conductivity of the batch reached 3.6 g L^{-1} NaCl.

For the variable salinity conditions, it was obtained by the mixing of real urban wastewater (collected at the Los Vados full-scale wastewater treatment plant, located near Granada, Spain) with salinity amended tap water (50 mS cm^{-1} in order to avoid excessive dilution of real urban wastewater) in a mixing tank. The mixing was controlled electronically by a conductivity module TOPAX LF1 (Lutz-Jesco GmbH, Wedemark, Germany) connected to a conductivity sensor that measured the conductivity of the mixing tank wastewater and its temperature continuously, which also activated two Watson Marlow peristaltic pumps that provided real urban wastewater or salinity-amended tap water to the mixing tank depending on the desired salinity with respect to the cycle time.

A representation of the salinity conditions is given in Figure S1. The systems were operated in similar conditions except for the influent composition. The hydraulic retention time was 6 h and the total solids concentrations in the bioreactors were controlled to provide a total concentration of around 2500 mg L^{-1}.

Furthermore, different physico-chemical parameters were measured in the influent wastewater and the effluent of the MBBR-MBR system such as the five-day biochemical oxygen demand (BOD$_5$) and chemical oxygen demand (COD), according to the standard protocols and inorganic nitrogenous ions ammonium, nitrite, and nitrate through ionic chromatography analysis [6,7].

2.2. Biomass Collection

Samples of the mixed liquor from the membrane tank and membrane-attached biomass (biofouling) were extracted from the systems operating once every two weeks during steady-state operational conditions (one-month period). The sampling protocols followed those described in Gonzalez-Martinez et al. [8]. Thus, 100 mL of mixed liquor were collected for this purpose. Additionally, a given part of the membrane was sonicated in order to detach its fixed biomass and then detached biomass was placed in 0.9% NaCl saline solution. Collected biomass samples of mixed liquor from the membrane tank and membrane-attached biomass were used for the isolation of cultivable bacterial cells and DNA extraction.

2.3. Culture, Isolation, and Identification of the Bacterial Strains with Biomineralization Capacity

Biomass from biofouling samples was used for the isolation of bacterial strains capable of calcium carbonate and phosphate crystals precipitation, according to the protocols given by Gonzalez-Martinez et al. [8]. Thus, two different media were used for crystal formation of calcium carbonate (MC) and struvite precipitation (ME). Both media consisted on 18 g L^{-1} agar, 10 g L^{-1} yeast extract, 5 g L^{-1} protease peptone, and 1 g L^{-1} glucose. The MC medium was added of 4 g L^{-1} calcium acetate while ME medium was added with 8 g L^{-1} magnesium acetate. The components of the media were autoclaved at 112 °C for 20 min by avoiding the autoclaving of calcium acetate with the rest of the MC medium, which added it to the other components after the autoclave process. The pH of the media was 7.2 and was adjusted using 0.1 M KOH solution.

Biofouling samples (1 mL) were serially diluted and spread on struvite and calcium carbonate culture media. For each dilution and each media, five replicates were aerobically incubated at 25 °C for 12 days and checked every 24 h for the presence of minerals in the media. Colonies associated with minerals formation were then isolated in their respective medium of growth for their taxonomic identification. Bacterial strains isolated from the biofouling samples with a struvite or calcium carbonate bio-precipitation capacity were surface-inoculated onto MC and ME solid media, aerobically incubated at 25 °C, and examined every 24 h (during 12 days) with an optical microscope for the presence of biomineralization processes. The experiments were carried out in triplicate and were repeated twice. Moreover, culture media inoculated with autoclaved cells and un-inoculated culture media were included in all experiments as a control.

2.4. Environmental DNA Extraction and Massive Parallel Sequencing Procedure

Samples were collected from the systems and processed for extraction of environmental DNA. Samples were centrifuged at 3500 rpm during 10 min at room temperature and the pelleted biomass was kept at −20 °C for further DNA extraction using a FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) and the FastPrep 24 (MP Biomedicals, Inc., Leicestershire, UK) apparatus by following the instructions given by the manufacturers of the DNA extraction kit. The environmental DNA was kept at −20 °C while being sent to the Research & Testing Laboratory (Lubbock, TX, USA) for the massive parallel sequencing procedure.

The environmental DNA samples underwent a massive parallel sequencing for the identification of their bacterial community structure using the Illumina MiSeq equipment and the Illumina MiSeq Reagents Kit v3 (Illumina, Inc., San Diego, CA, USA). The sequencing procedure targeted the V1-V2-V3 hypervariable regions of the domain *Bacteria* using the primer pair 28F (5′-GAGTTTGATCNTGGCTCAG-3′)-519R (5′-GTNTTACNGCGGCKGCTG-3′) [6,7,20]. The PCR conditions used for the Illumina MiSeq massive parallel sequencing were preheating at 94 °C during 120 s. Then the regions underwent 32 cycles of 94 °C for 30 s, 40 °C for 40 s, and 72 °C for 60 s as well as a final elongation at 72 °C for 300 s.

The raw data from the massive parallel sequencing has been uploaded to the SRA of the NCBI under the accession SRP145468.

2.5. Bioinformatics Pipeline

The raw data obtained from the massive parallel sequencing process was further treated to yield the bacterial community ecology of the biomass sampled using mothur [21] and UCHIME [22] software. First, paired-end reads were merged into contigs by avoiding the generation of ambiguous bases in the overlap region [23]. Then the sequences underwent a quality-trimming process consisting of the following steps: (i) removal of sequences with more than eight ambiguous bases or more than eight homopolymers, (ii) removal of sequences that failed to start at the position of the primer 28F after alignment with the SiLVA SEED release 128 database, or those that terminated further than the 95% of total aligned sequences. After this, sequences were pre-clustered in a two-difference threshold [24] and checked for chimeras using UCHIME and removing them. The non-chimeric sequences were then taxonomically affiliated using the SiLVA SEED release 128 database and those that were found to not belong to the domain *Bacteria* were eliminated. The remaining sequences were then used for the determination of the bacterial community structure of the biomass sampled. This was done by the construction of a Phylip distance matrix for the sequences in each sample, which was then used for the clustering of the sequences in each sample to operational taxonomic units (OTUs) in a 97% similarity threshold. A representative sequence was then selected for each of the OTUs and these representative sequences were classified using the SiLVA SEED release 128 database. The classified OTUs were then merged into a consensus taxonomy using an 80% consensus taxonomy threshold.

2.6. Ecological Analyses of the Massive Parallel Sequencing Samples: Analysis of Bacterial Ecological Coverage, Analyses of α-Diversity and β-Diversity of Bacterial Community Structure, Similarity Analysis of the Mixed Liquor and Biofouling Bacterial Community Structure, and SIMPER Analyses Comparing Mixed Liquor and Biofouling Communities

The ecological coverage of the domain *Bacteria* in the massive parallel sequencing was evaluated using a redundancy abundance-weighted coverage approach following Rodriguez-R & Konstantinidis [25]. For this purpose, NonPareil software was used for calculating the redundancy abundance-weighted coverage using query sets of 1000 sequences and considering the redundancy for sequences with at least 50% overlap and at least 95% similarity since these are the default parameters offered by the developers of the software [26].

The α-diversity analysis of the bacterial community structure of the massive parallel sequencing samples was conducted with PAST v3.0 [27], which was used for the calculating the species richness, Chao-1, Shannon-Wiener, Simpson, Pielou's evenness, and Berger-Parker indices through 9999 bootstrap replications. The β-diversity analysis of samples of interest was done by the calculation of the Morisita-Horn and symmetric indices through the packages vegan 2.0 and vegetarian implemented in R software [28].

The ecological data of the bacterial community structure of mixed liquor and biofouling samples was used for a similarity analysis to show differences among samples. The similarity analysis was done by the generation of a cluster dendrogram and a principal component analysis plot, which were calculated by Bray-Curtis distances using the vegan 2.0 package implemented in R software.

The SIMPER analyses were calculated using the Bray-Curtis distance and under 9999 bootstrap replications through the software PAST v3.0.

2.7. Characterization of Biominerals through X-Ray Diffractometry, Optic Microscopy, and Scanning Electron Microscopy

The minerals formed in the solid MC medium were extracted, according to Gonzalez-Martinez et al. [8] from such medium by cutting out pieces of the medium and placed boiling water to dissolve the agar. The precipitates were then washed using distilled water to remove impurities. Struvite crystals were obtained from the ME medium by using a small spatula and washed with distilled water to remove impurities. Both crystals types were air-dried at 37 °C for further analysis.

The characterization of the minerals was done using X-Ray diffractometry and scanning electron microscopy (SEM). The minerals were observed by using powder X-Ray diffractometry using a Philips PW 1710/00 diffractometer with a graphite monochromator automatic slit and CuKaα radiation. Data were collected for a 0.4 s integration time in 0.02 °C steps at 40 kV and 40 mA in a 2θ interval between 3 °C and 80 °C. Data was processed using the XPowder program for a qualitative and quantitative determination of the mineral composition. For quantitative analysis, full diffraction profiles of the experimental diffractograms were adjusted to weighed mixtures of the individual pattern phases. The crystalline mosaic size on hkl reciprocal vectors was obtained from full width at half of the maximum intensity after instrumental broadening and Kα2 corrections. The SEM of the minerals was made by carbon-coating samples and by using a high-resolution field emission scanning electron microscope Carl Zeiss, Supra 40 V (Carl Zeiss, Oberlocken, Germany). Selected samples were also analyzed for their chemical composition using energy dispersive X-ray (EDX) microanalysis (Aztec 350, Oxford Instruments, Abingdon, UK) and using the AMSCD mineral database.

3. Results

3.1. Physical-Chemical Parameters and Nutrient Removal

The removal of organic matter in the hybrid MBBR-MBR system subjected to variable salinity conditions was very good during the whole steady-state operation period with 99.51% removal of BOD_5 and 88.64% removal of COD for mean influent values of 293.33 ± 149.78 mg-BOD_5 L^{-1} and 588.36 ± 366.39 mg-COD L^{-1}. Total nitrogen elimination reached 36.83% removal for the influent mean value of 104.34 ± 22.25 mg-N L^{-1} [20]. For the constant salinity scenario, the removal rates of BOD_5 and COD were 98.09% and 88.55% for influent concentrations of 408.33 ± 46.18 mg-BOD_5 L^{-1} and 654.07 ± 154.57 mg-COD L^{-1}, respectively. In the case of total nitrogen removal, the influent mean value of 181.14 ± 28.59 mg-N L^{-1} yielded a 22.97% removal rate [6,7]. For both salinity conditions, the attached biofilm over the carrier media had small concentrations (< 20 mg L^{-1} and < 40 mg L^{-1} for the constant and variable salinity scenarios, respectively) with respect to the biomass concentrations in the MLSS (around 2500 mg L^{-1}).

The different salinity conditions showed an influence over the performance of the bioreactors in terms of total nitrogen removed. In this case, constant salinity conditions led to about 10% lower total nitrogen removal performance. On the other hand, the removal of organic matter was similar between the different salinity conditions. Therefore, the results suggested that maximum influent salinity was related to the removal of organic material and that influent salinity conditions (constant vs. variable) influenced the total nitrogen removal in the bioreactors. It is possible that constant salinity conditions exerted more pressure over the nitrifying and/or denitrifying microbial communities in the hybrid MBBR-MBR than the variable salinity conditions.

3.2. Ecological Coverage, α-Diversity and β-Diversity Analyses of the Bacterial Community Structure in the Mixed Liquor and Biofouling Samples

The data of the ecological coverage of the massive parallel sequencing samples is shown in Table S1. The mean coverage value was 94.19% and the lowest value was 89.69%. The values obtained showed that the massive parallel sequencing successfully covered the bacterial ecology diversity of the biomass analyzed.

The α-diversity analysis of the mixed liquor and biofilm samples analyzed is shown in Table S2. The chosen indices to observe α-diversity where the species richness, Chao-1, Shannon-Wiener, Simpson, Pielou's evenness, and Berger-Parker index. The species richness computes the number of species found in the biological sample. The Chao-1 index calculates the expected species richness of the sample. The Shannon-Wiener (H) and Simpson (1-D) indices are the Hill diversity indices of order 1 and order 2, respectively, and takes into account the diversity and evenness of the environment.

The Pielou's evenness shows the evenness of the samples. Lastly, the Berger-Parker index expresses the weighted true diversity and evenness of a sample.

Overall, the evenness of the variable salinity samples was lower than that of the constant salinity samples, which is shown by lower values of the Simpson and Pielou's evenness indices and higher values of the Berger-Parker index for the variable salinity scenario. In addition, the species richness along with the Shannon-Wiener and Chao-1 indices followed the same trend for constant and variable salinity scenarios than evenness indices.

Overall, the values of the Morisita-Horn indices were higher among variable salinity samples than among constant salinity samples (Figure 1). This was also found true for the symmetric index values. Morisita-Horn and symmetric indices have been reported as the best β-diversity estimators of dominant and rare phylotypes, respectively [29]. The variable salinity scenario showed higher similarity between biofouling and mixed liquor communities than the constant salinity scenario for dominant phylotypes. Biofouling had higher dissimilarity in dominant phylotypes than mixed liquor samples between the two salinity scenarios.
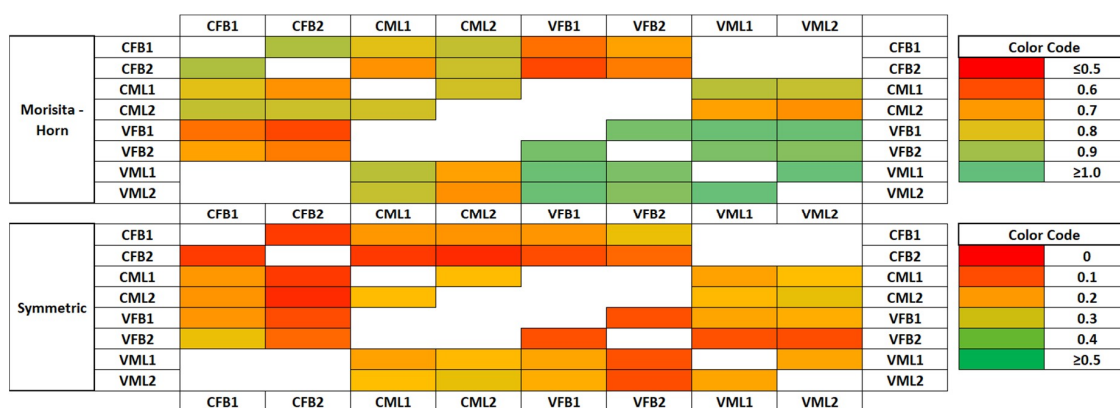
Figure 1. Heat map of β-diversity indices of pair of biological samples of interest (CFB: Constant salinity Fixed Biofilm, CML: Constant salinity Mixed Liquor, VFB: Variable salinity Fixed Biofilm, VML: Variable salinity Mixed Liquor).

3.3. Similarity Analysis of the Bacterial Community Structure of the Mixed Liquor and Biofouling Samples

The principal coordinates analysis plot and the cluster dendrogram ordinating the samples analyzed are shown in Figure 2. Both ordination methods showed two different groups separating the constant salinity and the variable salinity communities. The cluster dendrogram separated the communities of the two salinity scenarios at around 20% similarity, which showed that salinity conditions had a higher selection capacity over the bacterial community structure in the membrane fixed biomass and the membrane tank mixed liquor. As shown by β-diversity analysis, the constant salinity samples were different among them and clustered in a 30% to 60% similarity when compared to variable salinity conditions in which samples were clustered in the 60% to 80% similarity threshold.

Figure 2. Bray-Curtis similarity studies of the biological samples (top: cluster analysis, bottom: principal coordinates analysis).

3.4. Bacterial Community Structure of the Mixed Liquor and Biofouling Samples under Constant and Variable Salinity Conditions

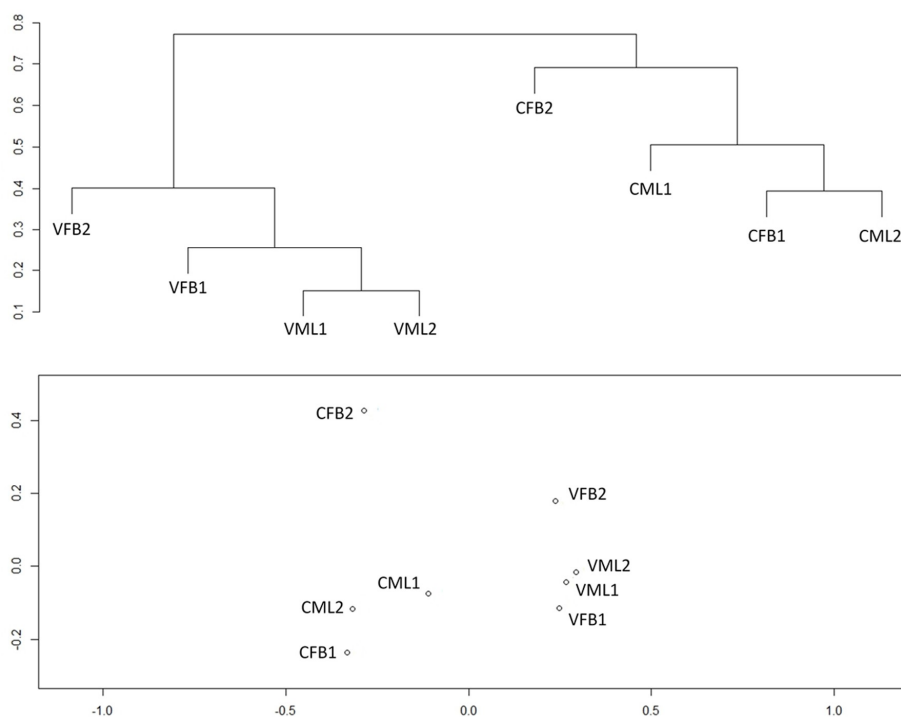The bacterial community structure of the mixed liquor and biofouling samples is shown in Figure 3. It was clear that certain bacterial genera were consistently found in mixed liquor and biofouling samples under both salinity conditions. Thus, *Rhodanobacter* (1.62% to 36.74%), *Ottowia* (2.23% to 8.32%), and *Dyella* (2.28% to 4.96%) were found in all samples studied while *Mycobacterium* (2.74% to 6.82%) and *Nitrobacter* (2.05% to 4.91%) were found in seven of the eight samples analyzed. Therefore, it could be suggested that these phylotypes are a major fraction of the microbiota involved in the MBBR-MBR system for the treatment of saline urban wastewater regardless of the influent salinity conditions.

As confirmed by α-diversity analysis, the variable salinity samples had a lower microbial diversity when compared to the constant salinity samples, which was caused by a strong dominance of *Rhodanobacter* (21.59% to 36.74%), *Mizugakiibacter* (3.77% to 4.39%), *Ottowia* (5.47% to 6.37%), and *Mycobacterium* (2.74% to 6.82%). Among these, *Gemmatimonas* (0.60% to 2.53%), *Thermomonas* (0.56% to 4.14%), and an uncultured *Comamonadaceae* clone (1.04% to 7.80%) were found only at constant salinity conditions. In addition, it should be highlighted that *Mizugakiibacter* (3.77% to 4.39%), *Comamonas* (3.3% to 4.56%), *Ferruginibacter* (2.59% to 7.71%), *Mycetocola* (2.20% to 3.24%), and *Cryocola* (2.78% to 4.51%) were detected only under the variable salinity scenario. On the contrary, a higher microbial diversity and evenness in the constant salinity samples was clearly defined by the ecological analysis, which shows a wide variety of different genera (Table S2). Under conditions of variable salinity, the genus *Ferruginibacter* (6.94% to 7.71%) was clearly more present in the biofouling than in the mixed liquor. On the other hand, genus *Rhodanobacter* was of lower relative abundance in the fixed biofilm conditions under constant salinity (1.62% to 3.30%) than for the rest of the samples.
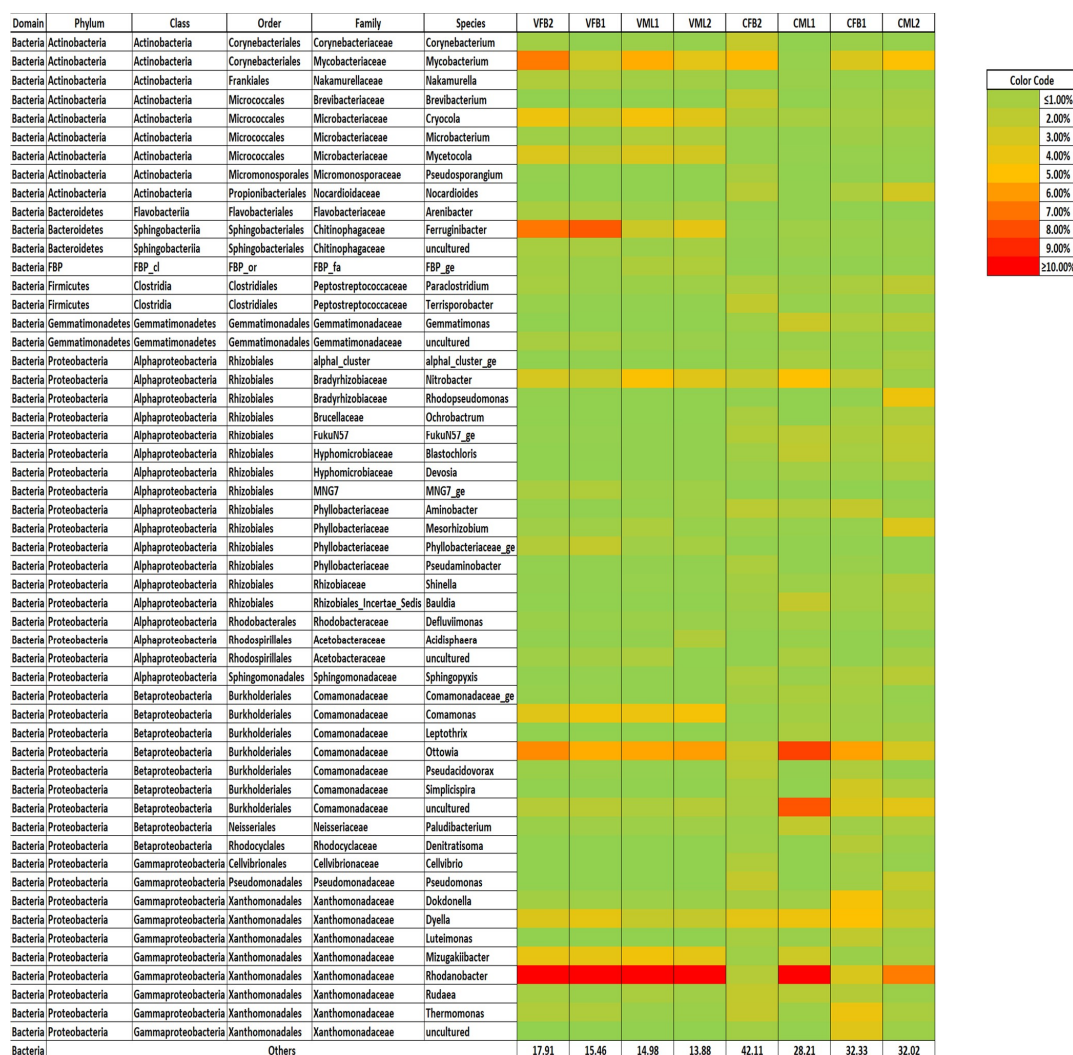
Domain	Phylum	Class	Order	Family	Species	VFB2	VFB1	VML1	VML2	CFB2	CML1	CFB1	CML2
Bacteria	Actinobacteria	Actinobacteria	Corynebacteriales	Corynebacteriaceae	Corynebacterium								
Bacteria	Actinobacteria	Actinobacteria	Corynebacteriales	Mycobacteriaceae	Mycobacterium								
Bacteria	Actinobacteria	Actinobacteria	Frankiales	Nakamurellaceae	Nakamurella								
Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Brevibacteriaceae	Brevibacterium								
Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	Cryocola								
Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	Microbacterium								
Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	Mycetocola								
Bacteria	Actinobacteria	Actinobacteria	Micromonosporales	Micromonosporaceae	Pseudosporangium								
Bacteria	Actinobacteria	Actinobacteria	Propionibacteriales	Nocardioidaceae	Nocardioides								
Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Arenibacter								
Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Ferruginibacter								
Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	uncultured								
Bacteria	FBP	FBP_cl	FBP_or	FBP_fa	FBP_ge								
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Paraclostridium								
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Terrisporobacter								
Bacteria	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	Gemmatimonas								
Bacteria	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	uncultured								
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	alphaI_cluster	alphaI_cluster_ge								
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Nitrobacter								
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Rhodopseudomonas								
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	Ochrobactrum								
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	FukuN57	FukuN57_ge								
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Blastochloris								
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Devosia								
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	MNG7	MNG7_ge								
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Aminobacter								
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Mesorhizobium								
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Phyllobacteriaceae_ge								
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Pseudaminobacter								
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Shinella								
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiales_Incertae_Sedis	Bauldia								
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Defluviimonas								
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Acidisphaera								
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	uncultured								
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingopyxis								
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonadaceae_ge								
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas								
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Leptothrix								
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Ottowia								
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pseudacidovorax								
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Simplicispira								
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	uncultured								
Bacteria	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Paludibacterium								
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Denitratisoma								
Bacteria	Proteobacteria	Gammaproteobacteria	Cellvibrionales	Cellvibrionaceae	Cellvibrio								
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas								
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Dokdonella								
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Dyella								
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Luteimonas								
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Mizugakiibacter								
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Rhodanobacter								
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Rudaea								
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Thermomonas								
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	uncultured								
Bacteria			Others			17.91	15.46	14.98	13.88	42.11	28.21	32.33	32.02

Color Code:
≤1.00%, 2.00%, 3.00%, 4.00%, 5.00%, 6.00%, 7.00%, 8.00%, 9.00%, ≥10.00%

Figure 3. Heat map of bacterial community structure of genera with at least >1% relative abundance in at least one sample.

Figure S2 showed that there were certain dominant phylotypes that caused the major dissimilarities between the mixed liquor and fixed biofilm samples for the constant and variable salinity scenarios. For the constant salinity conditions, the major contributors to dissimilarity were *Rhodanobacter* and *Mycobacterium*. For the variable salinity scenario, *Rhodanobacter* and *Mycobacterium* joined *Ferruginibacter* and *Ottowia*. Lower evenness at variable salinity conditions caused that contributions to dissimilarity were more marked for a few phylotypes than at constant salinity conditions. In general, *Rhodanobacter* had higher relative abundance in the mixed liquor (6.78% to 15.27%) than in the fixed biofilm (1.62% to 3.30%) under constant salinity (Figure 3). For the variable salinity conditions, this was also true. However, differences in relative abundance were less important (21.59% to 31.54% vs. 32.90% to 36.74%, respectively). *Mycobacterium* showed the opposite trend than that observed for *Rhodanobacter*. *Ottowia* showed higher relative abundance in the fixed biofilm under variable salinity conditions and higher relative abundance at the mixed liquor under constant salinity. *Ferruginibacter* had much higher (two-fold) relative abundance in the fixed biofilm than in the mixed liquor at variable salinity conditions.

3.5. Biomineral Precipitation by Bacteria Isolated from Fixed Biofilm under Constant and Variable Salinity Conditions

The formation of biofouling in the MBBR-MBR system was detected after 24 h under both experimental conditions (constant and variable salinity). Biofouling generation was easily detected by an optical microscope. Moreover, the formation of biominerals (calcium carbonate and struvite crystals) in artificial media (MC and ME solid media) by the bacterial strains isolated from the biofouling took place after three days of incubation time. However, no formation of crystals was detected in un-inoculated control media or in those culture media inoculated with a high concentration of dead cells, which lacked metabolic activity.

Images of the biominerals formed in ME and MC media by the strains isolated from the biofouling generated in the membranes under constant and variable salinity conditions are shown in Figure 4. The images showed that the minerals formed in ME were very similar between the constant and variable salinity conditions while the minerals formed in the MC had some slight differences among them.
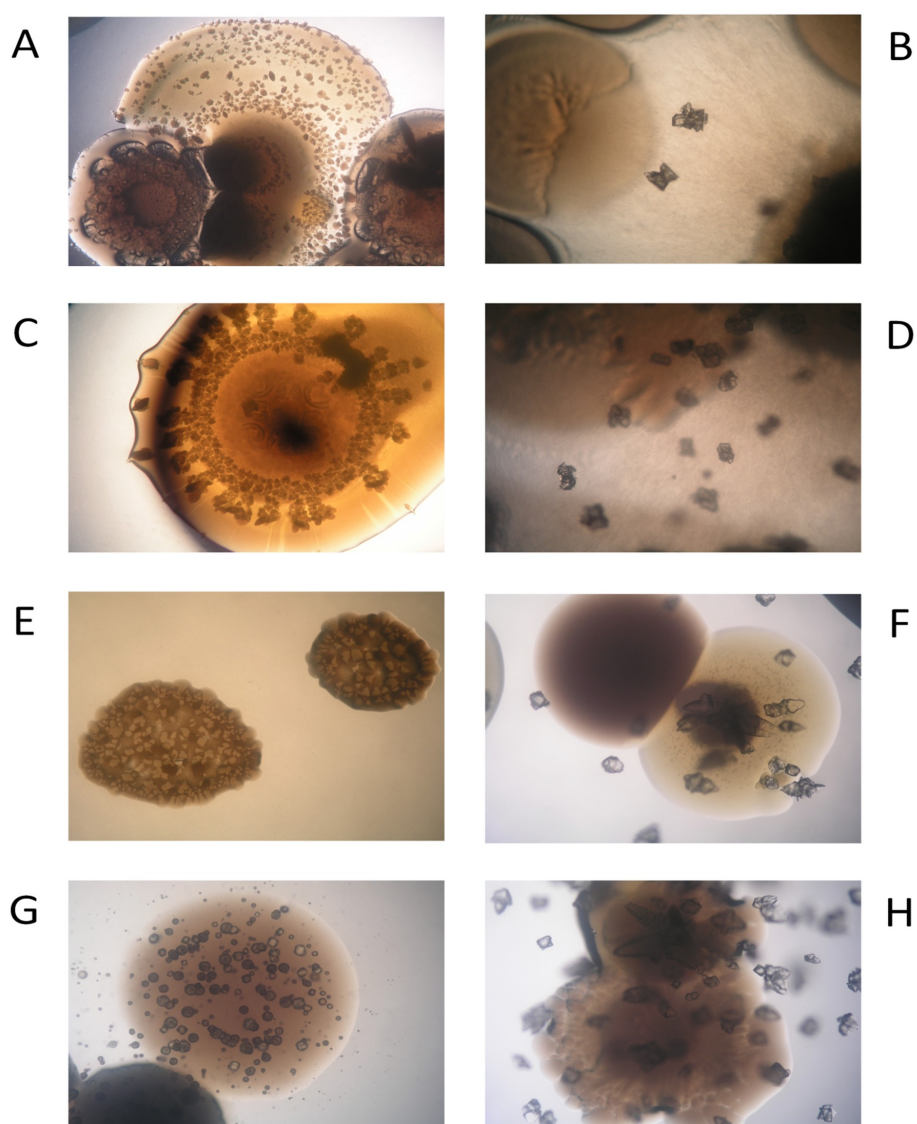
Figure 4. Optical microscopy images of biomineralization in culture media: (**A**,**C**) MC medium for constant salinity scenario, (**E**,**G**) MC medium for variable salinity scenario, (**B**,**D**) ME medium for constant salinity scenario, and (**F**,**H**) ME medium for variable salinity scenario.

The results of the SEM X-ray microanalysis showed that the minerals were mainly formed by the same principal components, which were Ca^{2+} for the MC medium and Mg^{2+} for the ME medium. SEM images of the minerals obtained from MC medium showed isolated or aggregated carbonate objects with spherical shapes, hereafter called bioliths (Figure 5), independently of the salinity conditions (constant and variable). In many cases, mineralized bacteria were evident on spherulites surfaces and a bacterial mold entirely covered the surfaces of certain bioliths (Figure 5). It can be observed in the SEM images of the spherulite surfaces (Figure 5) that carbonate precipitates were nucleated on bacterial nanoglobules (Figure 5A,B,D,E) thought to be the first stage of precipitation processes that are mediated by microbes [30].When crystal minerals produced in ME medium by the isolated strains from the membrane biofouling were examined under SEM, it was observed that phosphate minerals were not formed by the aggregation of mineralized cells (Figure 6) and, in such cases, bacterial cells contributed to the formation only by changing the environment as a consequence of their metabolic activity.
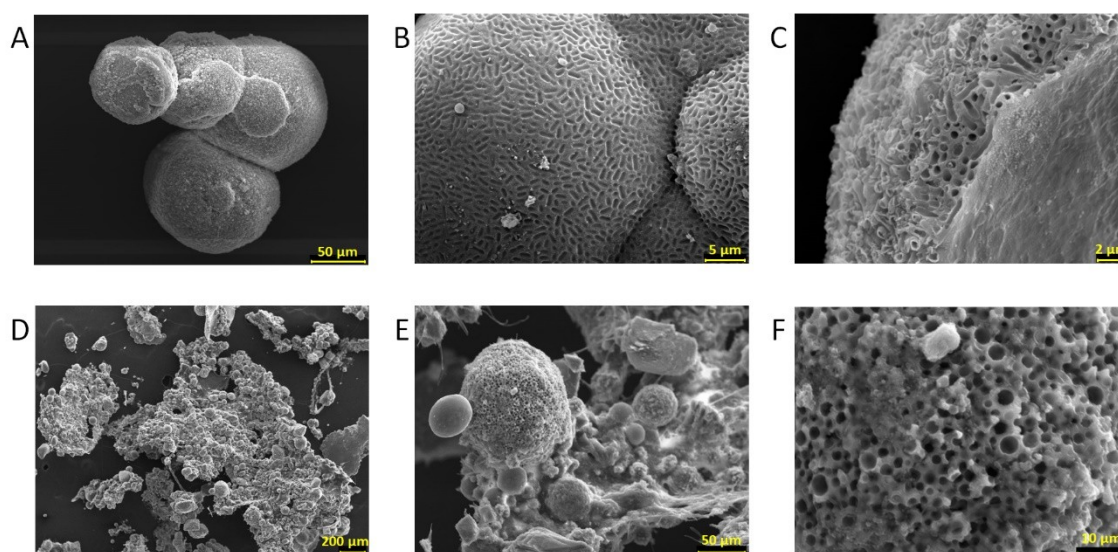
Figure 5. Scanning electron microscopy images of biominerals precipitated in the MC medium under constant (**A**–**C**) and variable (**D**–**F**) salinity conditions.
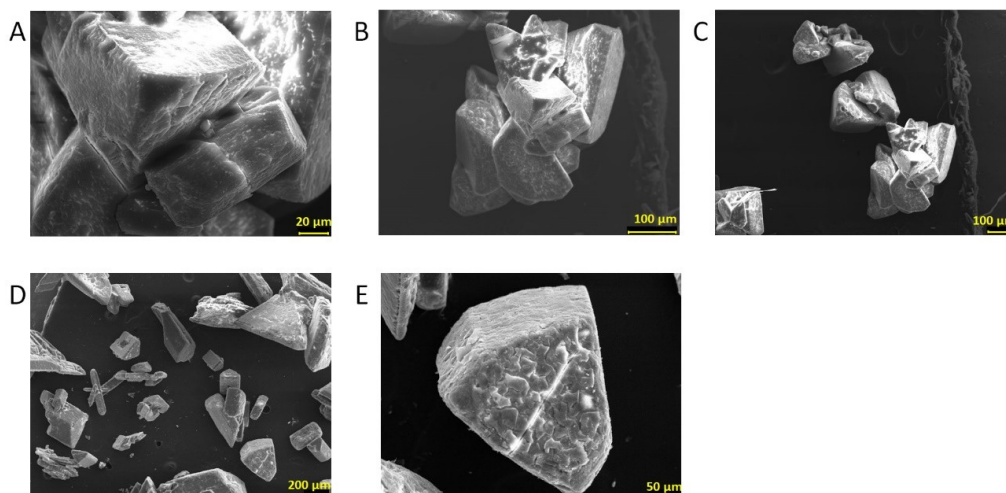
Figure 6. Scanning electron microscopy images of biominerals precipitated in the ME medium under constant (**A**–**C**) and variable (**D**,**E**) salinity conditions.

The X-ray diffractometry analysis results showed that the X-ray difractograms from precipitates in the MC medium by isolated strains from membrane biofouling under salinity conditions (constant and variable) were always calcite (Figure 7A,B). On the other hand, the minerals formed in ME medium by isolated strains from membrane biofouling at constant and variable salinity scenarios were closely related to K-struvite and struvite, respectively (Figure 7C,D).
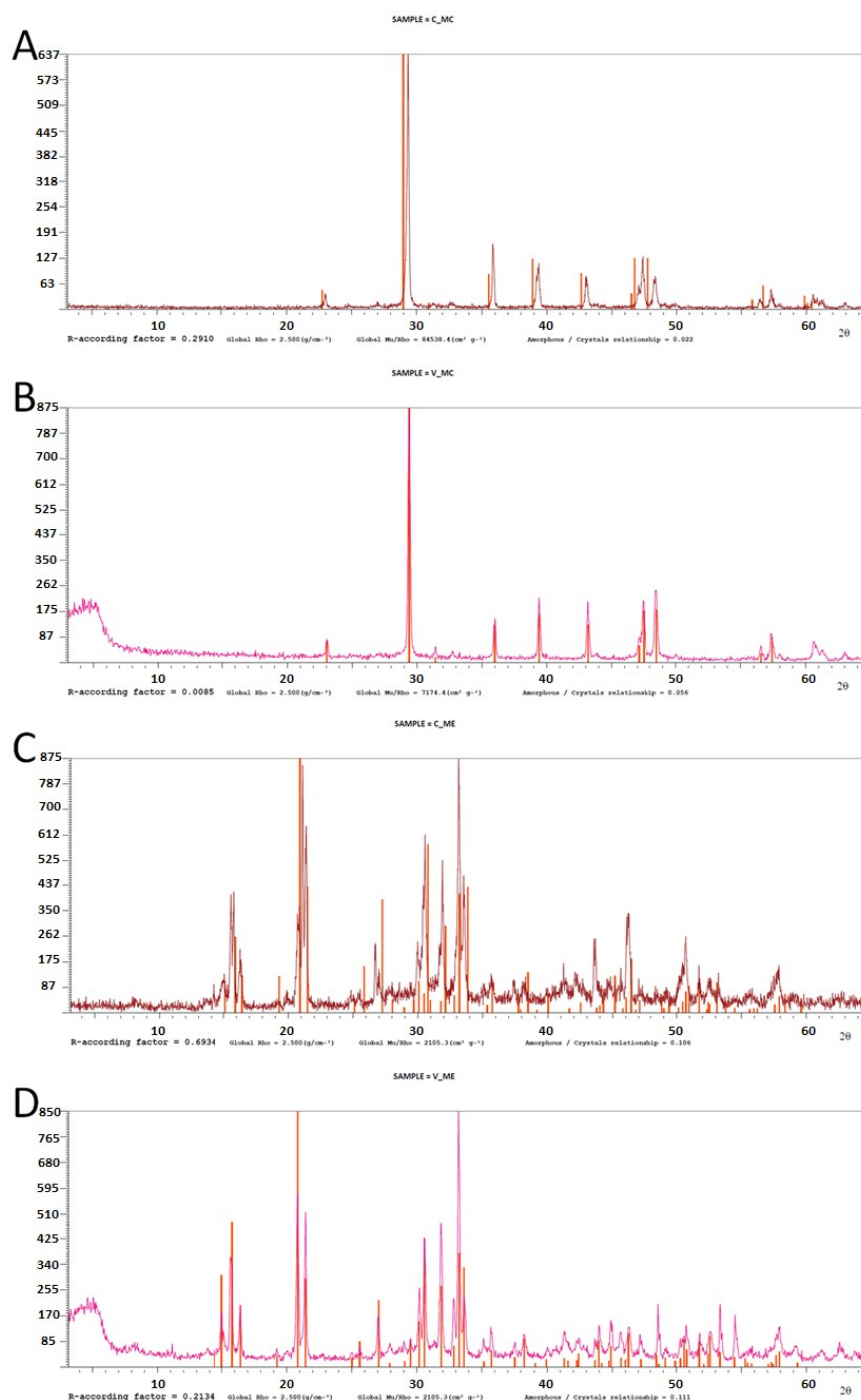
Figure 7. X-Ray diffractometry analyses of biominerals precipitated in the culture media: (**A**) MC medium for constant salinity conditions, (**B**) ME medium for constant salinity conditions, (**C**) MC medium for variable salinity conditions, and (**D**) MC medium for variable salinity conditions.

3.6. Identification of Bacterial Species Associated with Mineral Precipitation from the Biofouling Formed under Constant-Salinity and Variable-Salinity Conditions

Only one bacterial species, classified as *Citrobacter freundii* and associated with calcite precipitation capacity, was detected in MC medium for the constant salinity condition while, in ME medium under these same conditions, three strains were identified with K-struvite precipitation activity: *Citrobacter freundii*, *Bacillus licheniformis*, and *Bacillus pumilus*. For the variable salinity conditions, only one strain was found in MC medium being classified as *Brevibacterium linens* while the sole strain found in the ME medium with struvite precipitation activity was *Bacillus licheniformis*. The similarity values with NCBI sequences are given in Table S3.

For the constant salinity condition, the identified phylotypes with >1% relative abundance reported for calcite precipitation were *Nocardioides* (1.163% to 1.694%) [31], *Brevibacterium* (0.604% to 2.324%) [32], and *Pseudomonas* (0.671% to 2.284%) [33]. For the case of struvite precipitation, the identified phylotypes were *Corynebacterium* (0.492% to 2.374%) [34], *Brevibacterium* (0.604% to 2.324%) [35], and *Ochrobactrum* (0.671% to 2.284%) [15]. On the other hand, only one phylotype with >1% relative abundance in the variable salinity scenario was found with potential capabilities to precipitate calcite or struvite such as *Comamonas* (3.525% to 4.325%) identified for struvite precipitation [36].

4. Discussion

4.1. Physical-Chemical Parameters and Nutrient Removal

With respect to regular salinity operation, the bioreactors showed similar COD and BOD$_5$ removal while lower performance was observed in TN elimination [3]. The data suggested that amended-salinity wastewater could pressure nitrifying and/or denitrifying communities in the hybrid MBBR-MBR, which resulted in a TN removal efficiency loss. The absence of attached biofilm in the bioreactors showed that microbial communities in the bioprocesses could not form biofilms over the carrier surface in the presence of constant and variable 3.6 g L^{-1} NaCl maximum salinity. This result has been observed previously in hybrid MBBR-MBR systems [7] by proposing that salinity inhibits the ability of microorganisms in urban wastewater for the formation of attached biofilms. In practice, the hybrid MBBR-MBR systems operated under both scenarios of salinity operated as MBR systems.

4.2. Ecological Coverage, α-Diversity, and β-Diversity Analyses of the Bacterial Community Structure in the Mixed Liquor and Biofouling Samples

The α-diversity and β-diversity data suggested that, in the MBBR-MBR systems treating effluent with variable salinity conditions, the bacterial populations in the mixed liquor and in the biofouling had less diversity and evenness, which can be observed by the species richness, Chao-1, Shannon-Wiener, Simpson, Pielou's evenness, and Berger-Parker indices (Table S2) as well as higher similarity among dominant phylotypes, based on the Morisita-Horn index (Figure 1), than the bacterial populations present in the constant salinity scenario. Thus, it seemed that variable salinity conditions allowed for a more stringent selection of membrane biomass phylotypes.

4.3. Similarity Analysis of the Bacterial Community Structure of the Mixed Liquor and Biofouling Samples

The results obtained by the similarity analysis suggested that the influent conditions with respect to salinity loading (constant vs. variable) had a high influence over the bacterial community structure in the MBBR-MBR systems analyzed in this study, which is shown by the α-diversity and β-diversity analyses. In this sense, samples trended to cluster for similar influent salinity variations, which was more important in the ordination of samples than the nature of the biomass (Figure 2).

4.4. Bacterial Community Structure of the Mixed Liquor and Biofouling Samples under Constant and Variable Salinity Conditions

Dominant phylotypes found under both salinity conditions, *Rhodanobacter* and *Nitrobacter* (Figure 3), have been reported as the main nitrite oxidizers in hybrid MBBR-MBR systems treating salinity-amended wastewater [6]. In this context, *Rhodonobacter* is a microorganism that has the capacity to produce high amounts of EPS [37] and this fact could be of importance in the formation of membrane biofouling. Moreover, *Nitrobacter* has been identified as a microorganism involved in the formation of biofouling processes in MBR systems treating bathing wastewater [38]. On the other hand, microorganisms of the genus *Ottowia* has been described as halophilic phylotype in saline wastewater treatment systems [7] where it develops an important role in the formation of the floc biomass. In addition, members of the *Ottowia* genus have been reported to be involved in important metabolic activities such as heterotrophic ammonium oxidation and nitrite/nitrate reduction in biofilms [39]. Lastly, the *Dyella* genus has been found in membrane biocake and thus has been related to late biofouling processes of membrane treating wastewater [40]. With respect to important genus *Ferruginibacter*, Liu et al. [38] reported its important function in the fixed biofilm formation in membrane bioreactors where it contributes directly to the membranes' colonization and later to the establishment of a mature biofilm.

In the case of operation under regular salinity (around $1\,\mathrm{mS\,cm^{-1}}$ electric conductivity), there were huge differences in the biofouling community structure dominated by *Simplicispira*, *Rhodobacter*, *Comamonas*, and *Clostridium* [8].

The results of bacterial community structure analysis could suggest that salinity conditions (variable or constant) determined changes in the microbial community structure in the mixed liquor and in the membrane biofouling of the MBBR-MBR system possibly as a consequence of the different osmolarity produced during the operational conditions in the system. Moreover, β-diversity and similarity analyses confirmed this suggestion (Figures 1 and 2).

It was found that different salinity conditions resulted in similar organic matter removal performance but that total nitrogen removal was slightly higher for the variable salinity conditions. This could be caused by a difference in the nitrifiers and/or denitrifiers in the bacterial communities of the bioreactors at both salinity conditions. Among the dominant phylotypes found in both systems, it has been claimed that *Rhodanobacter*, *Nitrobacter*, and *Ottowia*, among others, have an important role in autotrophic nitrification kinetics in MBR and MBBR-MBR systems treating saline wastewater [7]. Additionally, *Rhodanobacter* has been associated with denitrification in MBR and MBBR-MBR systems [20]. The differential relative abundance of *Rhodanobacter* in the mixed liquor of the MBBR-MBR systems at different salinity conditions (6.78% to 15.27% under constant salinity, 32.90% to 36.74% under variable salinity) may be related to the differential total nitrogen removal. In this way, *Rhodanobacter* would have a very important ecological role in the nitrification and/or denitrification processes in MBBR-MBR systems treating saline wastewater.

The SIMPER analyses showed dissimilar representations of dominant phylotypes *Rhodanobacter* and *Mycobacterium* at both salinity conditions with *Mycobacterium* having more importance in the mixed liquor and *Rhodanobacter* in the biofouling, which occurred with *Ferruginibacter*. On the other hand, genus *Ottowia* was more represented in the mixed liquor than in the biofouling for the constant salinity conditions than for the variable salinity conditions. Taking into consideration that *Rhodanobacter* and *Ottowia* have been related to autotrophic nitrification kinetics [7], it is possible that their competition in the mixed liquor inside the MBBR-MBR systems had an effect over the total nitrogen removal of the bioreactors. Nevertheless, the results showed that possible performance of the MBBR-MBR treating saline wastewater was more affected by the relative abundance of major players in the nitrification and/or denitrification than by their differential presence between mixed liquor and biofouling, which was also suggested by the similarity analyses.

It has been found that the bacterial communities developing in membrane systems treating salinity wastewater are sensitive to the maximum influent salinity and its regime (constant or

variable) [41]. Regarding this information, it can be stated that the results obtained in this paper could be escalated to systems at full-scale, which operate under similar influent salinity conditions. In the same manner, the high variability of the full-scale effluents limits the application of these results to existing operating systems due to high sensitivity of bacterial communities in MBBR-MBR systems treating saline wastewater.

4.5. Biomineral Precipitation by Bacteria Isolated from Fixed Biofilm under Constant and Variable Salinity Conditions

The results obtained from the biomineralization experiment confirmed that the generation of biominerals was determined by the biological activity of the bacterial strains and that this is consequently a biomineralization process. Similar results have been previously described by Gonzalez-Martinez et al. [8]. The formation of crystals of struvite (ammonium-magnesium hydrated phosphate) or K-struvite (magnesium-potassium hydrated phosphate) should be associated with the characteristics of the bacterial strains isolated in the biofouling obtained under the different salinity conditions tested. Previous analyses of minerals precipitated in MC and ME media by bacterial strains isolated from MBBR-MBR systems operating at low salt concentration (around 1 mS cm^{-1} electric conductivity) demonstrated their capacity for bio-precipitation of struvite in the ME medium and calcite and vaterite in the MC medium [8]. This last mineral (vaterite) was never detected in our study.

4.6. Identification of Bacterial Species Associated with Mineral Precipitation from the Biofouling Formed under Constant-Salinity and Variable-Salinity Conditions

It is evident that the presence of high saline concentrations in wastewater originated in an important reduction in the biodiversity of cultivable strains with the capacity to precipitate minerals in culture media in the biofouling obtained from the MBBR-MBR system especially when compared with previous studies in MBBR-MBR systems with low saline concentration [8].

Citrobacter freundii strains have been isolated from a hypersaline marine environment [42]. *Citrobacter freundii* strains isolated from hypersaline coastal lagoons have been reported to mediate on calcite precipitation [43]. It is, therefore, important to point out that it is the first time that the formation of calcite crystals by *Citrobacter freundii* has been reported in engineering systems, which is important since its role in the formation of membrane biofouling in high salinity conditions is unknown. In addition, it is noteworthy that *C. freundii* was the only bacterial species isolated from membrane biofouling samples in carbonate precipitation media under conditions of constant salinity. The massive parallel sequencing procedure performed on the biofouling showed that the *Citrobacter* genus was present in the membrane biofouling samples at the constant salinity scenario in a range of relative abundance from 0.015% to 0.036%. These data suggested that, even though, from a quantitative point of view *C. freundii*, it did not represent a majority fraction of the microbiota present in biofouling, it did have a functionally important characteristic such as its ability to actively precipitate calcium carbonate crystals.

Genomic insights into the formation of calcite by *Brevibacterium linens* have shown that this species regulates three carbonic anhydrases in response to Ca^{2+} concentration in the precipitation medium [32]. The relative abundance of *Brevibacterium* in the biofouling of the variable salinity scenario ranged from 0.007% to 0.009%. Even though the *Brevibacterium* genus was found at higher values of 0.604% to 2.324% at the constant salinity scenario, none of its strains isolated from biofouling formed under these conditions was found to produce crystals. It could be suggested that salinity conditions (constant or variable) establish a process of microbial adaptation that determines the capacity of certain strains to precipitate with greater or lesser efficiency in different biominerals such as calcite. The ions' concentration in the environment plays an important role in these events, which is previously suggested by other authors [44].

The precipitation of calcite by *Bacillus licheniformis* is widely known both in natural and engineering systems. In this context, it has been proposed that *Bacillus licheniformis* biomineralization activity can help to raise the strength of cast concrete [45]. Nevertheless, the precipitation of

k-struvite and struvite by *Bacillus licheniformis* strains has never been reported. Particularly, in our study, *B. licheniformis* formed k-struvite and struvite under constant and variable salinity conditions, respectively. Furthermore, we must highlight that, in conditions of variable salinity, *B. licheniformis* was the only cultivable microorganism with the ability to form struvite minerals. The other *Bacillus* representative, *Bacillus pumilus*, has been reported for having a strong capacity for struvite crystallization from wastewater [33]. The relative abundance of *Bacillus* genus in the constant salinity scenario was from 0.015% to 0.029% while, for the case of the variable salinity scenario, ranged from 0.009% to 0.014%.

Following the measurement established by Gonzalez-Martinez et al. [8], the bacterial microbiota with biomineralization potential risk identified in the high constant salinity scenario was less than 10% of the total microbiota and less that 5% for the high variable salinity scenario in comparison with the microbiota with a biomineralization potential risk encountered at low salinity conditions for pure hybrid MBBR-MBR (around 20% of the total microbiota). In this sense, our studies seemed to suggest that the treatment of effluents with a high concentration of salts (especially with high variable concentrations) decreases the potential risk of formation of biominerals in biofouling processes in MBBR-MBR systems probably as a consequence of a decrease in microbial diversity in response to high environmental osmolarity.

It must be noted that the influent salinity conditions greatly affected the bacterial communities in membrane biofilm of MBBR-MBR systems [41]. In this sense, operation at higher salinities would certainly select for other bacterial species in membrane biofilm and, therefore, the bacterial strains with the capability for biomineralization as well as the nature of the biominerals formed would be different.

The evaluation of membrane biofouling in two hybrid MBBR-MBR systems subjected to different influent salinity conditions (constant at 3.6 g L^{-1} NaCl electric conductivity and tidal-like variational with a peak of 3.6 g L^{-1} NaCl) showed clear differences and thus the effect of the salinity conditions was demonstrated. The biofouling bacterial community had lower evenness and diversity under the variable salinity conditions, which is shown by α-diversity analysis. The variable salinity conditions also caused a greater similarity between mixed liquor and biofouling biomass than for the constant salinity scenario, which is observed by β-diversity analysis. The higher pressure of selection exerted by variable salinity conditions over constant salinity conditions were also demonstrated by ordination analysis based on Bray-Curtis distances. The bacterial community structure showed that *Rhodanobacter*, *Ottowia*, *Dyella*, *Mycobacterium*, and *Nitrobacter* were consistently found at high relative abundance across both salinity scenarios. Cultivation of bacterial strains with a capacity for calcium carbonate formation revealed that calcite precipitated for biomass adapted to both constant and tidal-like variable conditions, but, in the medium designed for phosphate precipitation, differences were found with mineralization of K-struvite and struvite for the constant and variable salinity scenarios, respectively. Only a few bacterial strains were identified as mineral-forming microorganisms and were related to *Citrobacter freundii*, *Bacillus pumilus*, *Bacillus licheniformis*, and *Brevibacterium linens*. The results obtained demonstrated that differences in salinity conditions affected the bacterial assemblage and biomineralization characteristics of the membrane biofouling.

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References

1. Gonzalez-Martinez, A.; Rodriguez-Sanchez, A.; Lotti, T.; Garcia-Ruiz, M.-J.; Osorio, F.; Gonzalez-Lopez, J.; van Loosdrecht, M.C.M. Comparison of bacterial communities of conventional and A-stage activated sludge systems. *Sci. Rep.* **2016**, *6*, 18786. [CrossRef] [PubMed]

2. Leyva-Diaz, J.C.; Calderon, K.; Rodriguez, F.A.; Gonzalez-Lopez, J.; Hontoria, E.; Poyatos, J.M. Comparative kinetic study between moving bed biofilm reactor-membrane bioreactor and membrane bioreactor systems and their influence on organic matter and nutrients removal. *Biochem. Eng. J.* **2013**, *77*, 28–40. [CrossRef]

3. Leyva-Diaz, J.C.; Gonzalez-Martinez, A.; Gonzalez-Lopez, J.; Muñio, M.M.; Poyatos, J.M. Kinetic modeling and microbiological study of two-step nitrification in a membrane bioreactor and hybrid moving bed biofilm reactor-membrane bioreactor for wastewater treatment. *Chem. Eng. J.* **2015**, *259*, 692–702. [CrossRef]

4. Leyva-Diaz, J.C.; Lopez-Lopez, C.; Martin-Pascual, J.; Muñio, M.M.; Poyatos, J.M. Kinetic study of the combined processes of a membrane bioreactor and a hybrid moving bed biofilm reactor-membrane bioreactor with advanced oxidation processes as a post-treatment stage for wastewater treatment. *Chem. Eng. Process.* **2015**, *91*, 57–66. [CrossRef]

5. Cortés-Lorenzo, C.; González-Martínez, A.; Smidt, H.; González-López, J.; Rodelas, B. Influence of salinity on fungal communities in a submerged fixed bed bioreactor for wastewater treatment. *Chem. Eng. J.* **2016**, *285*, 562–572. [CrossRef]

6. Rodriguez-Sanchez, A.; Leyva-Diaz, J.C.; Gonzalez-Lopez, J.; Poyatos, J.M. Performance and kinetics of membrane and hybrid moving bed biofilm-membrane bioreactors treating salinity wastewater. *AIChE J.* **2017**, *63*, 3329–3342. [CrossRef]

7. Rodriguez-Sanchez, A.; Leyva-Diaz, J.C.; Gonzalez-Martinez, A.; Poyatos, J.M. Linkage of microbial kinetics and bacterial community structure of MBR and hybrid MBBR-MBR systems to treat salinity-amended urban wastewater. *Biotechnol. Prog.* **2017**, *33*, 1483–1495. [CrossRef] [PubMed]

8. Gonzalez-Martinez, A.; Leyva-Díaz, J.C.; Rodriguez-Sanchez, A.; Muñoz-Palazon, B.; Rivadeneyra, A.; Poyatos, J.M.; Rivadeneyra, M.A.; Martinez-Toledo, M.V. Isolation and metagenomic characterization of bacteria associated with calcium carbonate and struvite precipitation in a pure moving bed biofilm reactor-membrane bioreactor. *Biofouling* **2015**, *31*, 333–348. [CrossRef] [PubMed]

9. Leyva-Díaz, J.C.; Martín-Pascual, J.; Muñío, M.M.; González-López, J.; Hontoria, E.; Poyatos, J.M. Comparative kinetics of hybrid and pure moving bed reactor-membrane bioreactors. *Ecol. Eng.* **2014**, *70*, 227–234.

10. Di Trapani, D.; Di Bella, G.; Mannina, G.; Torregrossa, M.; Viviani, G. Comparison between moving bed-membrane bioreactor (MB-MBR) and membrane bioreactor (MBR) systems: Influence of wastewater salinity variation. *Bioresour. Technol.* **2014**, *162*, 60–69. [CrossRef] [PubMed]

11. Leyva-Diaz, J.C.; Gonzalez-Martinez, A.; Muñio, M.M.; Poyatos, J.M. Two-step nitrification in a pure moving bed biofilm reactor-membrane bioreactor for wastewater treatment: Nitrifying and denitrifying microbial populations and kinetic modeling. *Appl. Microbiol. Biotechnol.* **2015**, *99*, 10333–10343. [CrossRef] [PubMed]

12. Dhami, N.K.; Reddy, M.S.; Mukherjee, A. Biomineralization of calcium carbonates and their engineered applications: A review. *Front. Microbiol.* **2013**, *4*. [CrossRef] [PubMed]

13. Rao, A.; Cölfen, H. Mineralization and non-ideality: On nature's foundry. *Biophys. Rev.* **2016**, *8*, 309–329. [CrossRef] [PubMed]

14. Rivadeneyra Torres, A.; Martinez-Toledo, M.V.; Gonzalez-Martinez, A.; Gonzalez-Lopez, J.; Martín-Ramos, D.; Rivadeneyra, M.A. Precipitation of carbonates by bacteria isolated from wastewater samples collected in a conventional wastewater treatment plant. *Int. J. Environ. Sci. Technol.* **2013**, *10*, 141–150. [CrossRef]

15. Rivadeneyra, A.; Gonzalez-Martinez, A.; Gonzalez-Lopez, J.; Martin-Ramos, D.; Martinez-Toledo, M.V.; Rivadeneyra, M.A. Precipitation of phosphate minerals by microorganisms isolated from a fixed-biofilm reactor used for the treatment of domestic wastewater. *Int. J. Environ. Res. Public Health* **2014**, *11*, 3689–3704. [CrossRef] [PubMed]

16. Gonzalez-Martinez, A.; Rodriguez-Sanchez, A.; Rivadeneyra, M.A.; Rivadeneyra, A.; Martin-Ramos, D.; Vahala, R.; Gonzalez-Lopez, J. 16S rRNA gene-based characterization of bacteria potentially associated with phosphate and carbonate precipitation from a granular autotrophic nitrogen removal bioreactor. *Appl. Microbiol. Biotechnol.* **2017**, *101*, 817–829. [CrossRef] [PubMed]

17. Radu, A.I.; Bergwerff, L.; van Loosdrecht, M.C.M.; Picioreanu, C. Combined biofouling and scaling in membrane feed channels: A new modeling approach. *Biofouling* **2015**, *31*, 83–100. [CrossRef] [PubMed]

18. Silva-Castro, G.A.; Uad, I.; Gonzalez-Martinez, A.; Rivadeneyra, A.; Gonzalez-Lopez, J.; Rivadeneyra, M.A. Bioprecipitation of calcium carbonate crystals by bacteria isolated from saline environments grown in culture media amended with seawater and real brine. *BioMed Res. Int.* **2015**. [CrossRef] [PubMed]

19. Silva-Castro, G.A.; Uad, I.; Rivadeneyra, A.; Vilchez, J.A.; Martin-Ramos, D.; Gonzalez-Lopez, J.; Rivadeneyra, M.A. Carbonate precipitation of bacterial strains isolated from sediments and seawater: Formation mechanisms. *Geomicrobiol. J.* **2013**, *30*, 840–850. [CrossRef]

20. Rodriguez-Sanchez, A.; Leyva-Diaz, J.C.; Gonzalez-Lopez, J.; Poyatos, J.M. Membrane bioreactor and hybrid moving bed biofilm reactor-membrane bioreactor for the treatment of variable salinity wastewater: Influence of biomass concentration and hydraulic retention time. *Chem. Eng. J.* **2018**, *336*, 102–111. [CrossRef]

21. Schloss, P.D.; Westcott, S.L.; Ryabin, T.; Hall, J.R.; Hartmann, M.; Hollister, E.B.; Lesniewski, R.A.; Oakley, B.B.; Parks, D.H.; Robinson, C.J.; et al. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* **2009**, *75*, 7537–7541. [CrossRef] [PubMed]

22. Edgar, R.C.; Haas, B.J.; Clemente, J.C.; Quince, C.; Knight, R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **2011**, *27*, 2194–2200. [CrossRef] [PubMed]

23. Unno, T. Bioinformatic suggestions on MiSeq-based microbial community analysis. *J. Microbiol. Biotechnol.* **2015**, *25*, 765–770. [CrossRef] [PubMed]

24. Huse, S.M.; Welch, D.M.; Morrison, H.G.; Sogin, M.L. Ironing out the wrinkles in the rare biosphere through improved OTU clustering. *Environ. Microbiol.* **2010**, *12*, 1889–1898. [CrossRef] [PubMed]

25. Rodriguez-R, L.M.; Konstantinidis, K.T. Nonpareil: A redundancy-based approach to assess the level of coverage in metagenomic datasets. *Bioinformatics* **2014**, *30*, 629–635. [CrossRef] [PubMed]

26. Rodriguez-R, L.M.; Konstantinidis, K.T. Estimating coverage in metagenomic data sets and why it matters. *ISME J.* **2014**, *8*, 2349–2351. [CrossRef] [PubMed]

27. Hammer, Ø.; Harper, D.A.T. *Palaeontological Data Analysis*; Wiley: Hoboken, NJ, USA, 2007.

28. Gonzalez-Martinez, A.; Garcia-Ruiz, M.J.; Rodriguez-Sanchez, A.; Osorio, F.; Gonzalez-Lopez, J. Archaeal and bacterial community dynamics and bioprocess performance of a bench-scale two-stage anaerobic digester. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 6013–6033. [CrossRef] [PubMed]

29. Barwell, L.J.; Isaac, N.J.B.; Kunin, W.E. Measuring β-diversity with species abundance data. *J. Anim. Ecol.* **2015**, *84*, 1112–1122. [CrossRef] [PubMed]

30. Rivadeneyra, M.A.; Martin-Algarra, A.; Sanchez-Roman, M.; Sanchez-Navas, A.; Martin-Ramos, J.D. Amorphous Ca-phosphate precursors for Ca-carbonate biominerals mediated by Chromohalobacter marismortui. *ISME J.* **2010**, *4*, 922–932. [CrossRef] [PubMed]

31. Busquets, A.; Fornós, J.J.; Zafra, F.; Lalucat, J.; Merino, A. Microbial communities in a coastal cave: Cova des Pas de Vallgornera (Mallorca, Western Mediterranean). *Int. J. Speleol.* **2014**, *43*, 205–216. [CrossRef]

32. Zhu, Y.; Ma, N.; Jin, W.; Wu, S.; Sun, C. Genomic and transcriptomic insights into calcium carbonate biomineralization by marine actinobacterium Brevibacterium linens BS258. *Front. Microbiol.* **2017**, *8*. [CrossRef] [PubMed]

33. Bai, Y.; Guo, X.; Li, Y.; Huang, T. Experimental and visual research on the microbial induced carbonate precipitation by Pseudomonas aeruginosa. *AMB Express* **2017**. [CrossRef] [PubMed]

34. Martín-Navarro, J.A.; Petkov-Stoyanov, V.; Gutiérrez-Sánchez, M.J.; Gordo-Flores, M.E. Struvite urolithiasis with Corynebacterium urealyticum infection: A case report. *Nefrologia* **2015**, *35*, 410–417. [CrossRef] [PubMed]

35. Soares, A.; Veesam, M.; Simoes, F.; Wood, E.; Parsons, S.A.; Stephenson, T. Bio-Struvite: A new route to recover phosphorus from wastewater. *Clean Soil Air Water* **2014**, *42*, 994–997. [CrossRef]

36. Rogers, C.J.; ten Broek, C.M.A.; Hodson, B.; Whitehead, M.P.; Schmerer, W.M.; Sutton, R. Identification of crystals forming on porcine articular cartilage: A new method for the estimation of the postmortem interval. *J. Forensic Sci.* **2014**, *59*, 1575–1582. [CrossRef] [PubMed]

37. Aqeel, H.; Basuvaraj, M.; Hall, M.; Neufeld, J.D.; Liss, S.N. Microbial dynamics and properties of aerobic granules developed in a laboratory-scale sequencing batch reactor with an intermediate filamentous bulking stage. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 447–460. [CrossRef] [PubMed]

38. Guo, J.; Xia, S.; Wang, R.; Zhao, J. Study on membrane fouling of submerged membrane bioreactor in treating bathing wastewater. *J. Environ. Sci.* **2008**, *20*, 1158–1167. [CrossRef]

39. Liu, T.; Mao, Y.; Shi, Y.; Quan, X. Start-up and bacterial community compositions of partial nitrification in moving bed biofilm reactor. *Appl. Microbiol. Biotechnol.* **2017**, *101*, 2563–2574. [CrossRef] [PubMed]

40. Lim, S.; Kim, S.; Yeon, K.; Sang, B.; Chun, J.; Lee, C. Correlation between microbial community structure and biofouling in a laboratory scale membrane bioreactor with synthetic wastewater. *Desalination* **2012**, *287*, 209–215. [CrossRef]

41. Rodriguez-Sanchez, A.; Leyva-Diaz, J.C.; Poyatos, J.M.; Gonzalez-Lopez, J. Influent salinity conditions affect the bacterial communities of biofouling in hybrid MBBR-MBR systems. *J. Water Process Eng.* **2018**, in press. [CrossRef]

42. Jacob, J.H.; Irshaid, F.I. Biochemical and molecular taxonomy of a mild halophilic strain of citrobacter isolated from hypersaline environment. *Res. J. Microbiol.* **2012**, *7*, 219–226. [CrossRef]

43. Guo, W.; Ma, H.; Li, F.; Jin, Z.; Li, J.; Ma, F.; Wang, C. *Citrobacter* sp. strain GW-M mediates the coexistence of carbonate minerals with various morphologies. *Geomicrobiol. J.* **2013**, *30*, 749–757. [CrossRef]

44. Rivadeneyra, A.; Gonzalez-Martinez, A.; Portela, G.R.; Martin-Ramos, D.; Gonzalez-Lopez, J.; Rivadeneyra, M.A. Biomineralisation of carbonate and sulphate by the halophilic bacterium Halomonas maura at different manganese concentrations. *Extremophiles* **2017**, *21*, 1049–1056. [CrossRef] [PubMed]

45. Krishnapriya, S.; Venkatesh Babu, D.L.; Arulra, G.P. Isolation and identification of bacteria to improve the strength of concrete. *Microbiol. Res.* **2015**, *174*, 48–55. [CrossRef] [PubMed]