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Composition and Molecular Identification of Bacterial Community in Seawater Desalination Plants

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Abstract

Biofouling is an important problem for reverse osmosis (RO) membrane manufacturers. Bacteria are mainly involved in generating fouling and obturating RO membranes. Insights into biofilm bacteria composition could help prevent biofouling, reduce the cost of using RO-fouling membranes and guarantee safe water. Culture-dependent and independent techniques were then performed in order to identify bacteria associated with RO membranes. Bacteria cultures described the presence of six pure colonies, four of which were identified through API testing. Based on 16s rRNA gene analysis, a predominant bacterium was identified and annotated as *Sphingomonas sp.* The 16s rRNA gene clone library, on the other hand, showed that the bacterium, *Pseudomonas marincola*, accounted for nearly 30% of the clone library, while the rest of bacteria were chimeras (62%) and non-representative species (3%). In conclusion, culture-dependent and independent approaches showed that two dominant bacteria were commonly observed in RO desalination membranes.

Keywords

Biofouling, Bacteria, 16s rRNA, Reverse Osmosis Membrane, *Pseudomonas sp.*, *Sphingomonas sp.*

1. Introduction

Sea water desalination is an increasing practice all over the world. Urban populations that live close to coastal areas can acquire significant quantities of fresh

water through desalination [1]. Reverse osmosis (RO) membranes perform an effective desalination method in terms of permeability, packing density, and fouling control [1] [2] [3]. Conversely, membrane fouling reduces membrane lifetime and increases cost-effective maintenance. In particular, organic fouling could result in water flux decline through RO membranes so complex structures are formed by dissolved organic matters in combination with other substances [4] [5] [6]. Unlike inorganic fouling caused by salt precipitation, organic fouling consists of marine organisms and their metabolic residues such as extracellular polysaccharides, proteins and lipids. Bacteria cells are enclosed in self-produced extracellular polymeric substances that adhere the bacteria to osmosis membranes [7] [8] [9].

Bacteria fouling is difficult to eradicate with pre-treatment methods, which has huge implications on operational costs. Several approaches based in dissecting fouled-RO membranes have given greater understanding of the specific origin and composition of biofilms.

Bacteria analyses of RO membranes have identified bacteria from phyla β-proteobacteria, γ-proteobacteria and α-proteobacteria, with representative genus of *Sphingomonas*, *Pseudomonas* and *Acidovorax* [10] [11]. This has allowed science to discover the types of bacteria that adhere, grow and participate in forming the biofilm on RO membranes. Biochemical analysis of fouled-RO membranes has also described conspicuous information on bacteria communities, although only between 0.01% and 3% of the population in natural environments has been described.

Notwithstanding the above, molecular identification based on 16s rRNA gene and the specific genes that encode both fouling and anti-fouling proteins can also reveal important information and allow for the development of biotechnological applications in the future. We hypothesised that appropriate identification of the dominant bacteria from biofouling, along with the study of gene behaviour has to improve RO-membranes designs in order to prevent biofouling. The objective of this work was to molecularly characterise bacteria associated with several kinds of RO membranes with culture-dependent and culture-independent techniques, based on region 16s rRNA.

2. Materials and Methods

2.1. Bacteria Detachment Procedure

Four reverse osmosis membranes were employed for the experiments (Hydranautics SCW4 plus, Dow Filmtec SW30HR LE-400, Nano H_2O Qfx SW 365 ES and Nitto SWC4+). Each membrane was removed from the pressure vessel once it completed its design life cycle at the desalination plant located on the east coast of Gran Canaria (Canary Islands, Spain). The main characteristics of the membrane are shown in **Table 1**.

The removed RO membrane was taken apart and opened in the laboratory. The membrane was then laid out and cut in 12 × 12 cm (approx. 8 pieces per

Table 1. Main characteristics of reverse osmosis membranes used in this study.

Membrane Type	Permeate Flow (m^3d^{-1})	Salt Rejection (%) Nominal Minimum	Configuration	Membrane Polymer	Nominal Membrane Area: (ft^2)	Reference
SCW4 plus	24.6	99.8 99.7	Spiral Wound	Composite Polyamide	400	Hydranautics. Nitto Group Co. Teaneck. NJ (USA)
SW30HR LE-400	28	99.80 99.95	Spiral Wound	Polyamide thin-film composite	400	Dow Filmtec Texas (USA)
Qfx SW 365 ES	47	99.75 99.70	8-inch Spiral Wound	Thin-film composite	365	NanoH_2O Inc. Los Angeles, CA (USA)
SWC4+	24.5	99.8 99.7	Spiral Wound	Composite polyamide	400	Nitto Group Co. Teaneck, NJ (USA)

membrane) to identify and characterise the bacteria with a 16s rRNA-molecular marker. These pieces were identified according to the membrane manufacturer and placed into beaker flask containing sterile phosphate buffered saline (PBS, pH 7.5).

Bacteria adhered to pieces of RO membranes were detached using ultrasounds bath, orbital shaking and scraping when required. Ultrasound bath was carried out for 1 min × 5 times each, at room temperature. Then orbital shaking was performed overnight at 23˚C. The pieces of reverse osmosis membranes were always submerged in phosphate buffered saline.

Two approaches were used for analysing the bacteria, biochemical identification (culture-dependent) and molecular identification (16s rRNA gene, culture-independent). In addition, pure bacteria cultures were also confirmed through 16s rRNA identification.

2.2. Bacteria Biochemical Identification

De-attached bacteria aliquots (200 µl) were plated in four different culture media. These were R2A Agar, seawater supplemented with agar, tryptone yeast agar prepared with seawater and marine agar. All the bacteria plates were then cultivated at 22˚C and all media and reagents were purchased from Scharlab (Barcelona, Spain).

Bacteria colonies with similar morphologies in terms of shape and colour were isolated from each plate and grown successively to attain the pure culture separately. The pure bacteria cultures were characterised with API 20 NE (BioMerieux, Craponne, France) to identify non-fastidious and non-enteric Gram-negative rods, combining 8 conventional tests and 12 assimilation tests. API 20 NE test were performed according to the instructions of the supplier. In addition, three independent biochemical reactions were performed: Gram staining, catalase and oxidase reagent strips (Scharlab, Barcelona, Spain).

2.3. Bacteria Molecular Identification

In order to characterise the bacteria molecularly, environmental DNA from de-

tached bacteria in the PBS solution was obtained from centrifugation at 15,000 g for 15 min. DNA was also obtained from pure-colonies from bacteria colonies isolated individually in different culture media as previously described. When bacteria biomass needed to be increased, pure colonies were cultivated in sterile PBS solution containing tryptone (10 $g \cdot l^{-1}$) and yeast extract (5 $g \cdot l^{-1}$) at 23˚C for 24 h. The culture was then centrifuged under the experimental conditions described above (*i.e.* 15,000 g for 15 min) and the resulting pellet was taken as template DNA.

DNA extraction was performed following the Murray and Thompson [12] procedure with modifications. DNA from each of the pure colonies and from detached bacteria pellets (sediments) were separately isolated. This way, the bacteria were homogenised in liquid nitrogen and then incubated in 800 µl of isolation solution containing 100 mM Tris-HCl (pH 8.2), 4 M NaCl, 20 mM EDTA, CTAB (2%, w/v), PVPP (0.1%, w/v), SDS (0.1%, w/v) and mercaptoethanol (2%) in a water bath at 65˚C for 1 h. A volume of chloroform was then added: isoamyl alcohol solution (24:1 v/v) was added and the samples were gently mixed by inversion at intervals of 20 s. The mixture was then centrifuged for 10 min at 3000 rpm in a Beckman Coulter Allegra X-22R centrifuge (Beckman Coulter Inc. Brea, CA USA). Successive washings with chloroform: isoamyl alcohol (24:1 v/v) solution were performed. The supernatant was then placed in a fresh tube and an equal volume of n-propanol (−20˚C) was added, mixed gently and centrifuged at 13,000 g for 30 min. The resulting pellet, containing DNA was washed with ethanol (80%, v/v, molecular grade), dried and suspended in sterile deionised water. DNA yield was assessed using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). All samples were in triplicate. Purity DNA was valued by smear absence migrating on a 0.8% (w/v) agarose gel.

After several attempts with various 16s rRNA gene primer designs, DNA (90 - 95 ng) was amplified using oligonucleotide pairs, 16s rRNA-F as a forward primer, and 16s rRNA-R as a reverse primer (**Table 2**).

Amplification was performed in a GenAmp 2400 thermal cycler (PerkinElmer Inc., USA) with 30 cycles consisting at 95˚C for 1 min, 55˚C for 1 min, and 72˚C for 1.5 min, followed by a final extension step of 5 min at 72˚C. Each PCR reaction mixture contained 0.5 U Takara Ex Taq DNA polymerase (TaKaRa Shuzo Co., Shiga, Japan), dNTP, 2.5 mM, Takara Ex Taq PCR buffer with $MgCl_2$, 10 µl, forward and reverse primers, 10 µM each and DNA template.

PCR products were visualised by agarose gel electrophoresis. In addition, amplification products ranging from 800 to 1200 bp were obtained and purified using the QIAEX agarose gel extraction kit (Qiagen Inc., Hilden, Germany). The fragments were then ligated to the pGEM-T-easy cloning vector (Promega, Wisconsin, USA) and cloned in JM109 cells according to the manufacturer's instructions (Promega). Plasmids were isolated using a plasmid purification kit (Qiagen Inc.). The insert in the plasmid was checked by PCR using primers M13F and M13R (Promega). The insert was then sequenced on both strands

Table 2. Sequences of the forward (F) and reverse (R) primers, for 16s rRNA gene. Sequences were retrieved from [13] [14].

Gene	Primer name	Sequence (5'-3')
16s rRNA	Forward	TTCGGAATAACAGTTG
	Reverse	CGGCTGGATCTAAGGA
	Forward	GAGTTTGATCCTGGCTCAG
	Reverse	ACG GHT ACC TTG TTA CGA CTT
	Forward	AGAGTTTGATCMTGGCTCAG
	Reverse	TACGGYTACCTTGTTACGACTT
	Forward	CCAGCAGCGCTAATACG
	Reverse	TACCAGGGTATCTAATCC

using an ABI-310 DNA automated sequencer (Applied Biosystems, Foster City, CA, USA) and BigDye Terminator v3.1. Nucleotide sequences were submitted to NCBI GenBank BLAST search and identified through similarity values. Alignment of 16s rRNA sequences was performed with ClustalX v.1.7 [15] using the default settings and was further refined by visual inspection. The alignment output was used to generate a phylogenetic tree based on the Maximum Likelihood method and General Time Reversibility model [16] as implemented in MEGA X [17]. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed [18]. Branches corresponding to partitions reproduced in less than 50% of the bootstrap replicates collapse. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [18]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences between sites (2 categories (+G, parameter = 0.1000)).

3. Results and Discussion

The microbial community of biofouling in RO membranes is barely known since many papers are based on bacteria which are easily cultivable on a nutrient rich artificial medium. As a benchmark, this paper focuses on isolates using approaches based on culture-dependent and culture-independent techniques through the clone library. Differences between the four reverse osmosis membranes analysed were not observed.

Although bacteria were able to grow in all the culture media—namely R2A Agar, seawater supplemented with agar, tryptone yeast agar prepared with seawater, and marine agar-, R2A Agar media showed the highest recovery of bacteria colonies and was used to differentiate bacteria. Thus, six different colonies were characterised and selected in order to obtain pure culture (**Table 3**).

Table 3. Pure colonies (colony number from 1 to 6) cultured on R2A Agar and distinctive morphological characteristics.

Colony number	Morphological Characteristics
1	Orange
2	White uniform
3	White with expansive growth
4	Transparent
5	Beige
6	Yellow

Biochemical tests performed with API 20 NE are shown in **Table 4**. Substrate assimilations were read after 24 and 48 h. The results were interpreted after 48 h using the identification software from the BioMérieux web site [19]. When required, API tests were carried out two or three times to corroborate ambiguous sugar results that were inconclusive at 42˚C. The results agreed with those previously reported in bacteria from sugar-starved habitats that were able to develop alternative metabolic pathways for sugars [20]. The API results also only showed gram-negative bacteria colonies (**Table 4**).

Although it was easy to grow the bacteria, the presence of gram-negative bacteria suggested that the natural bacteria communities were not fully represented. Hence, this work was also supported by culture-independent techniques. Khambhaty and Plumb [21] reported that a culture-based approach favoured the presence of bacteria such as proteobacteria over other bacteria groups. Moreover, over 99% of bacteria in seawater reverse osmosis membranes cannot be cultivated on nutrient rich artificial media [22].

Moreover, the API test allowed us to identify four out of six bacteria colonies at species level based on a >90% confidence level reported by the manufacturer (**Table 5**).

As **Table 5** shows, the bacteria species identified were *Vibrio metschnikovii*, *Aeromonas salmonicida ssp. salmonicida* w., *Brevundimonas vesicularis* and *Sphingomonas paucimobilis*. These biofouling bacteria were reported on the four RO membranes analysed, indicating that they probably adapted to attaching on RO membrane surfaces as they grew on different types of membranes. Although all these species were recognised as marine species, *Sphingomonas sp.* also constituted the dominant bacteria colonies. *Sphingomonas* has been described as the dominant bacteria on RO membranes of desalination plants and in water purification processes, as related with the formation of biofilms by this bacterium [11]. *Sphingomonas* also facilitates the adherence of other bacteria and encourages the maturing of the biofilm [23]. Thus, these results open the door to investigating bacteria communities involved in biofouling and their role in triggering a biofilm network.

Several *Sphingomonas* colonies were molecularly identified using the 16s rRNA gene. Using maximum likelihood, the analysis recovered a higher resolution

Table 4. Biochemical test from API 20 NE (BioMerieux, Craponne, France) in six pure bacteria colonies.

API test	Bacteria Colonies					
	1	2	3	4	5	6
Gram	–	–	–	–	–	–
Catalase	–	+	+	–	+	+
NO3	–	–	+	–	–	–
TRP	–	–	–	–	–	–
GLU	–	–	–	–	–	–
ADH	–	–	–	–	–	–
URE	–	–	–	–	–	–
ESC	–	–	+	+	–	+
GEL	+	–	+	+	+	–
PNPG	+	–	–	–	–	+
GLU	+	+	+	–	+	+
ARA	–	–	–	–	–	–
MNE	–	–	–	–	–	–
MAN	+	–	–	–	+	–
NAG	+	+	+	+	+	–
MAL	+	+	+	+	+	+
GNT	+	+	+	–	+	–
CAP	–	–	–	–	–	–
ADI	–	–	–	–	–	–
MLT	–	–	+	–	–	+
CIT	–	–	–	–	–	–
PAC	–	–	–	–	–	–
Oxidase	–	+	+	+	+	+

Table 5. Taxonomical identification, profile and confidence level, according to API 20 NE, corresponding to 6 pure colonies.

Colony number	Genus and Specie assignation	Profile	Confidence level %
1	*Vibrio metschnikovii*	Very good	99.9
2	–	Low discrimination	–
3	*Aeromonas salmonicida ssp salmonicida*	Good	97.6
4	*Brevundimonas vesicularis*	Acceptable	86.5
5	–	Unacceptable	–
6	*Sphingomonas paucimobilis*	Good	96.5

Table 6. Sequences of 16s rRNA-gene sequences corresponding to diverse *Sphingomonas* strains.

Species and strain	Accession No.
Proteobacteria phylum	
α-Proteobacteria	
Sphingomonadaceae family, Sphingomonas genus	
Sphingomonas abaci C42	AJ575817
Sphingomonas abaci C42	NR_042192
Sphingomonas abaci SS1-08	KU341393
Sphingomonas adhaesiva IFO 15099	NR_043391
Sphingomonas adhaesiva BPM19	MF289214
Sphingomonas aerolata R-36940	FR691420
Sphingomonas aerolata 1111TES25Y1	LN774415
Sphingomonas aquatilis MPR 1	KX110354
Sphingomonas aquatilis S7	KF542913
Sphingomonas asaccharolytica IFO 15499-T	Y09639
Sphingomonas asaccharolytica Gsoil 130	KY078832
Sphingomonas aurantiaca MA101b	AJ429236
Sphingomonas aurantiaca MA306a	AJ429237
Sphingomonas azotifigens NBRC 15497	AB217471
Sphingomonas azotifigens NBRC 15497	AB680881
Sphingomonas desiccabilis CP1D	NR_042372
Sphingomonas desiccabilis CP1DT	AJ871435
Sphingomonas echinoides S32312	AB649019
Sphingomonas echinoides NRRL B-3126	MG745876
Sphingomonas dokdonensis DS-4	NR_043612
Sphingomonas dokdonensis DS-4	DQ178975
Sphingomonas mali S32423	AB649020
Sphingomonas mali GM289	AB740933
Sphingomonas molluscorum An 18 (KMM 3882)	AB248285
Sphingomonas molluscorum EP2	MG778708
Sphingomonas panni T9BP11	JF459953
Sphingomonas panni L8-752	JQ659481
Sphingomonas panni T9BR13	JF459952
Sphingomonas parapaucimobilis JCM 7510	NR_115615
Sphingomonas parapaucimobilis JCM 7510T	D84525
Sphingomonas paucimobilis BN 2056	MG438514
Sphingomonas paucimobilis MFC-pH7 01	KY434108
Sphingomonas pituitosa NBRC 102491	NR_114119
Sphingomonas pituitosa EDIV	AJ243751
Sphingomonas pruni NBRC 15498	NR_113760
Sphingomonas pruni IFO 15498	NR_026373
Sphingomonas soli T5-04	AB166883
Sphingomonas soli NBRC 100801	AB681244
Sphingomonas wittichii RW1	NR_027525
Sphingomonas wittichii HJX9	KP979540
Sphingomonas sp. MAH-20	MH368767
Other Sphingomonadaceae genera	
Blastomonas aquatica PE 4-5	KJ528316

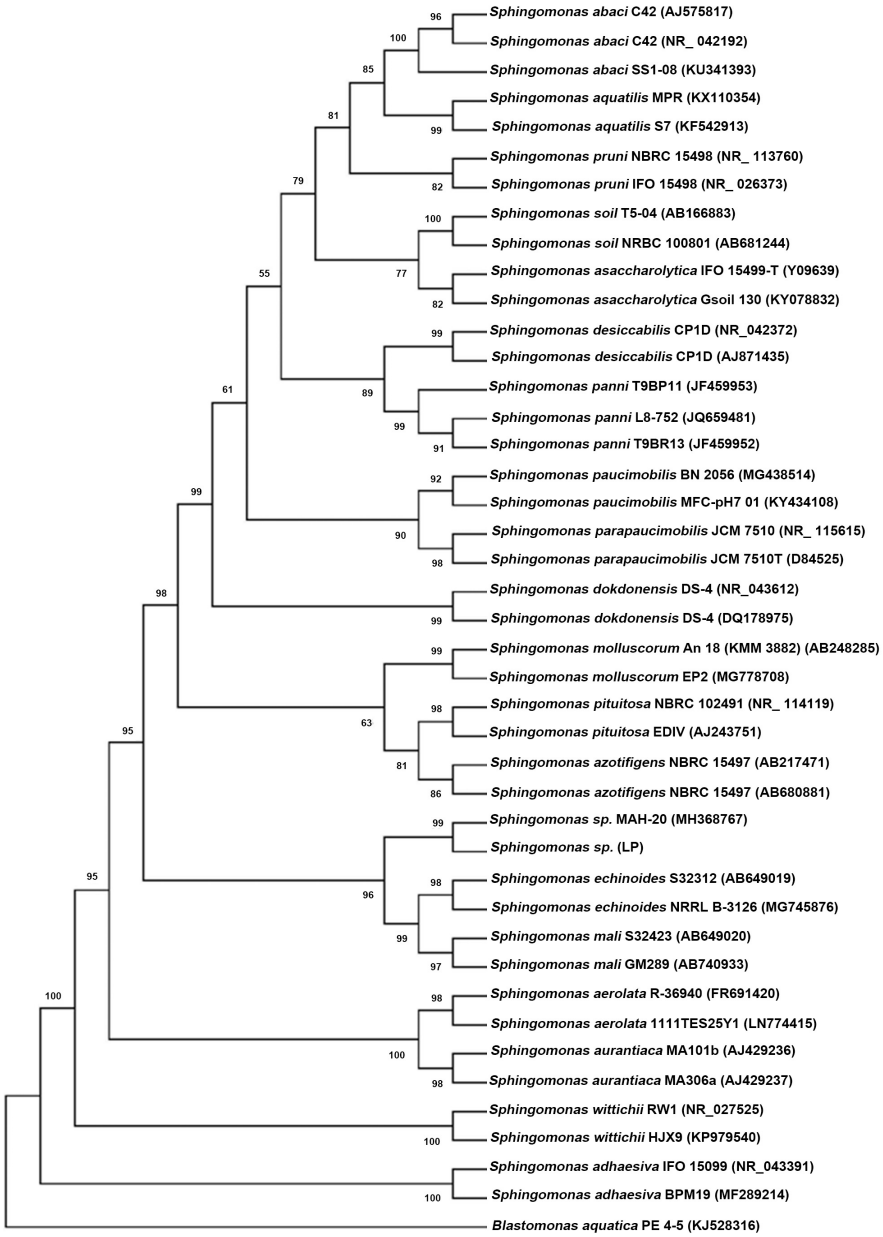

Figure 1. Maximum likelihood (ML) tree of *Sphingomonas sp*. inferred from 16s rRNA sequence calculated using general time reversible model plus gamma distribution (2 categories (+G, parameter = 0.0500)). Number in node represents values referring to sequence similarity. Bootstrap test was performed with 1000 replicates. LP in brackets indicates sequences analysed in the present study.

and grouped 16s rRNA *Sphingomonas* with another 43 16s rRNA gene sequences annotated as belonging to this genus (**Table 6**).

There were a total of 1023 positions in the final dataset. The phylogenetic analysis positioned the *Sphingomonas* sequences, from colonies determined biochemically, within the *Shingomonas sp*. genus. This result (**Figure 1**) demonstrated that the *Sphingomonas* obtained in this study could be regarded as representing a species within the genus.

Table 7. Sequences of 16s rRNA-gene sequences corresponding to diverse *Pseudomonas* strains.

Species and strain	Accession No.
Proteobacteria phylum	
γ-Proteobacteria	
Pseudomonadaceae family, *Pseudomonas* genus	
Pseudomonas aeruginosa H8	MG706125
Pseudomonas aeruginosa ACa02	KJ754135
Pseudomonas aeruginosa B2	KC633284
Pseudomonas argentinensis MSS-10	KM280652
Pseudomonas argentinensis AL243	MG819449
Pseudomonas argentinensis FPBBIH7	KU605764
Pseudomonas cuatrocienegasensis SR7-86	LN995508
Pseudomonas cuatrocienegasensis SR7-79	LN995501
Pseudomonas indica MBK3	MF682348
Pseudomonas indica VITPADJ5	KU598847
Pseudomonas indica NRCNA	MH917935
Pseudomonas indica NBRC	NR_114196
Pseudomonas marincola KMM 3042	NR_041592
Pseudomonas marincola K-W14	JQ799067
Pseudomonas nitroreducens R5-791	JQ659791
Pseudomonas nitroreducens R5-760-1	JQ659788
Pseudomonas nitroreducens R5-758-1	JQ659785
Pseudomonas nitroreducens R1-348	JQ659567
Pseudomonas synxantha X3-5-1	MK120107
Pseudomonas synxantha KGGI14	MH079449
Pseudomonas synxantha UCM B-399	MF196188
Pseudomonas synxantha IAM 12356	NR_043425
Other Pseudomonadaceae genera	
Azomonas agilis NBRC 102607	NR_114164

On the other hand, sequencing of 16s rRNA gene amplification fragments, assumed as operational taxonomic units (OTU) from independent cultures, revealed forty 16s rRNA gene fragments which were identified. Thirty percent of the total number of amplification fragments (total number of OTU, 40) was related to *Pseudomonas* species and 3 OTUs to *Sphingomonas*. The dominance of *Pseudomonas* may be explained by the association of these organisms with submerged surfaces. The remaining sequences were unrelated to other known bacteria and these were identified as non-representative. A total of 25 OTUs were detected as possible chimeras and were excluded from analysis.

Evolutionary analysis using the Maximum Likelihood method involved 24 nucleotide sequences (Table 7) with a total of 687 positions in the final dataset.

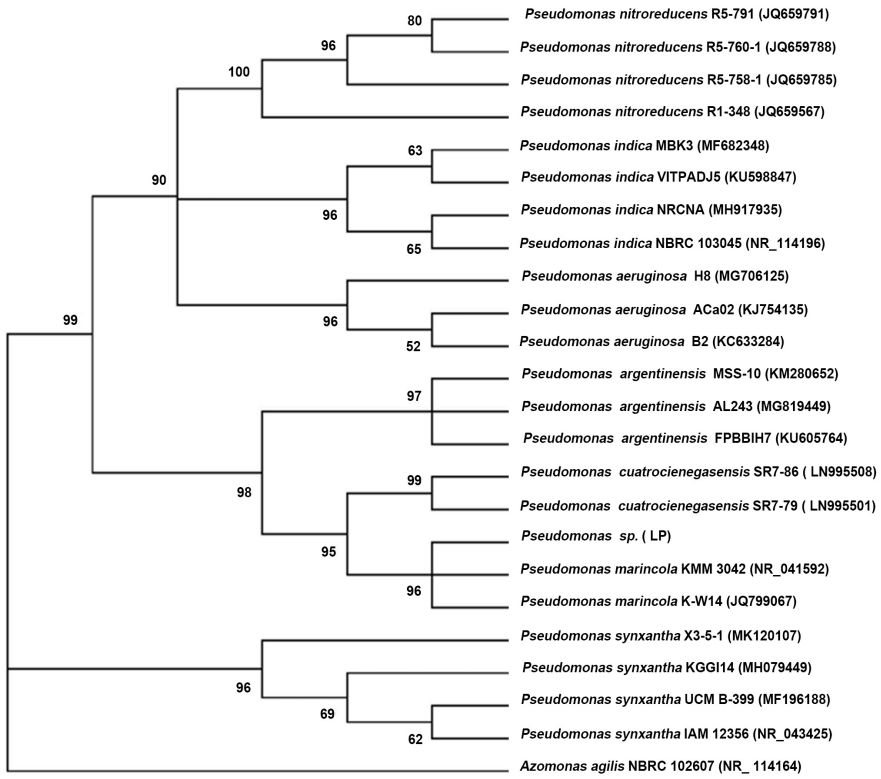

Figure 2. Maximum likelihood (ML) tree of *Pseudomonas sp.* inferred from 16s rRNA sequence calculated using general time reversible model plus gamma distribution (2 categories (+G, parameter = 0.1000)). Number in node represents values referring to sequence similarity. Bootstrap test was performed with 1000 replicates. LP in brackets indicates sequences analysed in the present study.

The phylogenetic analysis positioned the *Pseudomonas* sequences in a clade within *Pseudomonas marincola* (**Figure 2**). *Pseudomonas marincola* has been recently annotated as a marine species [22].

All in all, assuming the criterion for differentiating bacteria with a 16s rRNA gene sequence similarity value of over 95% [24], the phylogenetic trees of *Sphingomonas sp.* and *Pseudomonas marincola* were consistent with the molecular characterisation and their affiliations to the respective genus.

From an application point of view, it is worth mentioning that *Sphingomonas* and *Pseudomonas* have been described as containing an open reading frame coding for enzymes that are necessary for the initial stages of biofilm development [13]. Therefore, these molecular identifications open the door to studying the gene expression levels that encode the attachment protein and the biofouling potential of these bacteria. Gene expression levels of fouling and antifouling proteins under different experimental conditions (*i.e.* high-pressure water, permeate flow) can help to: 1) indicate a functional inclination to form biofilm on a reverse osmosis membrane, 2) discover how the membrane surface is colonised, and 3) determine how extracellular polysaccharides can help to initiate biofilm formation.

4. Conclusion

In conclusion, *Sphingomonas sp.* and *Pseudomonas sp.* assigned as *P. marincola*, have been identified on fouled marine reverse osmosis membranes. Culture-dependent and culture-independent approaches (clone library) showed that although the bacteria communities were not all identical, two dominant bacteria were commonly observed on the four RO membranes analysed.

Author's Contributions

P.G.J. conceived, designed and wrote the manuscript. M.C.A. conducted the phylogeny analysis. C.E.P and I.L.A. carried out microbiological assays. J.A.H.M. and J.R.B.R. analysed microbiological data. All the authors read and approved the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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