

# Composition and Molecular Identification of Bacterial Community in Seawater Desalination Plants

Pilar Garcia-Jimenez<sup>1\*</sup> , Marina Carrasco-Acosta<sup>1</sup>, Carlos Enrique Payá<sup>1</sup>, Irina Alemán López<sup>1</sup>, Juana Rosa Betancort Rodríguez<sup>2</sup>, José Alberto Herrera Melián<sup>3</sup>

<sup>1</sup>Department of Biology, Universidad de Las Palmas de Gran Canaria, Campus de Tafira, Las Palmas, Spain

<sup>2</sup>Department of Water, Instituto Tecnológico de Canarias, Playa de Pozo Izquierdo, Las Palmas, Spain

<sup>3</sup>Department of Chemistry, Universidad de Las Palmas de Gran Canaria, Campus de Tafira, Las Palmas, Spain

Email: \*pilar.garcia@ulpgc.es

**How to cite this paper:** Garcia-Jimenez, P., Carrasco-Acosta, M., Payá, C.E., López, I.A., Rodríguez, J.R.B. and Melián, J.A.H. (2019) Composition and Molecular Identification of Bacterial Community in Seawater Desalination Plants. *Advances in Microbiology*, 9, 863-876.

<https://doi.org/10.4236/aim.2019.910053>

**Received:** July 23, 2019

**Accepted:** October 18, 2019

**Published:** October 21, 2019

Copyright © 2019 by author(s) and Scientific Research Publishing Inc.

This work is licensed under the Creative Commons Attribution International License (CC BY 4.0).

<http://creativecommons.org/licenses/by/4.0/>



Open Access

## Abstract

Biofouling is an important problem for reverse osmosis (RO) membrane manufacturers. Bacteria are mainly involved in generating fouling and obtaining 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  $\beta$ -proteobacteria,  $\gamma$ -proteobacteria and  $\alpha$ -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<sub>2</sub>O 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 <sup>3</sup> d <sup>-1</sup> )	Salt Rejection (%) Nominal Minimum	Configuration	Membrane Polymer	Nominal Membrane Area: (ft <sup>2</sup> )	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 <sub>2</sub> O 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·l<sup>-1</sup>) and yeast extract (5 g·l<sup>-1</sup>) 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<sub>2</sub>, 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	AGAGTTTGATCMGGCTCAG
	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, Craaponne, France) in six pure bacteria colonies.

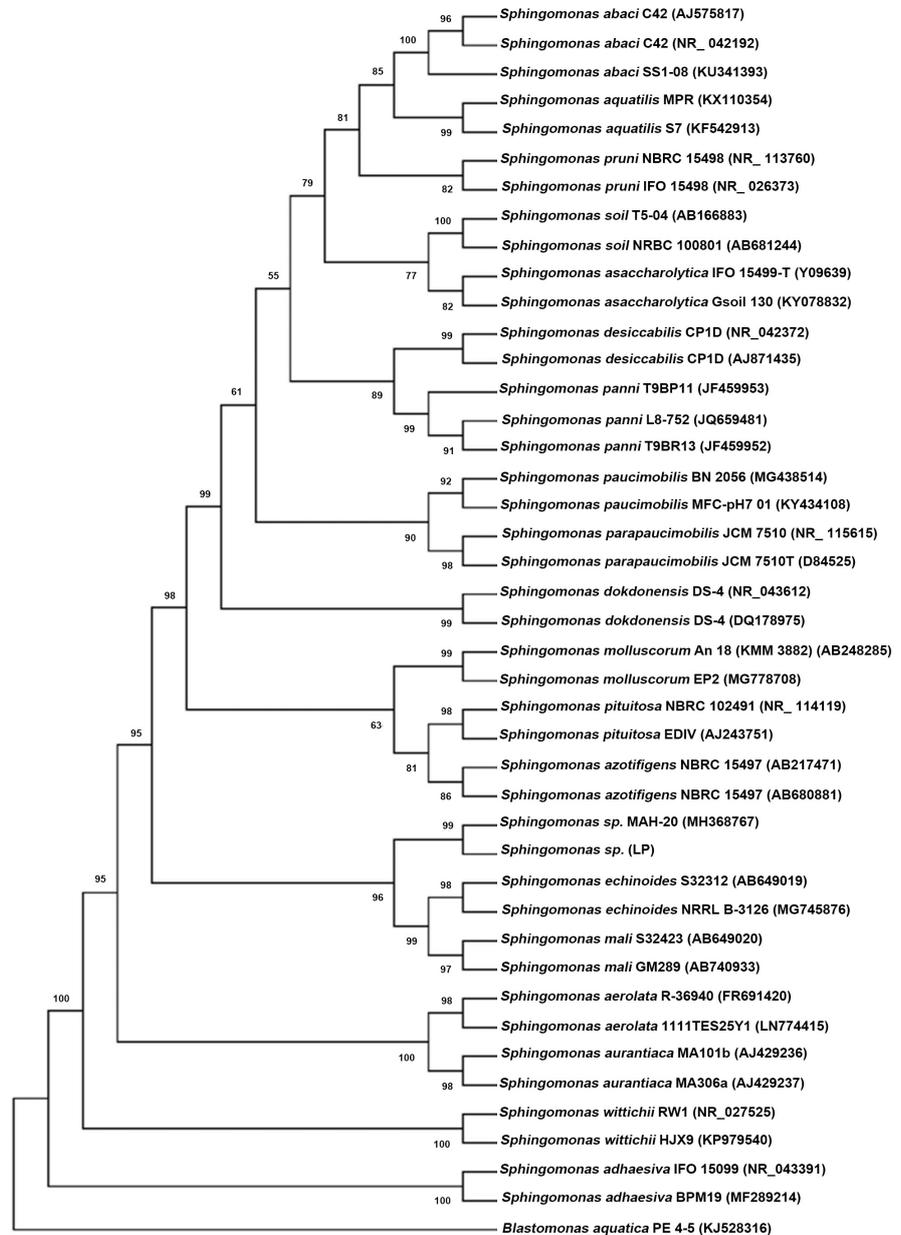
API test	Bacteria Colonies					
	1	2	3	4	5	6
Gram	-	-	-	-	-	-
Catalase	-	+	+	-	+	+
NO <sub>3</sub>	-	-	+	-	-	-
TRP	-	-	-	-	-	-
GLU	-	-	-	-	-	-
ADH	-	-	-	-	-	-
URE	-	-	-	-	-	-
ESC	-	-	+	+	-	+
GEL	+	-	+	+	+	-
PNPG	+	-	-	-	-	+
GLU	+	+	+	-	+	+
ARA	-	-	-	-	-	-
MNE	-	-	-	-	-	-
MAN	+	-	-	-	+	-
NAG	+	+	+	+	+	-
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	<i>Vibrio metschnikovii</i>	Very good	99.9
2	-	Low discrimination	-
3	<i>Aeromonas salmonicida ssp salmonicida</i>	Good	97.6
4	<i>Brevundimonas vesicularis</i>	Acceptable	86.5
5	-	Unacceptable	-
6	<i>Sphingomonas paucimobilis</i>	Good	96.5

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

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

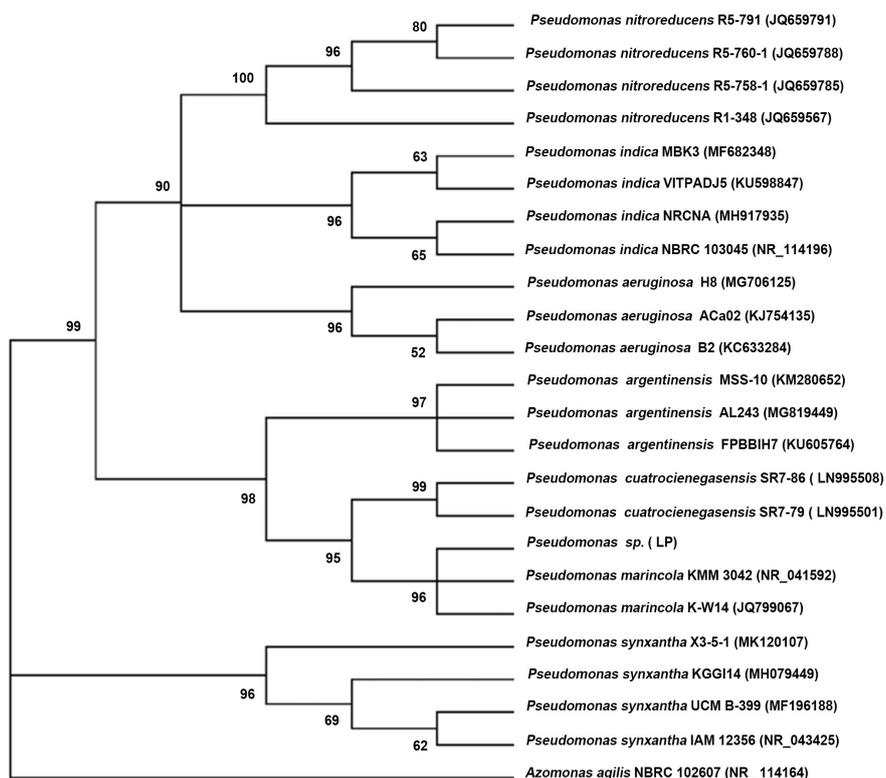
There were a total of 1023 positions in the final dataset. The phylogenetic analysis positioned the *Sphingomonas* sequences, from colonies determined biochemically, within the *Sphingomonas* sp. genus. This result (Figure 1) demonstrated that the *Sphingomonas* obtained in this study could be regarded 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.
<i>Proteobacteria</i> phylum	
<i>γ-Proteobacteria</i>	
<i>Pseudomonadaceae</i> family, <i>Pseudomonas</i> genus	
<i>Pseudomonas aeruginosa</i> H8	MG706125
<i>Pseudomonas aeruginosa</i> ACa02	KJ754135
<i>Pseudomonas aeruginosa</i> B2	KC633284
<i>Pseudomonas argentinensis</i> MSS-10	KM280652
<i>Pseudomonas argentinensis</i> AL243	MG819449
<i>Pseudomonas argentinensis</i> FPBBIH7	KU605764
<i>Pseudomonas cuatrocieneegasensis</i> SR7-86	LN995508
<i>Pseudomonas cuatrocieneegasensis</i> SR7-79	LN995501
<i>Pseudomonas indica</i> MBK3	MF682348
<i>Pseudomonas indica</i> VITPADJ5	KU598847
<i>Pseudomonas indica</i> NRCNA	MH917935
<i>Pseudomonas indica</i> NBRC	NR_114196
<i>Pseudomonas marincola</i> KMM 3042	NR_041592
<i>Pseudomonas marincola</i> K-W14	JQ799067
<i>Pseudomonas nitroreducens</i> R5-791	JQ659791
<i>Pseudomonas nitroreducens</i> R5-760-1	JQ659788
<i>Pseudomonas nitroreducens</i> R5-758-1	JQ659785
<i>Pseudomonas nitroreducens</i> R1-348	JQ659567
<i>Pseudomonas synxantha</i> X3-5-1	MK120107
<i>Pseudomonas synxantha</i> KGGI14	MH079449
<i>Pseudomonas synxantha</i> UCM B-399	MF196188
<i>Pseudomonas synxantha</i> IAM 12356	NR_043425
Other <i>Pseudomonadaceae</i> genera	
<i>Azomonas agilis</i> 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.

## Funding

This research was supported by the collaboration of Ministerio de Ciencia, Innovación y Universidades and from the Universidad de Las Palmas de Gran Canaria. (Grant CGL2016-78442-C2-2-R, GOBESP2017-04 ULPGC) to PGJ, and co-funded by ERDF funds, INTERREG MAC 2014-2020 programme, within the DESAL+ project (MAC/1.1a/094)". M.C.A was supported by a predoctoral fellowship granted by the University of Las Palmas de Gran Canaria (ULPGC2016).

## Conflicts of Interest

The authors declare no conflict of interest.

## References

- [1] Fritzmann, C., Löwenberg, J., Wintgens, T. and Melin, T. (2007) State-of-the-Art of Reverse Osmosis Desalination. *Desalination*, **216**, 1-76. <https://doi.org/10.1016/j.desal.2006.12.009>
- [2] Rautenbach, R. and Melin, T. (2003) Membranverfahren: Grundlagen der Modul- und Anlagenauslegung. 2nd Edition, Springer-Verlag, Berlin.
- [3] Belila, A., El-Chakhtoura, J., Otaibi, N., Muyzer, G., Gonzalez-Gil, G., Saikaly, P.E., van Loosdrecht, M.C.M. and Vrouwenvelder, J.S. (2016) Bacterial Community Structure and Variation in a Full-Scale Seawater Desalination Plant for Drinking Water Production. *Water Research*, **94**, 62-72. <https://doi.org/10.1016/j.watres.2016.02.039>
- [4] Naidu, G., Jeong, S., Kim, S.J., Kim, I.S. and Vigneswaran, S. (2014) Organic Fouling Behavior in Direct Contact Membrane Distillation. *Desalination*, **347**, 230-239. <https://doi.org/10.1016/j.desal.2014.05.045>
- [5] Shen, J. and Schafer, A.I. (2015) Factors Affecting Fluoride and Natural Organic Matter (NOM) Removal from Natural Waters in Tanzania by Nanofiltration/Reverse Osmosis. *Science of the Total Environment*, **527-528**, 520-529. <https://doi.org/10.1016/j.scitotenv.2015.04.037>
- [6] Ding, S., Yang, Y., Li, C., Huang, H. and Hou, L.A. (2016) The Effects of Organic Fouling on the Removal of Radionuclides by Reverse Osmosis Membranes. *Water Research*, **95**, 174-184. <https://doi.org/10.1016/j.watres.2016.03.028>

- [7] Frølund, B., Palmgren, R., Keiding, K. and Nielsen, P.H. (1996) Extraction of Extracellular Polymers from Activated Sludge Using a Cation Exchange Resin. *Water Research*, **30**, 1749-1758. [https://doi.org/10.1016/0043-1354\(95\)00323-1](https://doi.org/10.1016/0043-1354(95)00323-1)
- [8] Liu, H. and Fang, H.H. (2002) Extraction of Extracellular Polymeric Substances (EPS) of Sludges. *Journal of Biotechnology*, **95**, 249-256. [https://doi.org/10.1016/S0168-1656\(02\)00025-1](https://doi.org/10.1016/S0168-1656(02)00025-1)
- [9] Subbiahdoss, G., Zeng, G., Aslan, H., Ege Friis, J., Iruthayaraj, J., Zelikin, A.N. and Meyer, R.L. (2019) Antifouling Properties of Layer by Layer DNA Coatings. *Biofouling*, **35**, 75-88. <https://doi.org/10.1080/08927014.2019.1568417>
- [10] Ivnitsky, H., Katz, I., Minz, D., Volvovic, G., Shimoni, E., Kesselman, E. and Dosoretz, C.G. (2007) Bacterial Community Composition and Structure of Biofilms Developing on Nanofiltration Membranes Applied to Wastewater Treatment. *Water Research*, **41**, 3924-3935. <https://doi.org/10.1016/j.watres.2007.05.021>
- [11] Bereschenko, L.A., Heilig, G.H.J., Nederlof, M.M., van Loosdrecht, M.C.M., Stams, A.J.M. and Euverink, G.J.W. (2008) Molecular Characterization of the Bacterial Communities in the Different Compartments of a Full-Scale Reverse-Osmosis Water Purification Plant. *Applied and Environmental Microbiology*, **74**, 5297-5304. <https://doi.org/10.1128/AEM.00387-08>
- [12] Murray, M.G. and Thompson, W.F. (1980) Rapid Isolation of High Molecular Weight Plant DNA. *Nucleic Acids Research*, **8**, 4321-4325. <https://doi.org/10.1093/nar/8.19.4321>
- [13] Lee, J., Ren, X., Yu, H.W., Kim, S.J. and Kim, I.S. (2010) Membrane Biofouling of Seawater Reverse Osmosis Initiated by Sporogenic Bacillus Strain. *Environmental Engineering Research*, **15**, 141-147. <https://doi.org/10.4491/eer.2010.15.3.141>
- [14] Bernbom, N., Ng, Y.Y., Kjelleberg, S., Harder, T. and Gram, L. (2011) Marine Bacteria from Danish Coastal Waters Show Antifouling Activity against the Marine Fouling Bacterium *Pseudoalteromonas* sp. Strain S91 and Zoospores of the Green Alga *Ulva australis* Independent of Bacteriocidal Activity. *Applied and Environmental Microbiology*, **77**, 8557-8567. <https://doi.org/10.1128/AEM.06038-11>
- [15] Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: Improving the Sensitivity of Progressive Multiple Sequence Alignment through Sequence Weighting, Position-Specific Gap Penalties and Weight Matrix Choice. *Nucleic Acids Research*, **22**, 4673-4680. <https://doi.org/10.1093/nar/22.22.4673>
- [16] Nei, M. and Kumar, S. (2000) Molecular Evolution and Phylogenetics. Oxford University Press, New York.
- [17] Kumar, S., Stecher, G., Li, M., Knyaz, C. and Tamura, K. (2018) MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Molecular Biology and Evolution*, **35**, 1547-1549. <https://doi.org/10.1093/molbev/msy096>
- [18] Felsenstein, J. (1985) Confidence Limits on Phylogenies: An Approach Using the Bootstrap. *Evolution*, **39**, 783-791. <https://doi.org/10.1111/j.1558-5646.1985.tb00420.x>
- [19] Api Web, BioMérieux, Marcy-l'Étoile, France. <https://apiweb.biomerieux.com>
- [20] Bräsen, C., Esser, D., Rauch, B. and Siebers, B. (2014) Carbohydrate Metabolism in Archaea: Current Insights into Unusual Enzymes and Pathways and Their Regulation. *Microbiology and Molecular Biology Reviews*, **78**, 89-175. <https://doi.org/10.1128/MMBR.00041-13>
- [21] Khambhaty, Y. and Plumb, J. (2011) Characterization of Bacterial Population Associated with a Brackish Water Desalination Membrane. *Desalination*, **269**, 35-40.

<https://doi.org/10.1016/j.desal.2010.10.039>

- [22] Ferguson, R.L., Buckley, E.N. and Palumbo, A.V. (1984) Response of Marine Bacterioplankton to Differential Filtration and Confinement. *Applied and Environmental Microbiology*, **47**, 49-55.
- [23] Pollock, T.J. and Armentrout, R.W. (1999) Planktonic/Sessile Dimorphism of Polysaccharide-Encapsulated Sphingomonads. *Journal of Industrial Microbiology & Biotechnology*, **23**, 436-441. <https://doi.org/10.1038/sj.jim.2900710>
- [24] Stackebrandt, E. and Goebel, B.M. (1994) Taxonomic Note: A Place for DNA-DNA Reassociation and 16s rRNA Sequence Analysis in the Present Species Definition in Bacteriology. *International Journal of Systematic Bacteriology*, **44**, 846-849. <https://doi.org/10.1099/00207713-44-4-846>