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Salt pan brine water sulphated polysaccharides retrieved at pilot scale: ability to stimulate *in vitro* human macrophages and salmon head kidney cells

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ABSTRACT

Marine environments are the warehouse of a variety of novel bioactive compounds prone to be explored by food and feed industry. The growing interest in sulphated polysaccharides has led to the search for new sustainable sources, such as seawater. These compounds are naturally concentrated in salt pan brine water due to their evaporation by wind and sunlight. To take advantage of these sources, sulphated polysaccharides were concentrated from salt pan brine water using a scalable membrane ultrafiltration system with 30 and 100 kDa cut-off. This process allowed to concentrate ten times the polymeric material of brine water into 1.9 g/L, rendering a fluffy polysaccharide rich material after drying. It was mainly composed of 23 % (*w*/w) of uronic acids, 19 % of sulphate esters, and 34 % (w/w) of neutral sugars. This polymeric material has shown to stimulate *in vitro* both human macrophages and Atlantic salmon head kidney SHK-1 cells in a range of 6.25–50 μg/mL without toxicity, showing potential to be used in both human food and aquaculture feeding.

1. Introduction

Brine water is a by-product of traditional sea salt production. Along the process of salt production, with the evaporation of seawater and consequent concentration of dissolved compounds, there is a selective precipitation along the season and throughout the salt pan evaporation tanks. The cold water at the entrance of the salt pan goes slowly through the evaporation tanks, getting warm along the path. The crystallizer tanks, where occurs the final precipitation of NaCl [1], retain a high volume of brine water, rich in salt and highly sulphated polysaccharides [2]. The sources of these polysaccharides include a variety of microorganisms, seaweeds, other plants, and animals, widely distributed in marine environments [2]. Due to the potential immunomodulatory activities of brine water sulphated polysaccharides, observed by the stimulation of murine B cells [2], and the antiviral, anticoagulant, and antioxidant activities associated to most sulphated polysaccharides [3], it is worth to test the ability of brine water sulphated polysaccharides produced in a larger scale to stimulate other immune cells, both human and fish, and explore different applications.

The sulphated polysaccharides from salt pan brine water (200 mg/L) are the main component of polymeric material isolated by dialysis using membranes with 12 kDa cut-off [2]. In addition to sulphate groups

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(16–22 mol% of $-SO_3^{2}$), these polysaccharides are composed by uronic acids (UA, 35 mol%) and lower amounts of fucose, galactose, glucose, rhamnose, mannose, xylose, and arabinose (below 13 mol%) [2], which resembles the composition found in seawater and Atlantic Ocean salt polysaccharides [4]. Methylation analysis of salt pan brine water polysaccharides revealed that independently of the fractionation performed (graded ethanol precipitation, ion-exchange chromatography, and size-exclusion chromatography), all samples revealed a similar glycosidic linkage composition characteristic of sulphated polysaccharides, including rhamnans, fucans, mannans, xylomannans, glucuronomannans, galactans, glucans, and heteroglucans [2], also identified in sea salt from different regions in the Atlantic ocean [5], resultant from marine organisms such as seaweeds, microalgae, and invertebrates. Concerning the molecular weight distribution, it was described that half of the polysaccharides were spread through 120 to 400 kDa, allowing to infer also their size heterogeneity [2].

The heterogeneous composition found in brine water polymeric material also resembles carbohydrate composition found in exopolysaccharides from marine organisms and algae exudates [6,7], indicating that the polymeric material results from the concentration of compounds present in seawater. Notwithstanding, there is no information about the composition of brine water sulphated polysaccharides along the process of salt production.

In contrast to most marine sulphated and/or anionic polysaccharides that need extraction procedures and salting out fractionations, like fucoidans and alginates [7,8], brine water polysaccharides are readily solubilized in aqueous solutions. At a laboratorial scale, the dialysis process of brine water allows to remove the inorganic salts and obtain fractions rich in sulfated polysaccharides [2]. For scalability in food and biotechnological fields, where sustainability of water use is a requirement, tangential ultrafiltration is an alternative to dialysis. This is a faster process than dialysis that allows concentration, salt removal, and fractionation of biomacromolecules by size [9], including phenolic compounds [10], fucoidans [11], and galactomannans and arabinogalactans [12]. Polysaccharide molecular weight and charge significantly influence membrane fouling and material retention during ultrafiltration [13,14]. Depending on their availability as purified or raw materials, different biological properties, including immunostimulatory and pro-inflammatory ones can be obtained [13]. The parameters of ultrafiltration membranes, including pore size, material, and length of membrane surface area, and flow of ultrafiltration are among the parameters that influence the separation process. For these reasons, quantitatively comparisons of the different systems are not reliable [14] and there is a need to evaluate the reliability of obtaining bioactive salt pan polysaccharides by scaled-up processes.

In this work, brine water from traditional salt production ponds in Ria Formosa Natural Park, Portugal, were studied and processed in a pilot plant membrane system to obtain polymeric material concentrates. The potential of this material as immunostimulatory ingredient for food and feed was evaluated *in vitro* in immune cell lines, namely, human THP-1 derived macrophages and Atlantic salmon head kidney SHK-1 cell lines.

2. Material and methods

2.1. Salt pan water samples and materials

The water samples (5 L) were collected at the Necton, S.A. salt pan in Ria Formosa lagoon, Olhão, in the South Cost of Portugal (Atlantic Ocean) at three stages of salt production, start (13th July 2021), middle (27th August 2021), and end of the production season (10th September 2021), and in the different salt pan tanks: (1) entrance, (2) cold evaporator, (3) hot evaporator, and (4) crystallizer (Fig. 1). Brine water from the crystallizers at the end of 2020 (Brine 2020) and 2021 production (Brine 2021) were also collected. Both brine water samples were stored in intermediate bulk containers (IBC, 1000 L) up to one year before analysis and processing in the SANI membranes' system.

2.2. Isolation of polymeric material from brine water by dialysis membranes

The yield of polymeric material from each sample was obtained at analytical level by dialysis membranes (12 kDa cut-off), according to Fig. 2.a [2]. For each salt pan water sample, 3 replicas of 1 L were dialyzed and each retentate (polymeric material) was then frozen, freeze-dried, and kept in a desiccator until analysis.

2.3. Isolation of polymeric material from brine water by pilot-scale membranes

The polymeric material from Brine 2020 and Brine 2021 was isolated by a pilot-scale Vibro-I system from SANI Membranes® [15] equipped with three ultrafiltration membranes: (100 kDa LY-MAX, 30 kDa UH030, and 30 kDa NK-MAX) or three microfiltration membranes (0.2 μm Nadir MV020 T) (Fig. 2.**b**). The system has a tank of 80 L that allows to obtain 15–20 L of concentrated solutions, the minimum volume needed to circulate inside the system, and to recover the remaining volume as permeates. For the experiments of volumes higher than 80 L, 60–70 L were added stepwise when the volume inside the tank reached 15–20 L and the separation process continued in over tank mode, which

Fig. 1. Sampling points along Necton, S.A. salt pan tanks in Ria Formosa lagoon: **(1)** entrance, **(2)** cold evaporator, **(3)** hot evaporator, and **(4)** crystallizer. Image of Google Earth from 15th May 2021, at 37°01′28.18"N, 7°52′06.68"W coordinates.

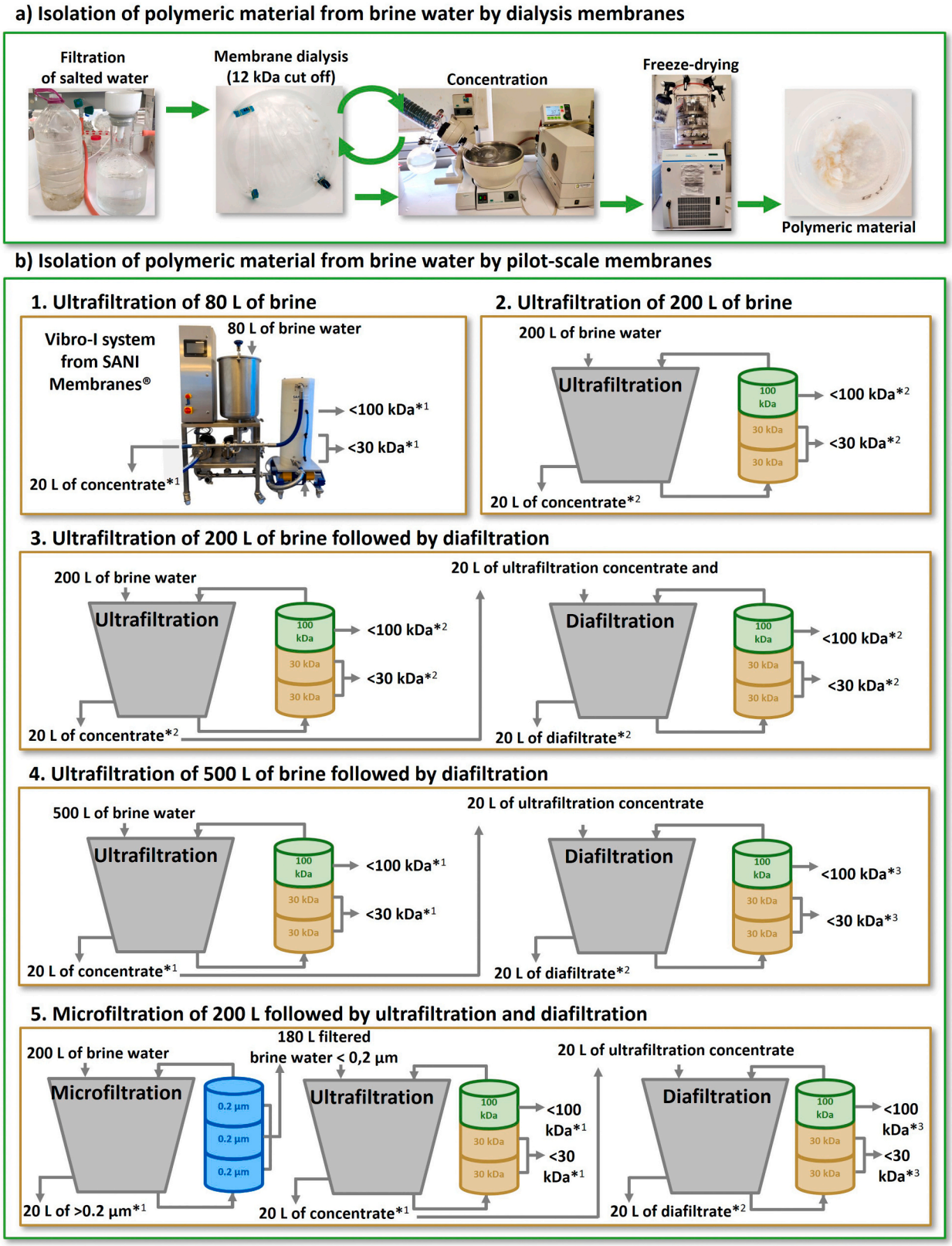

Fig. 2. Scheme of polymeric material isolation **a)** at analytical level by using dialysis membranes (12 kDa cut-off) and **b)** at pilot level by using SANI MEMBRANES system using ultrafiltration membranes with 30 kDa and 100 kDa cut-off and microfiltration membranes with 0.2 µm for the following experiments: **1.** Ultrafiltration of 80 L of brine; **2.** Ultrafiltration of 200 L; **3.** Ultrafiltration of 200 L followed by diafiltration; **4.** Ultrafiltration of 500 L followed by diafiltration; and **5.** Microfiltration of 200 L, followed by ultrafiltration and diafiltration. The way samples were collected is identified along the schemes: *1 Sampled at the end of the process. *2 Sampled along the process. *3 Not sampled.

is a batch mode where the media is gradually concentrated. Brine water components were obtained by five different experiments summarized as follows: **1.** Ultrafiltration of 80 L of brine; **2.** Ultrafiltration of 200 L; **3.** Ultrafiltration of 200 L followed by diafiltration; **4.** Ultrafiltration of 500 L followed by diafiltration; and **5.** Microfiltration of 200 L, followed by ultrafiltration and diafiltration (Fig. 2.b). In detail, in the Experiment 1 there was an ultrafiltration of 80 L of brine water added in one step to the tank, with the Vibro-I system working in continuous mode. In the Experiment 2 there was an ultrafiltration of 200 L of brine water added in 3 steps: 1 step of 80 L plus 2 steps of 60 L, with the Vibro-I system working in over the tank mode. This mode was used for the experiments that followed. In the Experiment 3 there was an ultrafiltration of 200 L of brine water added in 3 steps (1 step of 80 L plus 2 steps of 60 L). The 20 L of the ultrafiltration concentrate was then diafiltrated by addition of 70 L of tap water three times. A last diafiltration step was performed after removal of part of the diafiltrate and addition of 60 L of tap water to 10 L of diafiltrate. In the Experiment 4 there was an ultrafiltration of 500 L of brine water added in 8 steps (1 step of 80 L plus 7 steps of 60 L) and diafiltration of 20 L of ultrafiltration concentrate by addition of 60 L of tap water six times, followed by diafiltration of 20 L of diafiltrate by addition of 20 L of distilled water. In the Experiment 5 there was a microfiltration in 0.2 μm membranes of 200 L of brine water added in 3 steps (1 step of 80 L plus 2 steps of 60 L), followed by ultrafiltration of the 180 L of filtered brine added in 3 steps of 60 L. The 20 L of ultrafiltration concentrate obtained were diafiltrated by addition of 60 L of tap water five times, followed by diafiltration of 20 L of diafiltrate with 20 L of distilled water. The way the system works with ultrafiltration or microfiltration membranes is similar, except that after microfiltration, it was the permeate and not the concentrate that was used in the following steps. Concentrate and permeates were collected along the experiments at each step or at the end of the ultrafiltration or diafiltration and analysed for their salinity, macroelements, total carbohydrates, and amount of polymeric material by dialysis. The polymeric material of final concentrates/diafiltrates from each experiment was further characterized for their sulphate esters content, neutral sugars, and uronic acids.

2.4. Characterization of water samples and polymeric material

2.4.1. Carbohydrate analyses

Total carbohydrates of salt pan water samples and fractions of polymeric material obtained from brine water by pilot-scale SANI membranes were analysed according to phenol-concentrated sulphuric acid method [16]. The neutral sugars of polymeric material isolated after dialysis from salt pan water samples were analysed after 2 M trifluoroacetic acid hydrolysis for 1 h at 120 °C, acid evaporation, derivatisation to alditol acetates, and analysis by gas chromatography with flame ionization detector (GC-FID) [17,18]. To improve the hydrolysis of polysaccharides and the quantification of neutral sugars, the polymeric material was also hydrolysed with 2 M sulphuric acid for 2.5 h at 100 °C. Uronic acids (UA) were quantified by the 3-phenylphenol colorimetric method [19] and identified by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [17]. Unknown carbohydrates were calculated by the difference between total carbohydrates obtained by phenol-concentrated sulphuric acid method and the sum of neutral sugars and uronic acids.

2.4.2. Thermal gravimetric analysis (TGA)

Thermal gravimetric analysis (Seteram, setsys Ev 1750 ITGA) was performed to polymeric material stored at 25 °C with silica under oxygen atmosphere with a caudal of 200 mL/min. The temperatures ranged from room temperature (RT) to 600 °C at 10 °C/min. The moisture of polymeric material was determined by the weight loss between RT and 140 °C and the ashes content was determined by the remaining weight obtained after heating to 550 °C.

2.4.3. Elemental analysis

The elemental analysis was performed in a Truspec 630–200-200 elemental analyzer using 2 mg of each sample in duplicate. The operating temperature of combustion furnace was 1075 °C and the afterburner temperature was 850 °C. Thermal conductivity was used to detect the nitrogen and sulphate. Protein content was calculated through multiplication of %N by the nitrogen-to-protein conversion factor of 6.25 [2]. Sulphate ester residues were calculated through multiplication of %S by the conversion factor of 2.5.

2.4.4. Macroelement analysis

Quantification of macroelements (Na, Mg, K, Ca, and B) was performed by inductively coupled plasma optical emission spectroscopy (ICP-OES) in a HORIBA Jobin Yvon, model ACTIVA M. Samples were acidified prior quantification using HNO_3. Calibration curves were performed using six points, including 0.0 mg/L and multi-element calibration solutions were used for external calibration of ICP-OES. All standards solutions were prepared daily in HNO_3, a matrix that matches the sample to minimize matrix effects and for background correction. Calibration standards were analysed at regular intervals during analytical runs [20]. The wavelength used for the quantification of macroelements were Na 589.592 nm, Mg 279.800 nm, K 769.896 nm, Ca 422.673 nm, B 249.773 nm.

2.5. Principal component analysis (PCA)

Principal components analysis of salt pan water samples collected at the three stages of salt production and along salt pan tanks was applied to all components: moisture, ashes, protein, sulphate esters, unknown carbohydrates, uronic acids (UA), rhamnose (Rha), fucose (Fuc), arabinose (Ara), xylose (Xyl), mannose (Man), galactose (Gal), and glucose (Glc), using the values of μg/g of dry weight. The data matrix comprised 9 samples of polymeric material (3 repetitions) and 11 compounds, thus creating a matrix with 18 columns and 64 rows. The data matrix was analysed using MetaboAnalyst [21]. Data were normalized after auto scaling: mean-centring and division by the standard deviation of each variable.

2.6. In vitro immunostimulatory activity assays

2.6.1. Cell culture

THP-1 cells (American Type Culture Collection, ATCC, VA, USA) were maintained in RPMI 1640 medium, supplemented with 4 mM L-glutamine, 10 % foetal bovine serum (FBS), 1 % penicillin/streptomycin, 10 mM HEPES and 50 μM β-mercaptoethanol at 37 °C in a humidified atmosphere of 5 % CO_2. *Salmo salar* head kidney-1 (SHK-1) cells (European Collection of Authenticated Cell Cultures, ECACC) were cultured in Leibovitz L-15 medium supplemented with 15 % FBS and 1 % penicillin-streptomycin at 20 °C.

2.6.2. Cell viability

THP-1 cells were seeded in 96-well plates at a density of 1.0×10^5 cells/well. Phorbol 12-myristate 13-acetate (PMA, 50 nM) was added as a differentiation agent to obtain macrophages, as previously described [21,22]. After 24 h, this medium was discarded and replaced with fresh PMA-free medium for another 24 h period. Then, human macrophages were incubated with different concentrations of the polymeric material (6.25–50 μg/mL, experiment 3) in the presence and absence of polymyxin B (10 μg/mL) for 24 h. Additionally, SHK-1 cells were seeded in 96-well plates at a density of 6.0×10^4 cells/well for 24 h. Then, the cells were exposed to different concentrations of the polymeric material (12.5–50 μg/mL, experiment 3) with or without polymyxin B (10 μg/mL) for another 24 h period. After the exposure of cells to the polymeric materials, THP-1 and SHK-1 cell viability was determined by the ability of metabolically active cells to reduce MTT to formazan over the course of 2 h and 4 h, respectively. The formazan crystals were solubilized in

dimethyl sulphoxide and the absorbance was measured at 570 nm in a BioTek uQuant MXQ200 microplate reader. Results are expressed as a percentage of the respective control (medium, 100 % viability) and correspond to the mean ± standard error of the mean (SEM) of at least three independent experiments performed in triplicate.

2.6.3. Cytokines (IL-6 and TNF-α)

THP-1 monocytes were seeded in 96-well plates, differentiated into macrophages and treated, as described above. The concentrations of the pro-inflammatory cytokines IL-6 and TNF-α were determined in the supernatants of cell cultures by using human IL-6 and TNF-α duoSet ELISA kits, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Results correspond to the mean ± SEM of at least three independent experiments and are expressed in pg/mL. LPS O127: B8 at 0.1 and 1 μg/mL was used as positive control.

2.6.4. Intracellular ROS levels

The 2′,7′-dichlorofluorescein diacetate (DCFH-DA) diffuses into the cells and is deacetylated by esterases to a non-fluorescent DCFH, which is rapidly oxidized into a highly fluorescent 2′,7′-dichlorofluorescein (DCF) by several intracellular radicals. Therefore, the amount of ROS in the cell cytosol is directly correlated with fluorescence intensity. SHK-1 cells were seeded in 96-well black plates (6.0×10^4 cells/well) according to the above-mentioned conditions. After, cells were treated with different concentrations of polymeric material (12.5–50 μg/mL, experiment 3) in the presence and absence of polymyxin B (10 μg/mL) for 24 h. The cells were then washed with PBS and incubated with a solution of DCFH-DA (25 μM in PBS) for 45 min at 20 °C. The quantification of intracellular ROS was performed in a fluorescence microplate reader (Cytation™ 3, BioTek, Winooski, VT, USA) at 485/20 nm and 528/20 nm wavelengths for excitation and emission, respectively. Results were obtained as the percentage of increased fluorescence *vs* negative control (medium), and represent the mean ± SEM of at least three independent experiments performed in duplicate.

2.7. Statistical analysis

Statistical difference was analysed using ordinary One-Way ANOVA and Dunnett's multiple comparisons test, with a single pooled variance, comparing the mean of each column with the mean of a control column as indicated * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3. Results and discussion

3.1. Polymeric material composition of Ria Formosa brine water

At the start of the season of traditional salt production, the amount of polymeric material at the entrance of the salt pan (Fig. 1) accounted for 13 mg/L. The polymeric material contained 8 % of retained water, 13 % of ashes, and 27 % of protein (Table 1, start of season, column (1)). The amount of sulphated polysaccharides in the polymeric material, estimated as the sum of carbohydrates and sulphate esters, was 23 %. The amount of polymeric material increased in the first evaporator tanks to 52 mg/L and to 73 mg/L in the second evaporator tanks (Table 1, start of season, columns (2) and (3)), possibly due to lower volume of water in the second tanks. The content of retained water in the freeze-dried material increased to 11 % and 12 %, respectively, possibly due to the higher amount of salts present, estimated by the ashes (25–26 %) and sulphated polysaccharide content (41 %). On the contrary, protein decreased to 16 % and 15 %, possibly due to lower load of microorganisms [23].

At the middle of the season, water samples were collected in both first and second evaporator tanks and in the crystallizer tanks. The crystallizer tanks were only open after a period of concentration of seawater in the first and second evaporator tanks. In this middle part of the season, the amount of polymeric material in the first evaporator tanks decreased to 9 mg/L and to 36 mg/L in the second evaporator tanks (Table 1, middle of season, columns (2) and (3)). The decrease in polymeric material seems to reflect the increase of water stream along the salt pan through the evaporator tanks to the crystallizer tanks [1]. The freeze-dried material from the first evaporator tanks contained 12 % of retained water, 12 % of ashes, 29 % of protein, and 24 % of sulphated carbohydrates. The freeze-dried material from the second evaporator tanks contained a similar amount of retained water (11 %), twice the amount of ashes (22 %), a lower amount of protein (19 %), and a higher amount of sulphated polysaccharides (37 %). The composition of the polymeric material from the first evaporator tanks is similar to the composition of the material recovered at the entrance of the salt pan from the start of the season. Similarly, the composition of the polymeric material from the second evaporator tanks is similar to the composition

Table 1
- Polymeric material concentration and composition, recovered from brine water along salt production season (start, middle, and end) in salt pan tanks: (1) entrance, (2) cold evaporator, (3) hot evaporator, and (4) crystallizer.

Brine water	Start of season			Middle of season			End of season		
	(1)	(2)	(3)	(2)	(3)	(4)	(2)	(3)	(4)
Polymeric material (mg/L)	13 ± 1	52 ± 4	73 ± 9	9 ± 1	36 ± 4	133 ± 25	16 ± 1	58 ± 5	146 ± 12
Moisture (mg/g)	81 ± 4	110 ± 2	124 ± 2	119 ± 2	111 ± 2	123 ± 5	101 ± 3	106 ± 2	110 ± 1
Ashes (mg/g)	130 ± 2	245 ± 7	261 ± 7	115 ± 4	223 ± 6	253 ± 6	140 ± 3	248 ± 6	259 ± 7
Protein (mg/g)	265 ± 2	162 ± 5	153 ± 3	292 ± 2	192 ± 2	151 ± 2	319 ± 0	175 ± 3	164 ± 2
Sulphate esters (mg/g)	62 ± 6	76 ± 1	81 ± 3	34 ± 4	59 ± 1	65 ± 4	41 ± 2	69 ± 3	66 ± 4
Carbohydrates (mg/g)	166 ± 3	338 ± 3	326 ± 50	208 ± 17	315 ± 19	356 ± 48	146 ± 9	312 ± 16	359 ± 47
Uronic acids (mg/g)	64 ± 1	130 ± 14	125 ± 9	80 ± 7	121 ± 3	137 ± 11	56 ± 3	120 ± 7	138 ± 4
Neutral sugars (mg/g)	76 ± 19	131 ± 35	154 ± 38	65 ± 6	98 ± 8	121 ± 21	33 ± 19	76 ± 46	70 ± 42
Rha (mol%)	8 ± 4	5 ± 3	5 ± 3	5 ± 0	3 ± 0	3 ± 1	5 ± 2	3 ± 2	3 ± 2
Fuc (mol%)	19 ± 5	14 ± 3	14 ± 4	8 ± 0	5 ± 0	5 ± 2	8 ± 4	4 ± 3	5 ± 3
Ara (mol%)	18 ± 7	19 ± 9	20 ± 6	17 ± 2	19 ± 1	19 ± 3	15 ± 9	19 ± 13	19 ± 12
Xyl (mol%)	15 ± 4	16 ± 3	16 ± 1	22 ± 3	20 ± 1	21 ± 4	18 ± 13	20 ± 13	21 ± 13
Man (mol%)	12 ± 2	13 ± 3	13 ± 3	13 ± 2	12 ± 1	11 ± 1	15 ± 9	12 ± 7	11 ± 7
Gal (mol%)	14 ± 0	24 ± 2	22 ± 2	18 ± 1	28 ± 2	29 ± 3	18± 12	29 ± 16	29 ± 18
Glc (mol%)	14 ± 2	10 ± 1	9 ± 2	17 ± 2	14 ± 1	12 ± 1	21 ± 11	13 ± 7	11 ± 7
Unknown Carb. (%)	16	23	14	30	31	27	39	37	42

Yield of polymeric material obtained by dialysis with 12 kDa membrane cut-off; moisture of polymeric material when stored at 25 °C with silica estimated by thermogravimetric analysis (TGA, Fig. S1 in Supplementary material); ashes estimated by TGA analysis; protein equivalents by elemental analysis (%N x 6.25); carbohydrates by phenol-sulphuric acid method; sulphate as sulphate ester residues equivalents by elemental analysis (%S x 2.50); uronic acids by *m*-phenylphenol method; neutral sugars by GC-FID after 2 M TFA hydrolysis, 120 °C, 1 h; Unknown Carb. (%) percentage of unknown carbohydrates calculated by the difference of carbohydrates with uronic acids and neutral sugars.

of the material recovered at the first evaporator tanks in the start of the season. This emphasizes the effect of higher water stream along the evaporator tanks at the middle of the season of traditional salt production. The amount of polymeric material in the crystallizer tanks in the middle season is 133 mg/L (Table 1, column (4) from middle of season), 10 times higher than the amount of polymeric material of the seawater from the entrance at the start of season. The composition of polymeric material from the crystallizer resembled the one recovered from the second evaporator tanks at the start of the salt production season. As observed in the start of the season, along the salt pan tanks, ashes and sulphated polysaccharides increased twice, from 12 % to 25 % and 24 % to 42 %, respectively, and protein decreased to a half, from 29 % to 15 %.

At the end of the season of traditional salt production, the amount of polymeric material increased 1.7 times in the first evaporator tanks (16 mg/L), 1.6 times in the second evaporator tanks (58 mg/L), and 1.1 times in the crystallizer tanks (146 mg/L) in comparison with the amount of polymeric material at the middle season. The polymeric material composition resembled the composition of polymeric material from middle season. The higher amount of sulphated polysaccharides was recovered in the crystallizer tanks at the end of the season. These results show sulphated polysaccharides as the main component of the polymeric material of Ria Formosa brine water, as found in polymeric material from Aveiro salt pan water [2] and salts from Atlantic ocean [4].

The sulphated polysaccharides recovered in the crystallizer tanks at the end of the season, where higher amounts were recovered, were composed by 6.6 % of sulphate esters, 14 % of uronic acids, and 7.0 % of neutral sugar residue, namely Gal (29 mol%), Xyl (21 mol%), Ara (19 mol%), Man (11 mol%), Glc (11 mol%), Fuc (5 mol%), and Rha (3 mol %) (Table 1). These results show the structural heterogeneity of polymeric material from brine water, that was also found in polymeric material from Aveiro salt pan water [2] and salts from Atlantic ocean [4]. Notably, 42 % of unknown carbohydrates are present in these sulphated polysaccharides (Table 1), indicating that glycosidic linkages are not easily hydrolysed, possibly due to the presence of aldobiouronic acids [17,24], modified sugars, or accumulation of non-common carbohydrates, which were not quantified herein. Notwithstanding, a transversal feature of brine water sulphated polysaccharides was the negative charge provided by uronic acids and sulphate esters that accounted for 472 to 552 mg/g. In total, considering the 11 % of moisture and 16 % of protein, plus the 36 % of carbohydrates and 6.6 % of their sulphate esters, it is explained 70 % of the weight of the sample. Since these sulphated polysaccharides behave like salts in brine water, the increasing ashes content of polymeric material (130–259 mg/g) should be related to the counter ions of these negative charges, as observed in soluble charged marine polysaccharides [25]. Therefore, the amount of compounds related with polysaccharides is 4 times higher than the amount of protein present in the sample.

Given the heterogeneity of the samples, a partial component analysis (PCA) was performed with all the data from Table 1. It shows that the composition of polymeric material in concentrated brine water is different from the polymeric material in the more diluted brine water (Fig. 3a). The concentrated brine water had higher amount of uronic acids, sulphate esters, and ashes, and lower content of protein, Fuc, and Rha (Supplementary Material Fig. S2). As the brine water recovered in the crystallizer, from here called brine water, yielded a higher amount of sulphated polysaccharides and higher polymeric material, it has a higher potential to be explored.

To evaluate the reproducibility of the recovered polymeric material, brine water from Ria Formosa were collected at the end of 2020 and 2021 salt production seasons (Brine 2020 and Brine 2021, respectively).

Table 2
Polymeric material and sulphated polysaccharides, sulphate esters and uronic acid content of polymeric material from brine water and 20 L final concentrates from SANI membranes filtration system and permeates (< 100 kDa and < 30 kDa).

	Polymeric Material (mg/L)	Sulphated PS (mg/g)	Sulphate esters (mg/g)	Uronic acids (mg/g)
Brine 2020	171 ± 24	336 ± 32	46 ± 3	119 ± 3
Brine 2021	52 ± 2	363 ± 22	57 ± 3	116 ± 8
80 L ultrafiltration	138 ± 53	396 ± 47	44 ± 1	128 ± 7
<100 kDa permeate	28 ± 7	344 ± 11	55 ± 0	97 ± 6
<30 kDa permeate	32 ± 4	325 ± 40	51 ± 8	95 ± 5
200 L ultrafiltration and diafiltration	1369 ± 120	472 ± 39	91 ± 3	110 ± 3
500 L ultrafiltration and diafiltration	3140 ± 48	498 ± 52	89 ± 18	118 ± 3
200 L microfiltration, ultrafiltration, and diafiltration	277 ± 3	467 ± 46	81 ± 13	147 ± 3

Polymeric material concentration determined by dialysis with 12 kDa membrane cut-off; sulphated polysaccharides (PS) estimated by the sum of sulphate esters and carbohydrates determined by phenol-sulphuric acid method; sulphate as sulphate ester residues equivalents by elemental analysis (%S x 2.50); uronic acids by *m*-phenylphenol method; Neutral sugar composition is available at Table S1 from Supplementary Material.

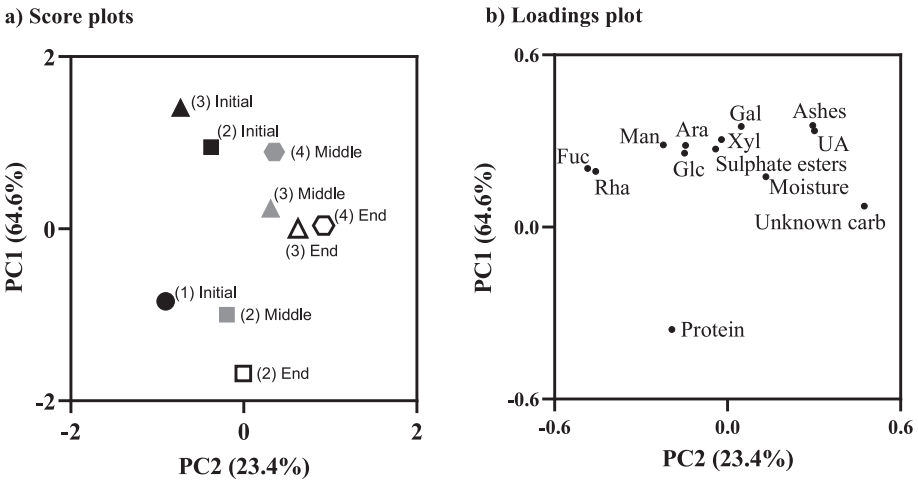

Fig. 3. Principal component analysis (PCA): a) score plot (PC1 *vs* PC2) and b) loadings plot (PC1 *vs* PC2) of polymeric material from brine water along salt pan tanks: (1) entrance (circle), (2) cold evaporator (evap., square), (3) hot evaporator (triangle), and (4) crystallizer (hexagon), during salt production season: initial (black colour), middle (grey colour), and end (white colour). UA-uronic acids.

The amount of polymeric material obtained ranged from 52 to 171 (Table 2). The differences observed can be due to the dilution of the material with water, although not excluding the interannual variations in organic matter that can give origin to the polymeric material. Carbohydrates composition resembled polymeric material recovered in the crystallizer in the end of salt production. These results indicate that the polymeric material recovered in Portugal salt pans (Ria Formosa herein and Aveiro in [2]) is similar and potentially have the same bioactivities, particularly, the ability to stimulate immune cells. To take advantage of this potential bioactive compounds, tangential ultrafiltration using a pilot-scale SANI membranes system was studied to obtain large scale sulphated polysaccharides with potential immunostimulatory activities.

3.2. Isolation of polymeric material from brine water by pilot-scale SANI membranes

A pilot-scale membrane system was used to concentrate the polymeric material while reducing the volume of brine water up to 25 times, reaching 15–20 L of concentrated solutions, the minimum working volume of the system. A total of five different experiments were performed: **1.** Ultrafiltration of 80 L of brine; **2.** Ultrafiltration of 200 L; **3.** Ultrafiltration of 200 L followed by diafiltration; **4.** Ultrafiltration of 500 L followed by diafiltration; and **5.** Microfiltration of 200 L, followed by ultrafiltration and diafiltration.

3.2.1. Ultrafiltration experiments (1 & 2)

To follow the diffusion of the salts through the membranes during the ultrafiltration process, macroelemental analysis was performed. It was observed along the process that the concentrated solutions had the same composition than the permeated water (Table 3) showing that salts freely diffused throughout the membranes without any restriction. Because the volume of permeate is much higher than the volume of retentate at the end of the process, it is inferred that the amount of salts diffused was much higher. On the contrary, the amount of carbohydrates showed an increase in the concentrated solutions of 1.2, 4.6, and 11.3 times in relation to brine when 80 L, 200 L, and 500 L of brine water were ultrafiltrated, respectively (Fig. 4.**a**). This corresponded to 2.3 g of carbohydrates in the 20 L of concentrated solution for the 80 L experiment (31 % of brine carbohydrates), 12.0 g for the 200 L (60 %), and 17.5 g for the 500 L (45 %). In accordance, the concentration of carbohydrates in the permeates decreased 4 times in relation to the brine for the 80 L experiment and 5 times for the two experiments with higher volumes. This corresponds to 1.9 g of carbohydrates in the 60 L of permeate for the 80 L experiment (26 % of brine carbohydrates), 4.5 g in the 180 L of permeate for the 200 L experiment (23 %), and 7.4 g in the 480 L of permeate for the 500 L experiment (19 %). Overall, from brine water, 57 % of carbohydrates were recovered in the 80 L experiment, corresponding to the sum of carbohydrates in the concentrate solution and permeate. In the 200 L experiment the recovery was 83 % and 64 % in the 500 L experiment. The increase of the yield from 80 to 200 L may be due to the better recovery of the carbohydrates retained in the membrane system, whereas the decrease of yield from 200 L to 500 L may be due to the aggregation of carbohydrates. Furthermore, the yield of carbohydrates recovered in the permeates show that more than 19 % of brine water carbohydrates have a molecular weight lower than 100 kDa, which is in accordance with the high abundancy of small carbohydrates observed in marine water [26].

As the concentrated solutions still contained a high concentration of salts (the same as the original brine water), to quantify the amount of polymeric material in both concentrate and permeate solutions, a dialysis step was performed in the laboratory. Polymeric material increased 1.2, 11.5, and 25.4 times (Fig. 4.b) when 80 L, 200 L, and 500 L of brine water were ultrafiltrated, respectively. The comparison of total carbohydrates with polymeric material shows that along the ultrafiltration process the percentage of carbohydrates in the polymeric material is 41 % (Fig. 4.c) and 53 % in the permeated samples, where carbohydrates of low molecular weight are present (Fig. 4.c). Despite the increase in carbohydrates and polymeric material of the concentrates after ultrafiltration, the dissolved solids in the concentrates were in the range of 380 to 430 g/L, similarly to the dry weight of brine water and permeates (Table 3). When compared to the value of 3 g/L of polymeric material present, this shows that there is still a high content of salt in the concentrates, representing the polymeric material about 1 %. To remove the salt, ultrafiltration experiments 3, 4, & 5 ended with a diafiltration.

Table 3
Macroelements (mg/L) present in brine water and samples from SANI membranes filtration system.

	Na	Mg	K	Ca	B
Brine water	40,825	51,310	13,970	176	149
Ultrafiltration of 80 L					
Concentrate	51,045	53,715	13,251	299	130
<100 kDa	51,201	52,305	13,045	287	126
<30 kDa	46,364	50,545	12,521	284	122
Ultrafiltration of 200 L					
Concentrate 1	44,000	37,106	10,434	140	115
<100 kDa	59,000	34,862	9623	128	94.0
<30 kDa	54,000	32,194	9018	122	87.0
Concentrate 2	41,000	40,507	11,279	125	128
<100 kDa	–	45,017	12,785	146	140
<30 kDa	–	38,451	10,990	123	120
Concentrate 3	48,000	41,397	11,625	110	126
<100 kDa	–	40,366	11,439	108	127
<30 kDa	–	41,872	11,820	109	131
Concentrate 4	45,000	42,644	11,851	110	128
<100 kDa	51,000	44,312	12,314	101	128
<30 kDa	60,000	46,602	13,120	105	127
Ultrafiltration and Diafiltration of 200 L					
Concentrate	45,000	45,000	12,000	100	130
Diafiltrate 4	160	52.8	4.38	210	0.17
<100 kDa	220	50.4	4.49	160	0.09
<30 kDa	240	53.5	5.42	160	0.11
PM (mg/g)	0.34	22.7	0.15	48.2	0.22
PM (mg/L)	0.47	31.1	0.21	66.0	0.30
Diafiltration of 500 L					
Concentrate	50,000	46,000	–	–	–
Diafiltrate 1	8300	9300	–	–	–
Diafiltrate 2	1500	1500	–	–	–
Diafiltrate 3	460	320	–	–	–
Diafiltrate 4	310	120	–	–	–
Diafiltrate 7	150	51.0	–	–	–
Dry (mg/g)	65.2	34.1	6.51	45.4	0.11
Microfiltration, ultrafiltration and diafiltration of 200 L					
>0,2 μm	31,700	57,900	16,700	99.6	201
<0,2 μm	34,100	62,000	18,300	88.4	216
Concentrate	32,300	58,800	16,500	94.2	201
<30 & < 100 kDa	33,600	59,100	17,100	90.6	211
Diafiltrate 1	5750	14,400	2900	129	36.9
Diafiltrate 2	260	940	72.0	85.0	0.97
Diafiltrate 3	188	52.0	3.00	132	0.062
Diafiltrate 4	167	44.0	3.00	132	0.059
Diafiltrate 5	177	62.0	7.00	132	0.104
Diafiltrate 6	50.0	18.0	2.00	45.5	0.032

PM: polymeric material isolated by dialysis of diafiltrate 4 and analysed after nitric acid digestion assisted by microwaves, results are expressed as mg/g of dry weight PM or as mg/L of diafiltrate 4, considering that it has 1.369 g/L of PM; Dry diafiltrate 7: oven dried and analysed after nitric acid digestion assisted by microwaves, with results are expressed as mg/g; –: not determined.

3.3. Ultrafiltration followed by diafiltration experiments (3 & 4)

The element analysis of samples from ultrafiltration of 200 L followed by diafiltration showed an increase in Ca concentration from 100 mg/L in the concentrated water after ultrafiltration to 210 mg/L in the solution after diafiltration. On the contrary, Mg decreased from 45 g/L to 0.05 g/L. The same trend was observed for Na, which decreased from 45 g/L to 0.16 g/L after diafiltration, B, which decreased from 130 mg/L to 0.2 mg/L, and K, which decreased from 12 g/L to 0.004 g/L (Table 3). These results show that the diafiltration of the concentrate removed the

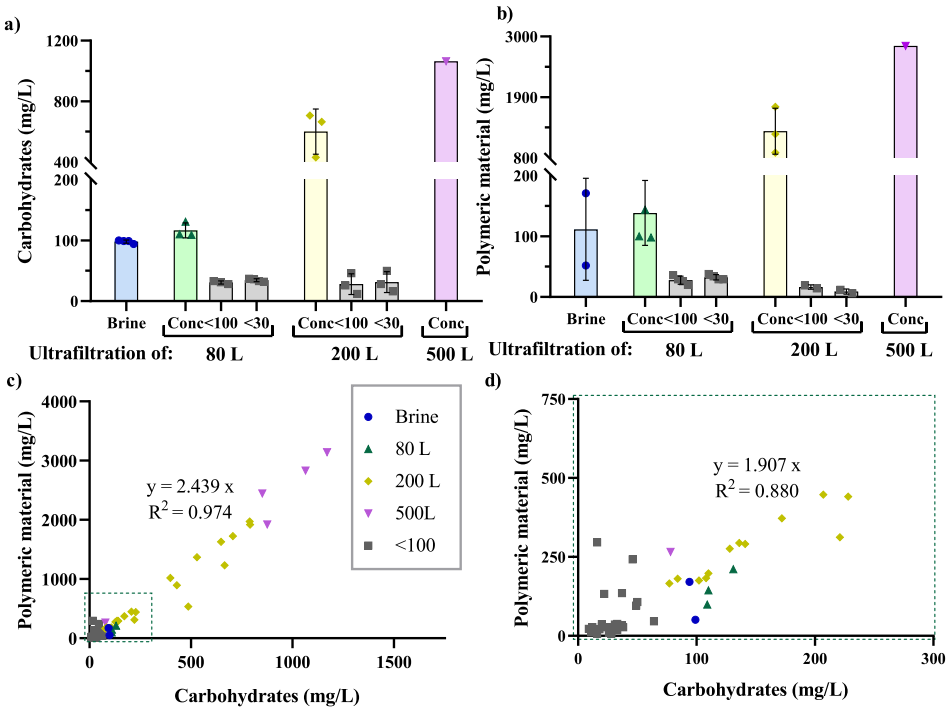

Fig. 4. Comparison of **a)** carbohydrates and **b)** polymeric material from samples obtained after ultrafiltration of 80 L, 200 L, and 500 L of brine water through pilot-scale SANI membranes system. Correlation of polymeric material with total carbohydrates of **c)** all 69 samples and **d)** 56 samples with less than 500 mg/L. Conc.: concentrate; < 100: permeate from membranes with 100 kDa cut-off; and < 30: permeate from membranes with 300 kDa cut-off.

main elements, except the Ca, indicating that the polymeric material is interacting with Ca [27]. The element composition analysis of the polymeric material isolated by ultrafiltration followed by diafiltration showed a content of 48.2 mg/g of Ca, confirming its retention. The polymeric material also contained 22.7 mg/g of Mg, and lower amounts of Na (0.34 mg/g), B (0.22 mg/g), and K (0.15 mg/g, Table 3), showing that Mg is also retained, although in a lower proportion than Ca [28].

The amount of polymeric material reached 1.9 g/L and 3.1 g/L after ultrafiltration followed by diafiltration of 200 L and 500 L, respectively. As the amount of the main elements was in the same order in both experiments, the diafiltrate from the 500 L experiment had a higher content of polymeric material in a dry weight basis (71 %), than in the experiment with 200 L (47 %). However, after processing 200 L of brine water into 20 L of diafiltrate, it was possible to obtain 40 g of polymeric material, whereas after processing 500 L, only 62 g were obtained. This shows that, although 2.5 times more brine were used in the 500 L experiment, the amount of polymeric material only increased 1.5 times. Although 500 L experiment allowed to obtain samples enriched in polymeric material, the 200 L experiment is more suitable when higher yields of polymeric material are wanted. The loss of polymeric material in the 500 L experiment can be due to agglomeration and membrane fouling.

3.4. Microfiltration before ultrafiltration and diafiltration experiment (5)

To prevent membrane fouling promoted by the presence of particles in suspension that are also concentrated during ultrafiltration, a tangential microfiltration with membranes of 0.2 μm was done before the use of ultrafiltration membranes. This step allowed to recover 180 L of filtered brine and 20 L of retentate. Element analysis of filtered brine water and retentate showed that they contained a composition similar to the brine water (Table 3), allowing to infer that the microfiltration step did not interfere with the element composition of the samples. However, the content of polymeric material in the retentate was 11 g in 20 L, which represents 50 % of the polymeric material present in the 200 L of brine water processed. This suggests that half of the polymeric material present in the brine water is part of the particles in suspension (cell debris, etc). The other half of the polymeric material from brine water was recovered in the 180 L of filtrate. Along the microfiltration step, it was observed a constant flow of polymeric material to the permeate, at a concentration of 74 mg/L (Fig. S3 in Supplementary material).

The ultrafiltration of the 180 L microfiltration permeate to 20 L, followed by diafiltration, allowed to recover only 46 % of the polymeric material from the filtered brine. This was lower than the 60 % recovered from the ultrafiltration of 200 L (experiment 3). It is possible that with the removal of particles by the microfiltration step some adsorbed polysaccharides were also removed or without particles in suspension the diffusion of polysaccharides with less than 100 kDa and 30 kDa through the membranes during the ultrafiltration and diafiltration steps was improved. Therefore, although the incorporation of microfiltration within the pilot-scale SANI membranes system ensured the elimination of particles, it could not to be suitable for the quantitative recovery of brine polysaccharides. Overall, the pilot-scale SANI membranes system allowed to increase more than 1000 times the amount of polymeric material in brine water dry weight by the removal of salt, ensuring the concentration of bioactive compounds for further food and feed applications.

3.5. Composition of polymeric material isolated from brine water by pilot-scale membranes

The polymeric material of the concentrates from the pilot-scale membrane experiments 1, 3, 4, & 5 and the polymeric material of the permeates of 30 and 100 kDa membranes of experiment 1 were analysed for their sulphated polysaccharide composition. The concentrate of 80 L (experiment 1) had 396 mg/g of sulphated polysaccharides, which included 32 % (*w*/w) of uronic acids and 23 % (w/w) of neutral sugars. Sulphate esters of these carbohydrates accounted for 11 % (w/w) (Table 2). Assuming that these sulphates were mainly esterified to neutral sugars, as observed for other marine sulphated polysaccharides [29], the degree of sulphation in moles was 0.8 for the concentrate of 80 L. The concentrates from 200 L and 500 L of brine were enriched in

sulphated polysaccharides by 20 % when compared with the concentrate of 80 L. Similarly, the sulphate esters present in the polymeric material increased 50–80 %. The degree of sulphation estimated was 1.0 for the concentrate of ultrafiltration of 200 L followed by diafiltration, 0.9 for the concentrate of ultrafiltration of 500 L followed by diafiltration, and 0.8 for the concentrate after microfiltration of 200 L, followed by ultrafiltration and diafiltration. These results show that the ultrafiltration of 200 L followed by diafiltration (experiment 3) was the procedure that allowed to obtain a higher amount of polymeric material, sulphated polysaccharides, and polysaccharides with higher degree of sulphation. In this sample, uronic acids accounted for 23 % (*w*/w), identified as GlcA and GalA by HPAEC-PAD, which also contribute to the negative charge of polysaccharides. Glc (24 mol%), Gal (20 mol%), Xyl (15 mol%), and Fuc (15 mol%) were the main other sugar components of this fraction. A similar composition was observed for the concentrate of ultrafiltration of 500 L followed by diafiltration (experiment 4) and for the concentrate after microfiltration of 200 L, followed by ultrafiltration and diafiltration (experiment 5). This sugars composition is also similar to those reported from Aveiro salt pan water [2] and salts from Atlantic Ocean [4].

The amount of polymeric material recovered in the experiment of 200 L ultrafiltration and diafiltration (experiment 3) was 32.38–33.18 g. This was calculated taking into account 180 L of ultrafiltration permeates (28–32 mg/L) and 20 L of diafiltrated concentrate (1369 mg/L). In total, it was recovered 5.0–5.8 g of polymeric material in permeates and 27.38 g of polymeric material in concentrate. Considering that 200 L of Brine 2020 have 34.2 g of polymeric material, 95–97 % of the brine polymeric material was recovered in experiment 3: 14.6 %–17.0 % in the permeates and 80.1 % in the concentrate. Therefore, the material that was recovered in the pilot scale represents 80 % of the material recovered at laboratory scale.

Giving the yields of polymeric material obtained in the experiments of pilot-scale SANI membranes and their similar composition, the polymeric material that results from the ultrafiltration of 200 L followed by diafiltration (experiment 3) was the one selected for the evaluation of the immunostimulatory activity experiments, as this procedure allowed to obtain a higher amount of polymeric material, sulphated polysaccharides, and polysaccharides with higher degree of sulphation.

3.6. Immunostimulatory activity

The immunostimulatory activity of polymeric material obtained

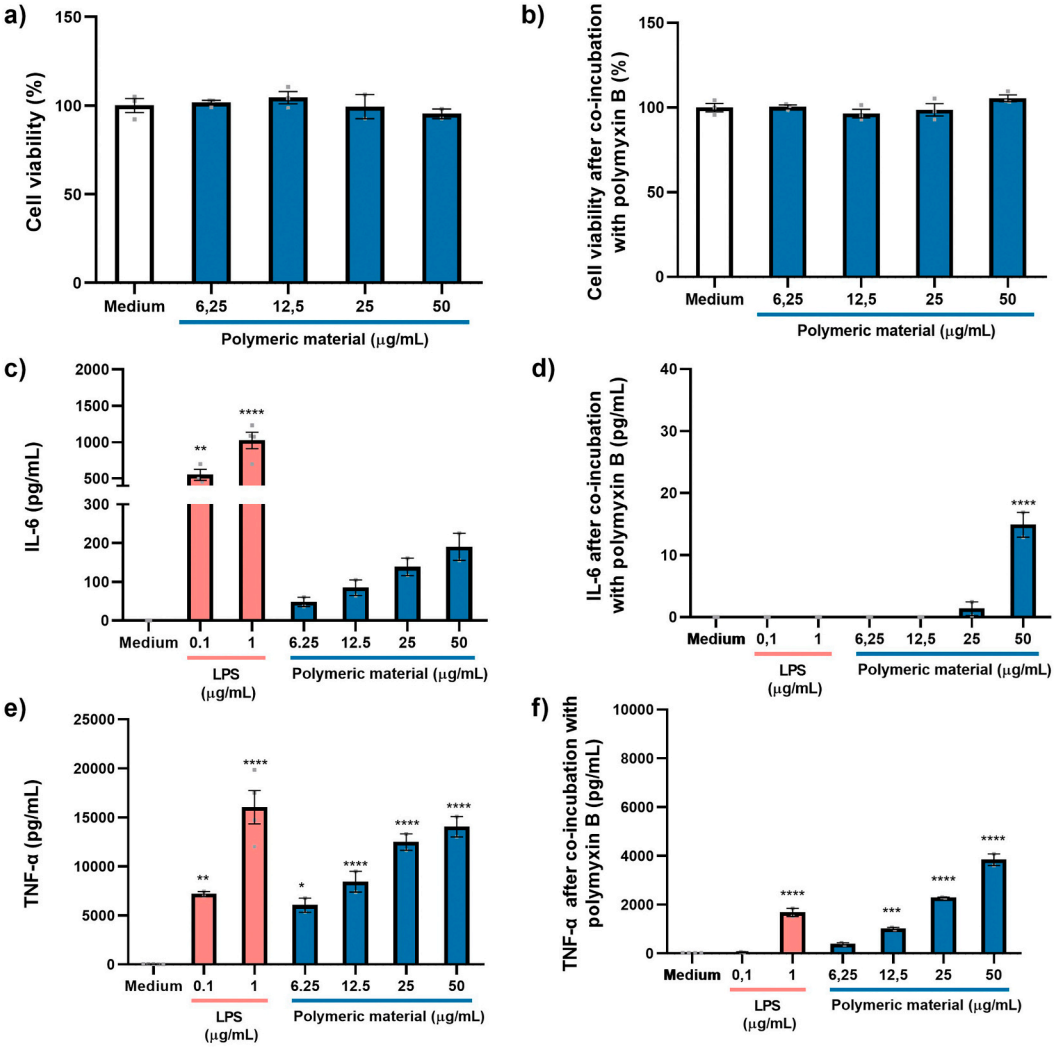

Fig. 5. Effect of polymeric material obtained from brine water (experiment 3) on **a-b)** viability, **c)-d)** induction of IL-6 and **e)-f)** induction of TNF-α in PMA-differentiated THP-1 cells, as a model for human macrophages, *co*-incubated without or with polymyxin B. Cells were treated for 24 h with 6.25–50 μg/mL of polymeric material from experiment 3. LPS O127:B8 (100 ng/mL and 1 μg/mL) was used as a positive control. Statistical difference was analysed using ordinary One-Way ANOVA and Dunnett's multiple comparisons test, with a single pooled variance, comparing the mean of each condition with the mean of the medium as indicated * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

from experiment 3 was evaluated in THP-1 cells differentiated with PMA, as a model for human macrophages, and in Atlantic salmon head kidney SHK-1 cells to evaluate polymeric material potential as immunostimulatory ingredients for food and feed. Viability of human macrophages was not affected by incubation with brine polymeric material in the range of 6.25–50 μg/mL (Fig. 5.a and b). In addition, polymeric material induced the expression of IL-6 and TNF-α 24 h after incubation (Fig. 5.c and e), even when co-incubated with polymyxin B (Fig. 5.d and f), an inhibitor of LPS-induced TLR4 activation. These results show that the polymeric material rich in sulphated polysaccharides has potential immunostimulatory activity, as observed for polymeric material isolated from Aveiro brine water [2] and sulphated polysaccharides isolated from marine organisms or chemically sulphated [3]. The activation of macrophages can contribute to host defence by stimulation of acute phase response and by triggering a pro-inflammatory response [30,31]. The activation of THP-1 cells by the polymeric material indicates that it could also be recognized by primary macrophages, such as those located in Peyer's Patches or in the gut lamina propria [32,33], which sample luminal contents [34]. The stimulation of gut macrophages could strengthen immunity in the intestinal tract and highlight the sulphated polysaccharides' potential to be used as functional food ingredients.

Viability of Atlantic salmon head kidney SHK-1 cells was also not affected by incubation with brine polymeric material in the range tested (12.5–50 μg/mL, Fig. 6.a). In addition, polymeric material (experiment 3) induced the production of ROS after 24 h incubation (Fig. 6.b). This *in vitro* immunostimulatory activity suggest that the polymeric material from brine water, rich in sulphated polysaccharides, are promising functional feed ingredients, competing with macroalgae sulphated polysaccharides that need extensive extraction and fractionation processes [35].

4. Concluding remarks

The results of this study show that it is possible to obtain sulphated polysaccharides from brine water at pilot scale using an ultrafiltration membrane system. The ten times concentration of brine water followed by diafiltration results in a sample depleted of most of the salts, comprising 1.9 g/L of polymeric material as 47 % of dry weight material, contrasting with the 400 g/L of salt in the starting brine water. The sulphated polysaccharides are composed of 23 % (*w*/w) of uronic acids, 34 % (w/w) of neutral sugars, and sulphate esters accounted for 19 % (w/w). This polymeric material can stimulate *in vitro* human macrophages to produce IL-6 and TNF-α and Atlantic salmon head kidney SHK-1 cells. Therefore, ultrafiltration of salt pan brine water allows to obtain sulphated polysaccharides that stimulate *in vitro* human macrophages and salmon head kidney cells showing their potential as immunostimulatory ingredients for food and feed applications.

CRediT authorship contribution statement

Writing-original draft preparation, SSF and MAC. Writing-review and editing, SSF, RBP, DB, HB, NF, AC, PF, BP, AMCR, JN, EP, MV, CN, MAC. Methodology, SSF, CN, MAC, RBP, MV, BP, AMCR. Investigation, SSF, RBP, DB, HB, NF, AC, BP. Validation, SSF, AC, PF, AMCR, JN, EP, MV, CN, MAC. Funding acquisition, AMCR, JN, EP, MV, CN, MAC. All authors have read and agreed to the published version of the

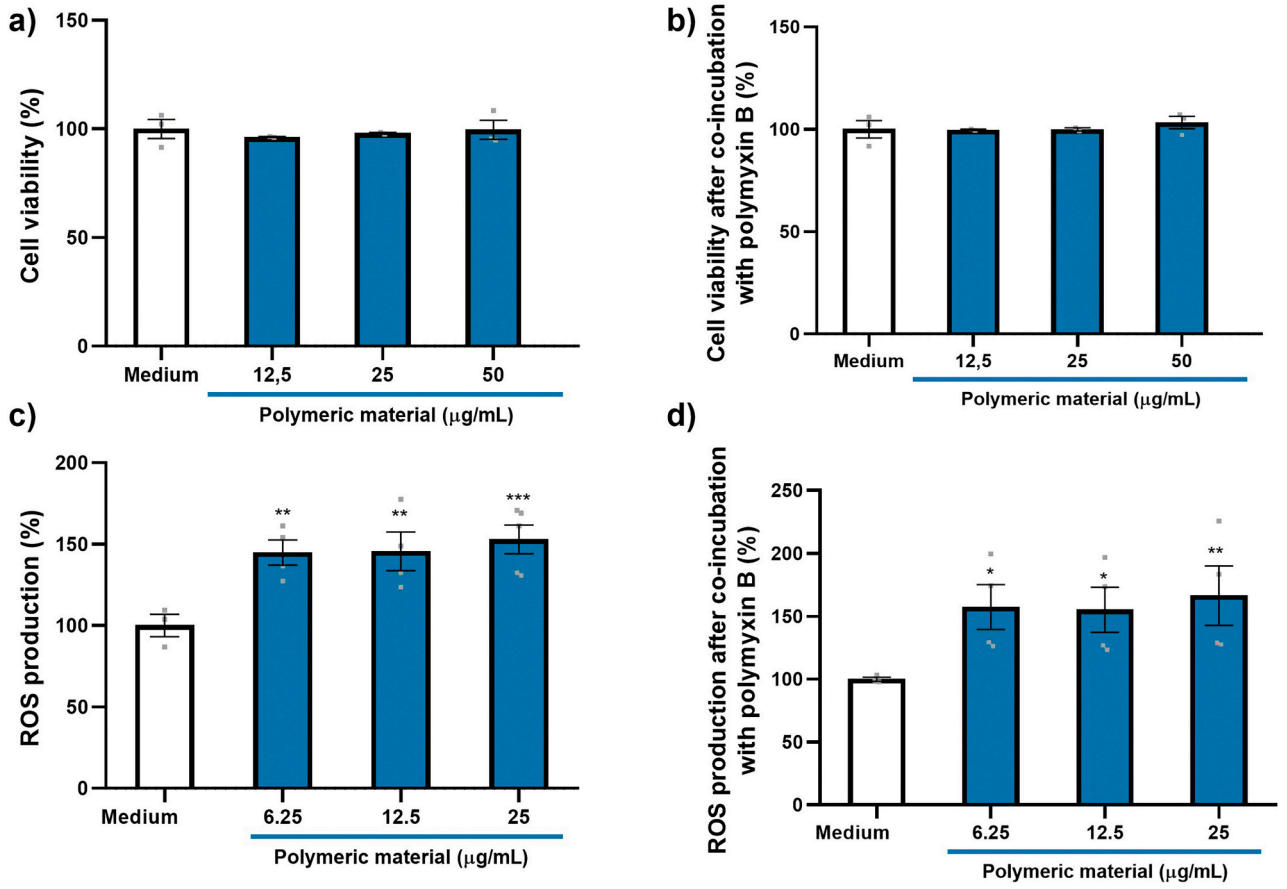

Fig. 6. Effect of polymeric material obtained from brine water (experiment 3) on **a-b)** viability and **c-d)** production of ROS in Atlantic salmon head kidney SHK-1 cells, as a model for fish immune cells, co-incubated without or with polymyxin B, respectively. Cells were treated for 24 h with 12.5–50 μg/mL of polymeric material (experiment 3). Statistical difference was analysed using ordinary One-Way ANOVA and Dunnett's multiple comparisons test, with a single pooled variance, comparing the mean of each condition with the mean of the medium as indicated * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2025.144506.

Data availability

Data will be made available on request.

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