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Two-Dimensional Isoelectric Focusing OFFGEL and Microfluidic Lab-on-Chip Electrophoresis for Assessing Dissolved Proteins in Seawater

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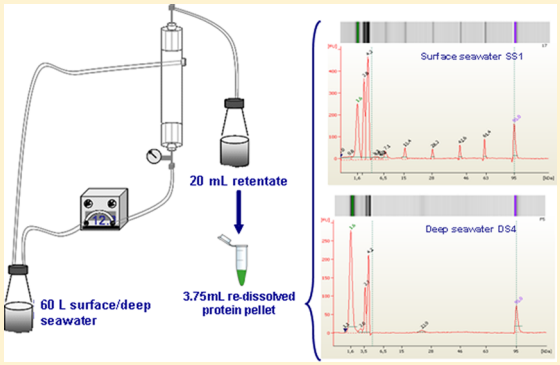
Supporting Information

ABSTRACT: Dissolved proteins were assessed in surface and deep seawater by two-dimensional isoelectric focusing (IEF) OFFGEL−lab-on-chip (LOC) electrophoresis after tangential flow ultrafiltration followed by centrifugal ultrafiltration (preconcentration factor of 3000). Dissolved protein isolation was performed by treating the ultrafiltrated retentate with cold acetone and also with chloroform as precipitating reagents. The best electrophoretic behavior of the isolated proteins was obtained after protein precipitation with chloroform before different rinsing stages for removing methanol and water interferences. Metals bound to proteins in the different OFFGEL fractions were assessed by inductively coupled plasma-optical emission spectrometry and electrothermal atomic absorption spectrometry, under optimized operating conditions. Experiments regarding stability of the metal-binding proteins [superoxide dismutase (SOD) and alcohol dehydrogenase (ADH) as protein models] showed the integrity of the Zn-binding SOD/ADH under the OFFGEL electrophoretic conditions. However, stability of Cu bound to SOD is not guaranteed. The first electrophoretic dimension (IEF OFFGEL) showed that dissolved proteins in surface seawater exhibit alkaline isoelectric points (pIs of 8.10 and 8.37) and also acid Ips (4.82, 5.13, 5.43, and 5.73), while LOC showed that the isolated proteins exhibit a spread molecular weight range (within 15 − 63 kDa); although, high molecular weights were the most commonly found. Regarding deep seawater, isolated proteins were of acid Ips (from 3.30 to 4.22) and low molecular weight (within the 21−24 kDa range). Elements such as Cd, Cu, Mn, and Ni were mainly associated with dissolved proteins of alkaline pIs in surface seawater, while Zn was mainly associated to proteins of acid pIs. However, only Cu and Mn were found to be bound to dissolved proteins of higher Ips in deep seawater, and the amount of Mn (from 68 to 84 μg L^{-1}) was higher than that found in dissolved proteins in surface seawater (22.4 μg L^{-1}).



The global carbon cycle is affected by the dissolved inorganic carbon (DIC) fraction and also by the fraction of dissolved organic carbon [DOC, or dissolved organic matter (DOM)].[1] DIC is the largest and best understood group of reactive carbon in the oceans.[2] Nevertheless, little is known about DOM, mainly because this fraction comprises a large group of coarsely identifiable substances which exhibit very different molecular weights.[1,2] Complex macromolecular compounds from biological and chemical degradation processes from biota are included in this latter fraction, which also encompass large biomolecules and degraded and reworked products derived from them.[3] Sugars, lipids, amino acids, and proteins are biochemical substances identified in seawater, and their presence is attributed to marine algae metabolism in the euphotic zone, as well as to microbial exudation and cellular lysis.[2] This class of substances, mostly found in surface seawater, shows a labile nature, which explains the important role of these reactive substances in influencing toxicity and bioavailability of nutrients and toxic contaminants.[4,5] However, some of these marine-derived polymeric materials (such as dissolved proteins) in deep seawater constitute the largest DOM biorefractory fraction, as explained by Powell et al.[6] based on two theories (i.e., physical protection and selective preservation), which were previously proposed by Hedges et al.[7] Dissolved proteins affect therefore the global carbon cycle through the long-term preservation of carbon and nitrogen in the ocean.[8] In addition, dissolved proteins have also been found to persist as discrete units in the environment[6,8] and are probably the most characterizable component of refractory marine DOM at the molecular level.[6]

Although there is some literature regarding marine DOM in surface and deep waters, as reviewed by Mecozzi et al.[3] and by Mopper et al.,[4] data concerning dissolved proteins are scarce. Pioneering works were developed by Tanoue who demonstrated the occurrence of proteins associated to the particulate organic matter (POM) fraction from intermediate and deep

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waters[9−11] and also as discrete dissolved units in oceanic waters.[11,12] Further studies suggested that bacterial porins (β barrel proteins)[13] and other major outer membrane proteins, such as OmpA-like proteins, of most pathogenic gram-negative bacteria[14] are the major source of dissolved proteins in seawater. Recent literature encompasses both particulate[15] and dissolved proteins,[6,16−18] as well as degradation products (peptides) dissolved in deep waters[19] or associated with POM.[20]

As in the case of other types of dissolved marine DOM, the assessment of dissolved proteins in seawater is difficult due to their inherent low concentration and the presence of large amounts of inorganic salts. Tangential flow ultrafiltration (UF) rather than solid-phase extraction (SPE) techniques are preferable when isolating marine DOM of high molecular weight,[4,21,22] resulting in a more appealing approach when dealing with dissolved proteins.[8] It must be pointed out that losses of marine DOM by adsorption onto the UF membranes within the 11−16% range commonly occur,[22,23] and as reported by Powell and Timperman,[16] this phenomena is especially important for dissolved proteins because of their highly adsorptive nature. Nevertheless, all the recent developments for the dissolved protein assessment are based on UF as a sample pretreatment.[6,11,14,16−19]

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE)[9−14,16] and a two-dimensional electrophoresis (2DE)[15,17,18] have been used for characterizing dissolved proteins. In general, 2D gel methods are time-consuming and require tedious procedures, and other 2D approaches for assessing proteins have been developed. With regard to marine proteomics, Powell et al.[6] have therefore applied a 2D (SCX and RPC) high-performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS) approach for dissolved proteins in seawater. Additionally, two-dimensional approaches based on the combination of IPG IEF [first-dimensional (1D) separation according to the proteins' pIs] and RPC (2D separation according to the proteins' molecular weights) have also been developed.[24,25] Recently, the introduction of preparative IPG IEF, where proteins can be recovered from the liquid phase (OFFGEL electrophoresis) avoids drawbacks associated with IPG gels, allowing the direct sampling of the isolated protein (1D separation) into HPLC instruments.[26] Further developments are focused on coupling OFFGEL electrophoresis to microfluidic chip-based liquid chromatography,[27] on increasing protein recoveries by cup loading instead of conventional in-tray rehydration,[28] and on using a 48-well setup instead of a 24-well setup for improving the separation of proteins with small pI differences.[28]

Although the high degree of automation of OFFGEL−lab-on-chip (LOC) electrophoresis and the avoidance of gel cutting and further protein extraction and cleaning up stages when performing 2D approaches, some limitations must be reported. First, once the OFFGEL fractions are loaded into the chips, they can not be recovered, which further limits analysis for protein identification and for elucidating metals bound to each separated protein. In these cases, 2DE are more advantageous because direct MS/MS protein identification after tryptic digestion of in-gel proteins can be performed and metals can be assessed by direct laser ablation (LA)-ICP-MS analysis of the dehydrated gels. In addition, the low volume of the OFFGEL fractions, and also the high salt content, can lead to problems when combining OFFGEL electrophoresis with chromatographic methods.

As previously reported, natural organic matter (NOM) plays a crucial role on the adsorption behavior and transport of trace metals in surface and deep water, as well as in soils and sediments, which conditions metals toxicity/bioavailability toward micro- and macro-organisms.[29,30] Several studies have therefore been performed to understand and characterize its interactions with metals in fresh waters,[29,31,32] and recent papers have also shown the association of bioactive elements (Cu, Fe, and Zn)[22,33] and other trace elements such as Ba, Co, Mn, and Sr[22] with marine DOM. As dissolved proteins are an important fraction of DOM, the characterization of metal-dissolved protein complexes in marine ecosystems is a topic of interest.

One of the aims of the current work has been the development and application of an IPG IEF OFFGEL electrophoresis method followed by microfluidic lab-on-chip (LOC) electrophoresis for assessing dissolved proteins in surface and deep seawater. Because proteins are recovered from liquid phase after IPG IEF OFFGEL electrophoresis, microfluidic devices can be used for direct sampling of the isolated proteins (1D separation). As shown by recent reviews,[34−37] LOC electrophoresis has gained popularity, driven by the increased need to create portable, fast, and low analyte consumption devices.[35] Therefore, the improved sensitivity of this 2D technique, in addition to the minimal sample consumption and the reduction of the number of handling steps inherent to conventional SDS−PAGE, makes this combination an appealing approach for the assessment of low-abundance proteins, such as dissolved proteins. In addition, since the electrophoretic operating parameters mainly condition the sample pretreatment, different strategies were tested and optimized to achieve an adequate protein pellet before two-dimensional IEF OFFGEL−LOC electrophoresis analysis. Finally, an additional goal of the current work has been the identification and quantification of trace metals bound to dissolved proteins. To the best of our knowledge, these two latter objectives have not yet been addressed.

EXPERIMENTAL SECTION

Apparatus. Protein pI-based fractionation with liquid-phase recovery in the 24-well setup (first-dimensional separation) was achieved by using the Agilent 3100 OFFGEL fractionator (Agilent Technologies, Santa Clara, CA). 2D electrophoresis was performed using the Agilent 2100 Bioanalyzer (Agilent Technologies) with the electrode cartridge for on-chip electrophoresis and fluorescence detection. A Perkin-Elmer model 1100B (Perkin-Elmer, Norwalk, CT) atomic absorption spectrometer equipped with an HGA-700 graphite furnace atomizer, deuterium background correction, and an AS-70 autosampler was used for determining cadmium, cobalt, chromium, copper, manganese, nickel, and lead bound to proteins of similar pIs (OFFGEL fractions). The sources of radiation were monoelement hollow cathode lamps (HCLs): chromium, copper, manganese, and nickel HCLs were from Cathodeon (Cambridge, U.K.), while cobalt and lead HCLs were the Lumina type from Perkin-Elmer. In all cases, HCLs were operated at the recommended manufacturer operating conditions. An Optima 3300 DV inductively coupled plasma (ICP)-atomic emission spectrometer (Perkin-Elmer) equipped with an autosampler AS 91 (Perkin-Elmer) and a Gem-Cone cross-flow nebulizer type (Perkin-Elmer) was used for iron and zinc determinations. The tangential flow ultrafiltration (UF) system consisted of a Masterflex I/P pump (Millipore, Bedford,

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MA), a Prep/Scale-TFF Cartridge (Millipore) with a polyethersulfone membrane (nominal MW cutoff 10 kDa), and a Pre/Scale-TFF Holder (Millipore) equipped with a pressure gauge. Centrifugal ultrafiltration was performed with an Alresa Digtor centrifuge (Madrid, Spain). Other laboratory devices were an ultracentrifuge Laborzentrifugen model 2K15 (Sigma, Osterode, Germany), a Heidoph shaker type Reax 2000 (Gemini B.V., Apeldoorn, Netherlands), and an ORION 720A plus pH meter with a glass−calomel electrode (ORION, Cambridge, U.K.).

Reagents and Material. Ultrapure water, resistance of 18 MΩ cm, was obtained from a Milli-Q water-purification system (Millipore). IPG Dry Strips (pH 3−10), OFFGEL Buffer 3-10, and Plus One DryStrip cover fluid used for pI protein fractionation were supplied by GE Healthcare Life Science (Uppsala, Sweden). The Protein OFFGEL stock solution was prepared by dissolving 25.2 g of urea (electrophoresis grade, Sigma Aldrich, St. Louis, MO), 9.1 g of thiourea (analytical grade, Sigma Aldrich), 600 mg of 1,4-dithiothreitol (DTT, electrophoresis grade, Merck, Darmstadt, Germany), 6 mL of glycerol solution (Merck), and 600 μL of OFFGEL Buffer (pH 3−10) in ultrapure water (final volume of 50 mL). This solution was used for protein pellet redissolution, and it allowed an adequate isoelectric focusing for OFFGEL electrophoresis separation. Sizing and analysis of proteins by LOC electrophoresis was performed with the Agilent protein 80 Kit Agilent Technologies (Waldbronn, Germany). This supply consists of protein chips containing an interconnected set of gel-filled microchannels that sieves proteins by size within the 5−80 kDa range (electrophoresis separation); Protein 80 Gel-Matrix and Protein 80 Dye Concentrate, which are used as a filling gel for chips; and Protein 80 Sample Buffer, which is used for preparing the denaturing solution. The kit also contains the Protein 80 Ladder, which consists of standard proteins with molecular weights of 6.5, 15, 28, 46, and 63 kDa (concentration of 600 ng μL^{-1} as a sum of all standard proteins) used for molecular weight assessment; and also, standard proteins coded as lower marker and upper marker, at concentrations of 1 and 60 ng μL^{-1}, respectively, used for protein quantification. Centrifugal ultrafiltration was performed with Vivaspin 20 ultrafiltration tubes (polyethersulfone membrane of 10 kDa molecular cutoff) from Sartorius Stedin Biotech (Goettingen, Germany). Seawater filtration was achieved using Millipore HAWP14250 0.45 μm mixed esters of cellulose membrane filters (140 mm diameter). Superoxide dismutase from bovine erythrocytes (SOD), and alcohol dehydrogenase from *Saccharomyces cerevisiae* (ADH) were from Sigma-Aldrich, and they were used when studying metal−protein stability during the OFFGEL electrophoresis procedure. Sodium azide (used for avoiding dissolved protein degradation), acetone, and methanol plus chloroform (used for obtaining protein pellet) were from Panreac (Barcelona, Spain), while 2-mercaptoethanol (BME), used when preparing the denaturing solution, was from Fluka (Vancouver, Canada). Sodium dodecyl sulfate (SDS), used for avoiding protein adsorption onto the UF membranes, was purchased from AppliChem (Darmstadt, Germany). Other reagents were high purity 69% nitric acid (Panreac), used for treating the OFFGEL fractions before ETAAS/ICP-OES measurements, sodium hydroxide (Merck) for UF membrane cleaning, and ammonium hydrogencarbonate (BDH, Poole, U.K.) for salt removal after retentate preparation by centrifugal UF. Element standard solutions (used for metal quantification) were prepared from cadmium, cobalt, chromium, copper, iron, manganese, nickel, lead, and zinc stock standard solutions (1.000 g L^{-1}) from Scharlau (Barcelona, Spain). The bovine serum albumin (BSA) standard (2 mg mL^{-1}) from Thermo Scientific (Rockford, IL) was used when performing analytical recovery studies.

Seawater Sample Collection. Three surface seawater samples (1−2 m depth, 60 L), coded as SS1, SS2, and SS3, and one deep seawater sample (50 m depth, 60 L), coded as DS4, were collected from the Ría de Arousa estuary (northwestern Spain) in precleaned 12 L nonmetallic free-flushing Niskin bottles attached to a 1015 rosette multibottle array (General Oceanics, Miami, FL). After collection, seawater samples were filtered (0.45 μm) and immediately subjected to the tangential flow ultrafiltration procedure.

Seawater Tangential Flow Ultrafiltration. In accordance with the manufacturer's instructions, the ultrafiltration system was cleaned by passing 2 L of 0.1 M NaOH at 45 ± 5 °C (this solution was recirculated for 60 min) and then by rinsing with 9 L of Milli-Q water, also at 45 ± 5 °C. Operating conditions for the tangential ultrafiltration process were described elsewhere,[22] although with slight modifications. Before ultrafiltration, filtered seawater samples (60 L) were treated with 19.5 g of sodium azide (concentration of 5.0 mM in the 60 L of seawater) and 6.0 g of SDS [concentration of 0.01% (m/v) in the 60 L of seawater][14,15,17−19] to prevent protein adsorption onto the ultrafiltration membrane.[16] The preserved seawater sample was then concentrated through a polyethersulfone membrane (size 0.6 m^2, nominal molecular mass cutoff of 10 kDa), until a volume of retentate (ultrafiltrate containing substances of molecular weight higher than 10 kDa) was obtained within the 400−600 mL range.

Retentate Centrifugal Ultrafiltration. The retentate fraction (volumes ranging from 400 to 600 mL) was further ultrafiltrated by centrifugal ultrafiltration (ultrafiltration tubes with polyethersulfone membrane of 10 kDa molecular cutoff). Before use, polyethersulfone membranes were rinsed by loading 20 mL of ultrapure water and centrifuging at 4000 rpm for 10 min. This treatment allows the removal of trace amounts of glycerine adsorbed onto the polyethersulfone membrane. The centrifugal ultrafiltration procedure consisted of subjecting successive 20 mL aliquots of retentate at 4000 rpm for centrifugation times within the 10−30 min range. Because of the high volume of retentate (400 to 600 mL), four different centrifugation tubes were needed for treating each sample (retentate). Centrifugation was performed until obtaining a volume of 5 mL of retentate in each membrane tube. Each 5 mL retentate was finally rinsed by loading 15 mL of a cleaning solution containing 0.01% (m/v) SDS and 35 mM ammonium hydrogencarbonate and centrifuging (4000 rpm) until 5 mL of clean retentate in each membrane tube was obtained. The four retained and clean 5 mL retentate aliquots were combined, and a retentate of 20 mL was finally obtained (preconcentration factor of 3000).

Protein Pellet Precipitation. Twenty different aliquots (1 mL) from the whole retentate (20 mL) were mixed with methanol (4 mL), chloroform (1 mL), and water (3 mL), inserting a vortex mixing stage after adding each solvent. The mixtures were then centrifuged at 4000 rpm for 10 min, and the upper methanol−water layer was discarded. The chloroform layer containing proteins was then vortexed with 4 mL of methanol, and after centrifugation (4000 rpm, 10 min), the liquid phase was discarded and the different precipitates were combined (protein pellet). Protein pellets were redissolved in 3

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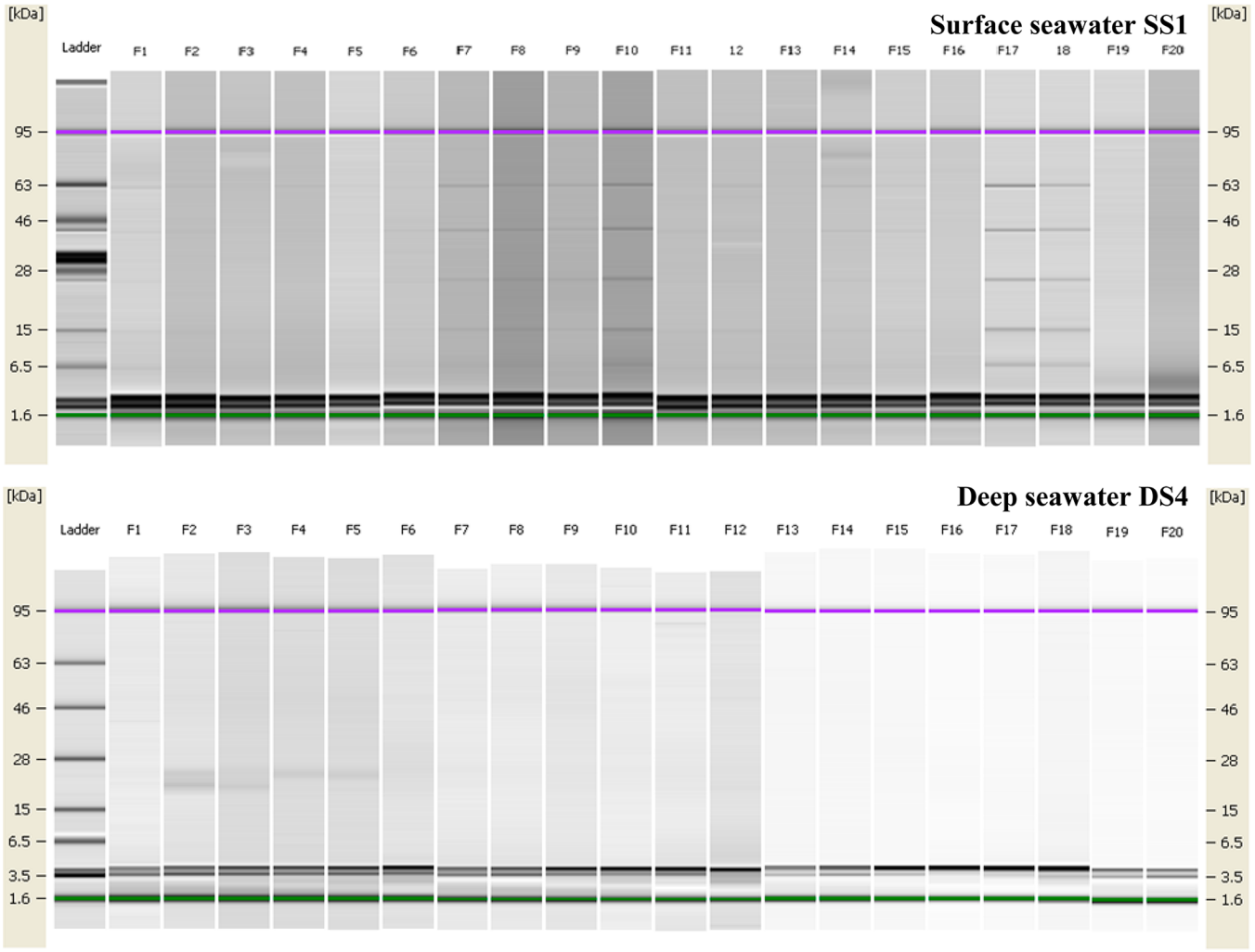

Figure 1. Gel-like images (fractions from 1 to 20) for dissolved proteins in surface and deep seawater samples: ladder lane corresponds to standard proteins of known molecular weight; F lanes correspond to OFFGEL fractions 1 to 20.

mL of protein OFFGEL stock solution (see Reagents and Material) plus 0.75 mL of ultrapure water before OFFGEL electrophoresis analysis.

Isoelectric Focusing OFFGEL Electrophoresis. IPG Dry Strips (24 cm, pH 3−10) were first rehydrated for 15 min by loading 40 μL of OFFGEL Rehydration solution (prepared by mixing 960 μL of Protein OFFGEL Stock solution and 240 μL of ultrapure water) into each 24 well. Isoelectric-focusing electrophoresis separation was then performed by loading 150 μL aliquots of the protein solution (protein pellet redissolved in Protein OFFGEL Stock solution−ultrapure water mixtures, as shown in Protein Pellet Precipitation) into each well. Protein separation in accordance with their pIs was performed by focusing with a maximum current of 50 μA (200mW) and typical voltages ranging from 500 to 4500 V (voltage to time ratio of 64 kV h^{-1}). Protein separation zones were maintained at a constant voltage of 500 V (20 μA and 50 mW).

Lab-on-Chip Electrophoresis. After pIs proteins separation (first dimension), the recovered fractions were further separated according to their molecular weights by LOC electrophoresis (second dimension). OFFGEL fractions (4 μL) were combined with 2 μL of the denaturing solution (1 μL of BME plus 28.6 μL of Protein 80 Sample buffer) in 0.5 mL Eppendorf tubes. The latter solution contains a lower (1.6 kDa) and an upper (95 kDa) marker, which allows the correct alignment of each lane. Sample tubes were then heated at 95 °C in a thermostatted water bath for 5 min and after cool down, 84 μL of ultrapure water was added before vortexing. A similar procedure (6 μL of Protein 80 Ladder solution) was performed when preparing the ladder (mixture of proteins exhibiting different molecular weights). The standard protein ladder is loaded on each chip (commonly coded as "lane L"), and allows an estimation of the appropriate molecular weight of the separated proteins. This solution also contains the lower marker and the upper marker proteins of known molecular weight at concentrations of 1 ng μL^{-1} and 60 ng μL^{-1}, respectively, used for protein quantification. Before loading the samples (6 μL) and the ladder (6 μL) into the chips, they were filled with 12 μL of the Gel-Dye Mix (G/D) and Destaining solution (DS). The G/D and DS solutions were prepared following manufacturer's recommendations: the DS solution consists of 650 μL of Protein 80 Gel-Matrix previously filtered and centrifuged; whereas, the G/D solution is a mixture of 650 μL of Protein 80 Gel-Matrix (also filtered and centrifuged) with 25 μL of Protein 80 Dye concentrate. LOC electrophoresis (protein detection by laser-induced fluorescence) was performed under recommended manufacturer's conditions: the system automatically fixes parameters such as voltage (a value

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within the 50−1500 V range), current (within the −20 to +20 μA range), and LED wavelength (a value from 458 to 482 nm), in accordance with the chip type (protein 80 kit in the current application). The Agilent 2100 Bioanalyzer software controls data collection, reporting and interpretation functions, and protein quantification is performed, taking into account the upper marker concentration of 60 ng μL^{-1}.

ETAAS Measurements. Cadmium, cobalt, chromium, copper, manganese, nickel, and lead were determined in the OFFGEL fractions containing proteins. These elements were assessed by ETAAS under optimized graphite furnace temperature programmes (Table S1 of the Supporting Information). OFFGEL fractions (100 μL) were first made up to 500 μL with 2% (v/v) nitric acid. ETAAS determinations involved a further dilution by mixing 50 μL of the acidified OFFGEL fraction, 25 μL of 500 mg L^{-1} palladium nitrate, and 25 μL of 200 mg L^{-1} magnesium nitrate, as chemical modifiers, and 100 μL of ultrapure water. Different reagent blanks were also analyzed, and negligible background signals were recorded. Determinations were performed using aqueous standards matched with the Protein 80 OFFGEL standard solution (100 μL), plus 2% (v/v) nitric acid (400 μL). Calibrations for Co, Cr, Cu, Ni, and Pb covered concentrations up to 20 μg L^{-1}, while calibrations were performed up to 10 μg L^{-1} and 4 μg L^{-1}, for Mn and Cd determinations, respectively. The LODs (3 SD criterion, SD standard deviation of eleven measurements of a reagent blank) referred to the OFFGEL fraction (1:5 dilution) and expressed as micrograms per liter were 0.33, 2.9, 12, 2.6, 4.8, 11, and 12 μg L^{-1} for Cd, Co, Cr, Cu, Mn, Ni, and Pb, respectively. Similarly, the LOQs (10 SD criterion) were 1.1, 9.8, 41, 8.8, 16, 35, and 41 μg L^{-1} for Cd, Co, Cr, Cu, Mn, Ni, and Pb, respectively.

ICP-OES Measurements. Iron and zinc in the OFFGEL fractions from seawater, and also copper and zinc when performing metal-binding protein stability studies, were determined by ICP-OES (axial configuration), using the operating parameters listed in Table S2 of the Supporting Information. Determinations were performed by using aqueous standards matched with Protein 80 OFFGEL standard solution (0.5 mL) made up to 5 mL with 1% (v/v) nitric acid after adding the adequate standard volumes when analyzing OFFGEL fractions from seawater. Calibrations covered Fe and Zn concentrations within the 0−2 mg L^{-1} range. Volumes of 200 μL of OFFGEL fractions were mixed with 1800 μL of 1% (v/v) nitric acid and directly nebulized in the ICP torch. With regard to metal−protein stability studies, they matched calibrations with Protein 80 OFFGEL standard solution (500 μL), variable volumes of standard solutions (covering concentrations from 0 to 2 mg L^{-1}), and dilution to 10 mL with 1% (v/v) nitric acid. Metal-binding proteins were analyzed after dilution of 100 μL of each OFFGEL fraction with 1900 μL of 1% (v/v) nitric acid. The LODs (3 SD criterion) expressed as μg L^{-1} were 0.11, 0.006, and 0.015 mg L^{-1} for Fe, Cu, and Zn, respectively. Similarly, the LOQs (10 SD criterion) were 0.37, 0.02, and 0.05 mg L^{-1} for Fe, Cu, and Zn, respectively.

RESULTS AND DISCUSSION

Preliminary Studies for Dissolved Marine Protein Precipitation. Dissolved proteins were isolated by tangential ultrafiltration (as described in Seawater Tangential Flow Ultrafiltration) followed by centrifugal ultrafiltration of the retentate fraction (see Retentate Centrifugal Ultrafiltration). A general protocol for protein precipitation based on ice-cold acetone (protein pellet formation at −20 °C for 2 h)[38] was first applied (4 mL of precipitating solution for every 1 mL of retentate). Preliminary experiments by loading small amounts of the obtained protein pellet (commonly from 5 mL of ultrafiltrated retentate) directly redissolved with 3.8 mL of Protein OFFGEL stock solution, showed long separation times (approximately 72 h) for completing the IEF OFFGEL electrophoresis (first dimension). These problems can be attributed to the presence of other compounds in the redissolved protein pellet. This fact was confirmed when loading the whole redissolved pellet (from 20 mL of retentate), which led to problems for adjusting the electrical current, and the IEF OFFGEL electrophoresis could not be initialized.

To obtain a purified and SDS-free protein pellet, an alternative method consisting of removing methanol and water-soluble interferences was tested.[6] The method requires the addition of methanol, water, and chloroform and the final protein precipitation in the separated chloroform layer (Protein Pellet Precipitation). The protein pellet redissolved in the Protein OFFGEL stock solution led to free-interference IEF OFFGEL separation after 36 h, even when obtaining the protein pellet from the whole retentate (20 mL).

Table 1. pI Values, Molecular Weights, and Relative Concentrations of Dissolved Proteins in Surface and Deep Seawater

sample	OFFGEL fraction	pI	MW (kDa)	relative concentration (ng μL^{-1})
SS1	F7	4.82	15−63	119
	F8	5.13	15−63	4.4
	F9	5.43	15−63	5.9
	F10	5.73	15−63	103
	F17	8.10	15−63	188
	F18	8.37	15−63	53.5
DS4	F2	3.30	21−24	58.3
	F3	3.61	21−24	19.1
	F4	3.91	21−24	13.7
	F5	4.22	21−24	15.9

Two-Dimensional IEF OFFGEL-LOC Electrophoresis. *Molecular Weight Repeatability.* The repeatability of the molecular weight assessment was studied by loading the ladder solution (containing standard proteins with molecular weights of 6.5, 15, 28, 46, and 63 kDa) eight times. After LOC electrophoresis, the calculated molecular weights were 6.5 ± 0.1, 15 ± 0.1, 28 ± 0.2, 46 ± 0.2, and 63 ± 0.08, which offer RSD values of 2, 0.7, 0.6, 0.4, and 0.1% for standard proteins of molecular weights of 6.5, 15, 28, 46, and 63 kDa, respectively. Good repeatability when assessing the molecular weights of the isolated proteins (OFFGEL fractions F7 to F10, F17, and F18 in surface seawater, and F2 to F5 in deep seawater) is therefore expected.

IEF OFFGEL-LOC Analytical Recovery. Because certified reference material for proteins, and especially for dissolved proteins, are not available, the accuracy of the overall IEF OFFGEL-LOC electrophoresis method was assessed by the analytical recovery approach. Therefore, the IEF OFFGEL of an albumin aqueous standard solution (0.20 mg of albumin) was run twice. After LOC electrophoresis, albumin was indentified in the OFFGEL fraction 10 (pI of 5.73). Because the separation process was performed under reductive conditions (use of DTT), the molecular weight found for albumin (66.5 kDa) was shifted to 72.4 kDa. This fact can be

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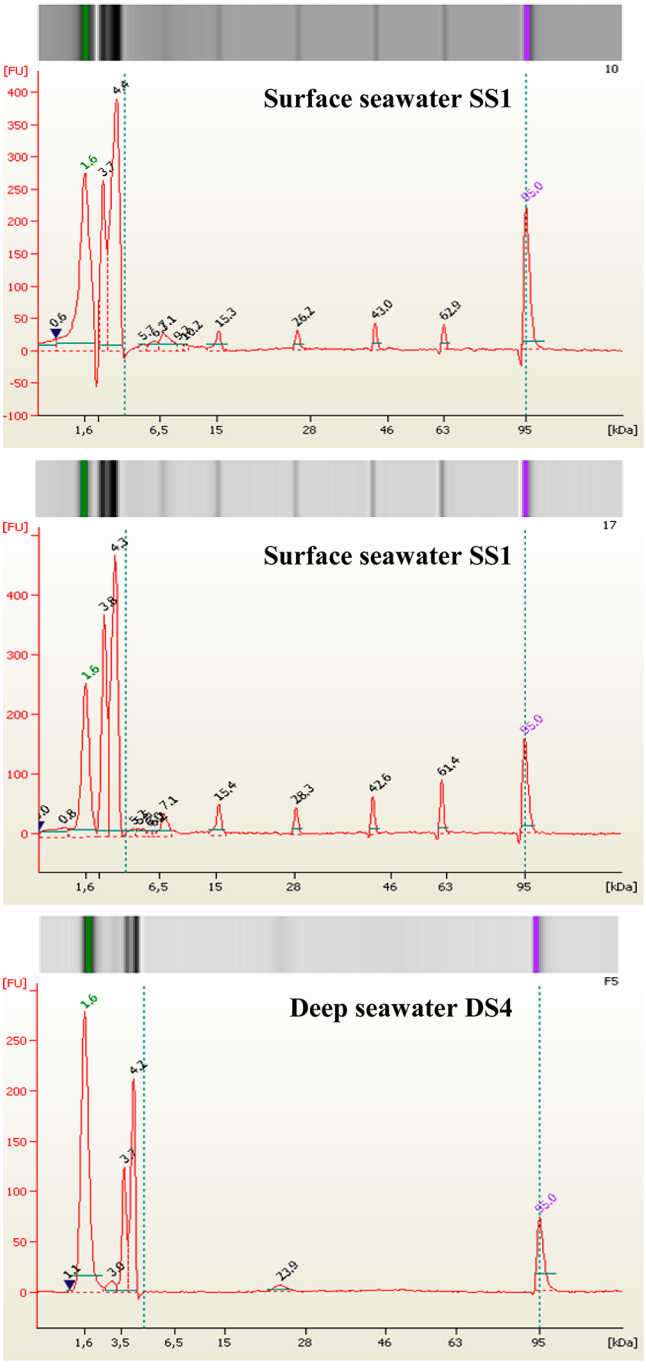

Figure 2. Electropherograms of selected OFFGEL fractions for dissolved proteins in surface (F10 and F17) and deep (F5) seawater samples.

attributed to the disulfide bridges breakdown, which leads to a different migration behavior.[39] By performing LOC electrophoresis in triplicate, a mean molecular weight of 72.4 ± 0.19 kDa (n = 6) was measured for albumin, whereas the albumin mass measured was 0.22 ± 0.011 mg (n = 6), offering a mean analytical recovery of 109 ± 6% (n = 6). Therefore, albumin is not partially retained onto the IPG dry strips and remains in the liquid fraction. Good analytical recovery can therefore be expected for dissolved proteins.

Metal Binding Proteins Stability Studies. Data reported by Jiménez et al.[40] show the influence of different PAGE conditions when assessing two metal-binding proteins with different metal−protein affinities such as superoxide dismutase, SOD (two units of molecular weight of 16.2 kDa for each one and the presence of Cu and Zn), and alcohol dehydrogenase, ADH (tetramer of molecular weight of 141 kDa containing four equal subunits of 32.5 kDa and the presence of Zn).

To test the effect of the denaturing OFFGEL conditions on the stability of the metal−protein bindings, experiments by loading SOD and ADH aqueous solutions (2.8 and 3.1 mg, respectively, in 4.0 mL of Protein OFFGEL stock solution) were performed. Regarding SOD, LOC electrophoresis showed the presence of this protein in OFFGEL fractions 9 to 12 (pIs of 5.43, 5.73, 6.04, and 6.34), with a molecular weight of 18.6 kDa, quite similar to the theoretical value (16.2 kDa). The determination of Zn in all 24 OFFGEL fractions proved the presence of Zn in the OFFGEL fractions containing SOD (fractions 9 to 12). The Zn concentrations in these four fractions were 6.9 ± 0.2, 26.6 ± 1.1, 3.0 ± 0.1, and 0.7 ± 0.1 mg L^{-1}, respectively, and negligible Zn concentrations were found in the remaining OFFGEL fractions. A mass balance study, implying the Zn concentration in the SOD solution (2.2 ± 0.08 mg g^{-1}), and the Zn concentration as a sum of Zn concentrations in fractions 9 to 12 (2.4 ± 0.05 mg g^{-1}), shows a Zn percentage of 109%. However, Cu was not found in the OFFGEL fractions containing SOD (fractions 9 to 12), but it was found in fractions 22 (11.4 ± 0.4 mg L^{-1}) and 23 (6.10 ± 0.03 mg L^{-1}). Similarly, the Cu content in a SOD solution (2.3 ± 0.1 mg g^{-1}), the negligible Cu concentrations in the OFFGEL fractions 9 to 12 (presence of SOD), and the presence of Cu in fractions 22 and 23 (absence of SOD, 0.80 ± 0.02 and 0.40 ± 0.002 mg g^{-1}, respectively) suggest that the Cu binding SOD is completely broken down under the used OFFGEL conditions. In addition, a Cu percentage of 53% is found in fractions 22 plus 23, which means that the remaining Cu released from SOD must be spread among other OFFGEL fractions. Electropherograms when subjecting the OFFGEL fractions 22 and 23 to LOC electrophoresis showed a signal for low molecular weights (6.5 kDa). The proteinaceous and/or amphoteric nature of these compounds can be responsible for complexing the major amount of released Cu. In addition, the presence of Cu is not attributed to metal contamination because analysis of the OFFGEL stock standard solution and OFFGEL fractions from ultrapure water subjected to the whole process gave negligible metal concentrations.

OFFGEL results when analyzing ADH showed the presence of this protein in the fractions 9, 10, 11, 12, 13, and 14 (pIs of 5.43, 5.73, 6.04, 6.34, 6.74, and 7.1, respectively), whereas LOC electrophoresis gave a molecular weight of 39.8 kDa, quite similar to the theoretical value (32.5 kDa). The determination of Zn showed the presence of this metal in the OFFGEL fractions 9, 10, and 11 (5.2 ± 0.06, 22.2 ± 0.3, and 2.30 ± 0.07 mg L^{-1} respectively), fractions which contain the ADH protein. In addition, negligible Zn concentrations were found in the remaining OFFGEL fractions. Results were confirmed by a mass balance study after analyzing an ADH solution for Zn (2.10 ± 0.06 mg g^{-1}) and comparing it to the Zn concentrations as a sum of Zn concentrations in fractions 9, 10, and 11 (2.10 ± 0.08 mg g^{-1}), which offered a Zn percentage of 100%.

Therefore, Zn-binding SOD/ADH appears to offer high affinity, whereas Cu-SOD offers a weak metal−protein affinity. In general, OFFGEL conditions are less drastic than those inherent to conventional PAGE,[40] and at least the integrity of the Zn-binding SOD/ADH appears to be guaranteed.

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Table 2. Concentrations of Metal Binding Dissolved Proteins in Surface and Deep Seawater

	SS1 metal concentrations (μg L^{-1})								
	Cd	Co	Cr	Cu	Fe[a]	Mn	Ni	Pb	Zn[a]
F7	<1.1	<9.8	<41	<8.8	3.1 ± 0.1	<16	<35	<41	<0.05
F8	<1.1	<9.8	<41	<8.8	2.9 ± 0.1	<16	<35	<41	<0.05
F9	<1.1	<9.8	<41	<8.8	3.5 ± 0.1	<16	<35	<41	<0.05
F10	2.8 ± 0.4	<9.8	<41	<8.8	2.8 ± 0.1	<16	<35	<41	1.0 ± 0.1
F17	3.1 ± 0.6	<9.8	<41	12.0 ± 1.0	2.6 ± 0.1	<16	<35	<41	<0.05
F18	2.0 ± 0.3	<9.8	<41	20.5 ± 1.2	2.6 ± 0.1	22.4 ± 1.2	43.6 ± 2.5	<41	<0.05
	DS4 metal concentrations (μg L^{-1})								
	Cd	Co	Cr	Cu	Fe[a]	Mn	Ni	Pb	Zn[a]
F2	<1.1	<9.8	<41	<8.8	<0.37	<16	<35	<41	<0.05
F3	<1.1	<9.8	<41	<8.8	<0.37	<16	<35	<41	<0.05
F4	<1.1	<9.8	<41	<8.8	<0.37	84.1 ± 4.4	<35	<41	<0.05
F5	<1.1	<9.8	<41	20.3 ± 1.2	<0.37	68.1 ± 2.2	<35	<41	<0.05

[a]Expressed as mg L^{-1}.

Application. *Dissolved Proteins Assessment.* Three surface seawater samples and one deep seawater sample were analyzed for dissolved proteins by 2D IEF OFFGEL–LOC electrophoresis after tangential and centrifugal ultrafiltration and marine protein precipitation. Proteins were detected in OFFGEL fractions 7, 8, 9, 10, 17, and 18 (pIs of 4.82, 5.13, 5.43, 5.73, 8.10, and 8.37) in surface seawater SS1 (Figure 1), whereas deep seawater DS4 analysis (Figure 1) showed the presence of dissolved proteins in OFFGEL fractions 2, 3, 4, and 5 (pIs of 3.30, 3.61, 3.91, and 4.22). The obtained pIs of dissolved proteins in surface seawater are similar to those offered by Yamada and Tanoue,[17] after conventional 2D electrophoresis, who have reported dissolved proteins of pIs within the 5.0–8.0 range. However, the pIs of the isolated proteins in the deep seawater are lower than those reported by the same authors for deep seawaters (from 5.1 to 8.7).[18] A possible explanation must be the different depth of the sampled seawaters, between 150 and 1000 m in depth in Yamada and Tonoue's studies,[18] and 50 m in depth in the current study.

Proteins were not detected in any OFFGEL fractions obtained from surface seawater samples coded as SS2 and SS3. After LOC electrophoresis of OFFGEL fractions from seawater sample SS1, several proteins exhibiting different molecular weights were measured (values are listed in Table 1). As an example, Figure 2 shows the electropherograms when analyzing the OFFGEL fractions 10 and 17 (surface seawater SS1) and OFFGEL fraction 5 (deep seawater DS4). The isolated protein-offered molecular weights ranging from 15 to 63 kDa in most of the OFFGEL fractions, values more similar than those reported by Yamada and Tanoue, were molecular weights from 20 to 48 kDa,[17] and between 16 and 48 kDa.[18] Regarding dissolved proteins in OFFGEL fractions from the deep seawater sample DS4, molecular weights ranged from 21 to 24 kDa. Although a small number of samples have been analyzed, dissolved proteins in deep seawater samples are of lower molecular weight (and also different pIs) that those determined in surface seawater. The molecular weights of the isolated proteins are also lower that those reported by Yamada and Tanoue, when analyzing dissolved proteins of deep seawater between 31 and 48 kDa.[18] Similarly, it must be pointed out that the deep seawater analyzed in the current work (50 m depth) is quite different than those deep seawater samples analyzed by Yamada and Tanoue[18] of depths within the 150–1000 m range.

Table 1 also lists the relative protein concentrations of each OFFGEL fraction. With regard to the surface seawater sample (SS1), a high concentration was measured for protein content in the OFFGEL fraction 17 (relative concentration of 188.2 ng μL^{-1}). Other OFFGEL fractions showed protein concentrations from 4.4 ng μL^{-1} (OFFGEL fraction 8) to 120 ng μL^{-1} (OFFGEL fraction 7). Protein concentrations in the deep seawater (DS4) varied from 13.7 ng μL^{-1} (OFFGEL fraction 4) to 58.3 ng μL^{-1} (OFFGEL fraction 2).

Quantification of Metals Bound to Dissolved Proteins. The concentrations of trace elements (Table 2) were assessed in all the OFFGEL fractions (see ETAAS Measurememts and ICP-OES Measurements). With regard to the surface seawater SS1, cadmium has been found to be associated to some of the protein content in OFFGEL fractions 10, 17, and 18; whereas, copper was mainly associated to some of the proteins in OFFGEL fractions 17 and 18 (alkaline pIs). Manganese and nickel were mainly found to be bound to certain proteins in OFFGEL fraction 18 (alkaline region), while zinc is associated with certain proteins of acid pIs (OFFGEL fraction 9 and 10). Finally, iron was detected in all OFFGEL fractions. With regard to deep seawater DS4, Cu was found to be bound to protein content in OFFGEL fraction 5; whereas, Mn was quantified in proteins of OFFGEL fractions 4 and 5. Therefore, dissolved proteins in surface seawater appear to offer a larger binding capacity for trace elements than dissolved proteins in deep seawaters. In general, elements such as Cu, Fe, Mn, Ni, and Zn have been found to be bound to dissolved proteins. These elements are considered to be essential for life, and they are integrated in different biological macromolecules.[41] However, it must pointed out that certain dissolved proteins in surface seawater are also associated with toxic elements such as cadmium.

With regard to stability of the metal-binding dissolved proteins, OFFGEL fractions 22 and 23 were analyzed for Cu, Fe, Mn, Ni, and Zn, and negligible concentrations of these elements were found. Therefore, the used OFFGEL operating conditions appear to guarantee the stability of the metal-binding dissolved proteins. Experiments that could show the nature of the metal-binding proteins (metal–protein complexes

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or metalloproteins) have not been developed in the current work. However, because the metal ions found are not usually integrated in the amino acids present in proteins, the isolated metal-binding proteins must be metal−protein complexes.[42]

CONCLUSIONS

Dissolved proteins were efficiently isolated from surface and deep seawater after successive tangential ultrafiltration and centrifugal ultrafiltration of the retentate fraction (10 kDa cut off). The overall procedure involves a preconcentration factor of 3000 (60 L of seawater to 20 mL of ultrafiltrated retentate). An improved method based on methanol/chloroform/water was optimized for achieving an adequate protein pellet for IEF OFFGEL and LOC electrophoresis. The optimized procedure guaranteed a successful isoelectric focusing and further proteins' separation in the basis of their different molecular weights. LOC electrophoresis was demonstrated to not be affected by the incubation time and temperature, and best performances were achieved by subjecting the redissolved protein pellet at a temperature of 95 °C for only 5 min before analysis. Most of the isolated proteins found in surface seawater offer alkaline pIs (8.10 and 8.37) and molecular weights between 15 and 63 kDa. These proteins were found to be mainly associated with several trace elements such as Cd, Cu, Fe, Mn, and Ni. Dissolved proteins exhibiting acid pIs, from 4.82 to 5.73 (15 − 63 kDa), were also detected in surface seawaters, and Cd, Fe, and Zn were found to be bound by them. Dissolved proteins in deep seawaters offered acid pIs (3.30−4.22) and lower molecular weights (within the 21−24 kDa range). In addition, low concentrations of essential elements such as Cu and Mn were also found. Limitations of OFFGEL−LOC electrophoresis, regarding protein identification as well as metal bound to dissolved proteins, will lead us to develop further studies by 2DE for protein identification by MS/MS techniques, as well as metal determination in the isolated proteins by LA-ICP-MS.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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