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Assessment of metals bound to marine plankton proteins and to dissolved proteins in seawater

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Abstract

Studies based on laser ablation – inductively coupled plasma – mass spectrometry (LA-ICP-MS) have been performed to assess metal bound to dissolved proteins and proteins from marine plankton after two-dimensional polyacrylamide gel electrophoresis (2D PAGE). Dissolved proteins were pre-concentrated from surface seawater (60 L) by tangential ultrafiltration with 10 kDa molecular weight cut-off (MWCO) membranes and further centrifugal ultrafiltration (10 kDa) before proteins isolation by methanol/chloroform/water precipitation**.** Proteins isolation from plankton was assessed after different trichloroacetic acid (TCA)/acetone and methanol washing stages, and further proteins extraction with a phenol solution. LA-ICP-MS analysis of the electrophoretic profiles obtained for dissolved proteins shows the presence of Cd, Cr, Cu, and Zn in five spots analyzed. These proteins exhibit quite similar molecular weights (within the 10 – 14 kDa range) and pIs (from 5.8 to

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7.3). Cd, Cr, Cu, and Zn have also been found to be associated to proteins isolated from plankton samples. In this case, Cd has been found to be bound to proteins of quite different molecular weight (9, 13 and 22 kDa) and pIs (4.5, 5.2, 5.5, and 10). However, trace elements such as Cr, Cu and Zn appear to be mainly bound to plankton proteins of low molecular weight and variable pI..

Keywords

2D polyacrylamide gel electrophoresis, laser ablation inductively coupled plasma mass spectrometry, metal-binding proteins, phytoplankton, dissolved proteins

1. Introduction

Plankton plays an important role in the ocean's carbon cycle. Phytoplankton in surface seawater, mainly photosynthetic cyanobacteria and single-celled algae [1], is responsible for about half the photosynthetic fixation of carbon (primary production) on Earth, and it is a primary producer of dissolved organic matter (DOM) in oceans [1,2]. DOM assessment in marine ecosystems is important because of the complexing properties of DOM for several dissolved substances which conditions the fate of trace metals and hydrophobic organic contaminants by marine biota [3].

In addition to various macro- and micro- nutrients, non-essential or toxic metals are present at low concentrations in seawater. Trace metals can be captured by phytoplankton, and introduced into the marine food chain by producing extracellular organic matter with metal complexing properties [4]. Some metals, such as Cu, Zn, Fe, Cr and Co, are essential micronutrients for phytoplankton and act as co-factors in different enzymatic reactions. As an example, Fe is involved in phytoplankton growth; whereas, Cu plays an important role in photosynthesis. Other metals such as Pb and Cd are non-essential or toxic. These elements may displace bioactive trace metals in enzymes and thus they can alter the proper enzymes

47 activity. Obviously, these changes produce significant effects at the different trophic levels in
48 the aquatic food chain [5,6]. Therefore, metal-proteins complexes and metalloproteins are an
49 important fraction of DOM as well as important constituents in marine plankton. Metal-
50 binding proteins with high-affinity interactions are considered as metalloproteins, while
51 metal-binding proteins with low-affinity interactions (easily broken) are referred to metal-
52 protein complexes. Both, metalloproteins and metal-protein complexes, can be involved in
53 complex biochemical reactions and can participate in several biological functions [7,8]. For
54 this reason, metal-binding proteins assessment in phytoplankton, as well as the determination
55 and characterization of DOM and dissolved proteins in water are issues of interest.
56 One or two-dimensional polyacrylamide gel electrophoresis (1D or 2D-PAGE) with
57 subsequent laser ablation inductively coupled plasma mass spectrometry (LA–ICP–MS) has
58 been increasingly used for the analysis of metalloproteins and metal-protein complexes [9].
59 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is commonly used
60 for the separation of protein mixtures on the basis of their molecular size. This technique
61 applied in the one-dimensional mode has also been used for isolating dissolved proteins, but
62 it fails when resolving complex protein mixtures [10-12]. In this case, 2D-PAGE is a
63 powerful and sensitive technique for separating individual proteins from complex samples. In
64 the first dimension, proteins are separated according to their isoelectric point (pI) by
65 isoelectric focusing (IEF); whereas, separation according to the molecular weight (Mw) is
66 achieved in the second dimension. 2D-PAGE has been used for characterizing Mws of both
67 particulate and dissolved proteins in surface seawater, and proteins from marine plankton
68 [13-15].
69 The choice of adequate sample preparation methods is important for obtaining reliable results
70 by electrophoresis analysis. Different protein extraction procedures from plankton have been
71 reported [16-19]. Jiménez et al. [16] have found that a treatment with a buffer solution

consisting of 25mM Tris–HCl and a commercial cocktail protease inhibitor, followed by protein precipitation with acetone was the most efficient procedure for protein extraction from plankton. Recently, García-Otero et al. [17] have proposed a protein extraction method for plankton which combines different washing stages with trichloroacetic acid (TCA)/acetone, and with methanol, before proteins extraction with phenol. Regarding dissolved proteins, main problems arise from the low concentrations in seawater and from the presence of high levels of dissolved salts. Therefore, pre-concentration methods for isolating the dissolved proteins from the saline matrix are needed. The literature shows that tangential ultrafiltration (UF) procedures are more adequate than solid phase extraction (SPE) methods for isolating dissolved compounds of high molecular weight such as dissolved proteins [10,11,13-15,20,21]. The selection of UF membranes of 10 kDa molecular weight cut-off guarantees the successful isolation of dissolved proteins in a minimum volume of retentate, which offers high pre-concentrations factors [22]. Further salts removal is also needed when performing chromatographic and electrophoresis analysis [22]. When dealing with dissolved protein, the formation of the protein pellets guarantees that most of dissolved salts remains in the liquid phase of the retentate. However, some protein precipitation procedures such as those based on ice-cold acetone (protein pellet formation at –20°C for 2 h) [23] have proved to be unsuccessful for further protein separation by OFFGEL electrophoresis [24]. In these cases, trichloroacetic acid as a precipitating reagent [10,20], and combined procedures based on water and methanol soluble interferences (salts) removal before protein precipitation by chloroform [20] have offered reliable electrophoresis separations [24].

The assessment of metals bound to proteins is commonly performed by direct analysis of gels by LA-ICP-MS. In addition to the development by Jiménez et al. [16] for assessing metals bound to proteins from plankton, LA-ICP-MS methods after 1D and 2D electrophoresis have been performed for detecting P in standard phosphorylated proteins [25], for assessing Cu-,

Zn- and Fe-containing human brain proteins [26], Zn-containing proteins in slug (*Genus Arion*) tissues [27], Se-containing proteins from sunflower leaves [28], Cd- and Zn-binding proteins in *Spinacia oleracea* [29], and determining Cu, Fe, Zn, Mn and Pb in metalloproteins from rat kidney [30]. Other developments involve the assessment of metal-humic acid complexes [31].

There are few developments regarding metal bound to proteins from plankton. Jiménez et al. [16] have recently applied LA-ICP-MS to assess metal bound to plankton proteins after 1D electrophoresis. However, this application has been performed with a plankton-based certified reference material (BCR-414), and the assessment of metal bound to proteins from fresh marine plankton remains un-reported. In addition**,** although various published papers on dissolved proteins characterization in seawater by 1D and 2D electrophoresis can be found in the literature, LA-ICP-MS developments focused on determining/identifying metals bound to dissolved proteins have not yet been addressed**.** The aim of the current work has been the application of LA-ICP-MS for determining metal-containing proteins from fresh marine phytoplankton and dissolved proteins in seawater. Detection/determination of trace metals associated to the isolated proteins has been performed after 2D-PAGE. Possibilities and problems found when applying the proposed methodology for assessing metals binding dissolved proteins and proteins from fresh phytoplankton are fully discussed.

2. Experimental

2.1. Apparatus

LA-ICP-MS measurements were performed with an UP-213 Nd-YAG LA system operating at 213 nm (New Wave Research, Huntingdon, UK) coupled to an ICP-MS (Elan 6000, Perkin Elmer Sciex, Toronto, Canada). Hitachi double–beam spectrophotometer model U–2010 (Hitachi, Berkshire, UK) equipped with 10 mm quartz cells was used for all UV–visible measurements. Isoelectrofocusing was performed with a Protean IEF System from Bio-Rad

(Hercules, CA, USA), and second dimension was run in a Protean XL (BioRad). The gels were vacuum dried before LA-ICP-MS measurements with a model 583 gel drier (Bio-Rad). The tangential flow ultrafiltration (UF) system consisted of a Masterflex I/P pump (Millipore, Bedford, MA, USA), a Prep/Scale-TFF Cartridge (Millipore) with a polyethersulfone membrane (nominal MW cut-off 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 centrifuge Centromix (Selecta, Barcelona, Spain), a Reax top shaker from Heidoph (Schwabach, Germany), and an Basic 20 pH–meter with a glass–calomel electrode (Crison Instruments S.A., Barcelona, Spain).

2.2. Reagents and material

Ultrapure water, resistance 18 MΩcm, was obtained from a Milli-Q water-purification system (Millipore). Centrifugal ultrafiltration was performed with Vivaspin 20 ultrafiltration tubes (polyethersulfone membrane of 10 kDa molecular cut-off) from Sartorius Stedin Biotech (Goettingen, Germany). Seawater filtration was achieved by using Millipore HAWP14250 0.45 μm mixed esters of cellulose membrane filters (140mm diameter). Total protein assessment was performed with a Bradford protein assay containing Coomassie Brilliant Blue G-250 and bovine serum albumin (BSA) from Thermo Scientific (Rockford, IL, USA). IPG Dry Strips (7 cm, pH 3-10; and 10 cm, pH 3-10), and Plus One DryStrip cover fluid (GE Healthcare Life Science, Uppsala, Sweden) were used for pI protein fractionation. Buffer solution (10mL) for equilibration after IEF was prepared with 6M urea, 0.375M Tris-HCl (pH 8.8), 20% (v/v) glycerol, 2% (w/v) sodium dodecyl sulphate (SDS), 0.002% (w/v) bromophenol blue and 100mg DTT (all reagents from Sigma Aldrich, St. Louis, MO, USA)

SDS gel (10mL) was prepared with 2.3 mL of water, 5 mL of 30% acrylamide mix (Bio Rad), 2.5 mL of 1.5 mol L-1 Tris-HCl pH 8.8 (Sigma-Aldrich), 0.1 mL of 10% SDS (Sigma-

147 Aldrich), 0.1 mL of 10% ammonium persulfate (Bio Rad) and 0.004 mL of
148 tetramethylethylenediamine (TEMED) (Bio Rad). Trichloroacetic acid, chloroform and
149 acetone were from Panreac (Barcelona, Spain). Tris(hyddroxymethyl)aminomethane (Tris),
150 sodium dodecyl sulphate (SDS), glycine, ammonium persulfate, and N,N,N′, N′-
151 tetramethylethylenediamine (TEMED) were from Sigma Aldrich. Phenol solution,
152 equilibrated with 10 mM Tris-HCl (pH 8.0, 1 mM EDTA) was provided by Sigma Aldrich.
153 2-DE rehydration solution was prepared with 5 M urea, 2 M thiourea, 2 mM tributyl-
154 phosphine (Sigma-Aldrich), 65 mM DTT, 65 mM CHAPS (Sigma-Aldrich) , 0.15 M NDSB-
155 256, 1 mM sodium vanadate (Sigma-Aldrich) , 0.1 mM sodium fluoride (Sigma-Aldrich), and
156 1 mM benzamidine (GE Healthcare Life Science). The running buffer (4L) was prepared with
157 glycine (576g) (Sigma-Aldrich), Tris-HCl pH 8.8 (121.1g) and SDS (40g). The SDS buffer
158 for protein extraction from plankton was prepared with 30%(m/v) sucrose (AppliChem
159 (Darmstad, Germany), 2%(m/v) SDS, 0.1 M Tris-HCl, pH 8.0, 5%(v/v) 2-mercaptoethanol
160 (Fluka, Vancouver, Canada) and protease inhibitor cocktail (Roche Diagnostics, Mannhein,
161 Germany). Sodium hydroxide (Merck), used for UF membrane cleaning, and ammonium
162 hydrogencarbonate (BDH, Poole, UK), for salt removal after retentate preparation by
163 centrifugal UF, were used when treating seawater. Kit for silver staining polyacrylamide gels
164 was purchased from Bio Rad. Element standard solutions (used for metal quantification) were
165 prepared from cadmium, cobalt, chromium, copper, iron, manganese, nickel, lead, and zinc
166 stock standard solutions (1.000 g L^{-1}) from Scharlau (Barcelona, Spain).
167
168 **2.3. Sample collection**
169 Surface seawater samples (1-2 m depth, 60L) were collected from the Ría de Arousa estuary
170 (north-western Spain) in pre-cleaned 12L non-metallic free-flushing Niskin bottles attached

171 to a 1015 rosette multibottle array (General Oceanics, Miami, FL, USA). After collection,
172 samples were filtered (0.45µm) and immediately subjected to the tangential flow UF.
173 Fresh plankton samples, consisting of mixture of microalgae (phytoplankton), were collected
174 in a clam hatchery located at the Ría de Arousa estuary in pre-cleaned 5L bottles. After
175 collection, plankton was concentrated by removing the supernatant after centrifugation at
176 4000 rpm for 15 min at 20ºC.

177 **2.4. Protein extraction**

178 *2.4.1. Extraction of dissolved proteins from seawater samples*

179 Filtered seawater samples (60L) were treated with sodium azide (final concentration referred
180 to 60 L of seawater of 5.0 mM) for avoiding sample degradation, and also with SDS (final
181 concentration referred to 60 L of seawater of 0.01% (m/v)) for minimizing proteins
182 adsorption onto the UF membrane. Preserved samples were then subjected to tangential flow
183 UF through a polyethersulfone membrane (size 0.6 m^2, nominal molecular mass cut-off of 10
184 kDa) until recovering approximately 400–600mL of retentate (ultrafiltrate containing
185 substances of molecular weight higher than 10 kDa). The UF system was previously cleaned
186 by passing 2L of 0.1M NaOH at 45 ± 5ºC, followed by rinsing with 9L of Milli-Q water, also
187 at 45 ± 5ºC. After tangential flow UF, the retentate was further concentrated and desalted
188 with a cleaning solution containing 0.01% (m/v) SDS and 35mM ammonium
189 hydrogencarbonate by centrifugal ultrafiltration (ultrafiltration tubes with polyethersulfone
190 membrane of 10 kDa molecular cut-off), until obtaining a concentrated and desalted retentate
191 of 20 mL (pre-concentration factor of 3000). Finally, protein pellets were obtained by
192 precipitation with chloroform after water and methanol soluble interferences removal [24].
193 The protein pellet was partially dried under a N_2 steam, and then re-dissolved in 50 µL of the
194 buffer solution for equilibration after IEF.

195 *2.4.2. Extraction of proteins from plankton samples [17]*

196 A portion of concentrated plankton sample was first grinded into a fine powder in a mortar
197 and pestle under liquid nitrogen. Six independent portions of 0.1g of the grinded plankton
198 were introduced into 2mL tubes, and the sample was subjected to different washing stages
199 before protein extraction. First, 2mL of 10%(v/v) TCA in acetone were added, and after
200 vortexing for a few seconds, and further centrifugation at 10000 rpm, 4ºC for 3 minutes, the
201 supernatant containing interfering substances was discarded. Then, washing was performed
202 with 2mL of 0.1 M ammonium acetate in 80/20 methanol/water mixture (vortex shaking,
203 centrifugation at 10000 rpm, 4ºC for 3 minutes, and supernatant discarding), followed by a
204 final washing with 2mL of a 80/20 acetone/water solution, and final oven-drying at 50ºC for
205 at least 10 minutes. Protein extraction was performed by adding 0.4 mL of phenol (pH 8.0)
206 plus 0.4mL of SDS buffer (30%(m/v) sucrose, 2%(m/v) SDS, 0.1 M Tris-HCl, pH 8.0,
207 5%(v/v) 2-mercaptoethanol and protease inhibitor cocktail) into each tube. The mixture was
208 vortexed thoroughly and was kept at room temperature for 5 minutes. The phenol phase was
209 then separated by centrifugation (10000 rpm, 4ºC) for 3 minutes, and the upper phenol phase
210 containing proteins was transferred to a 2mL tube. The phenol phase was finally treated with
211 5 volumes of cold methanol containing 0.1M ammonium acetate (approximately 2mL), and
212 the mixture was stored at -20ºC for 2 hours to overnight. Precipitated proteins were recovered
213 by centrifugation at 2500 rpm for 5 minutes (4ºC), and successively washed with 100%
214 methanol and 80/20 acetone/water. Protein pellet obtained was finally dried under a N_2
215 stream, and re-dissolved in 300 µL of 2-DE rehydration solution.

216 **2.5. Protein quantification by the Bradford assay**

217 Total protein determination in the re-dissolved protein pellets was assessed by the Bradford
218 method, which uses Coomassie Brilliant Blue G-250 as a chromogenic reagent (protein-dye
219 complex with maximum absorbance at 595nm). Calibration was performed with BSA as a
220 protein standard prepared in the 2-DE rehydration solution (stock concentration of 1000 µg

221 mL^{-1}). The standards were prepared by diluting the stock standard solution with the 2-DE
222 rehydration solution before mixing with the chromogenic reagent. Preparation of standard
223 was as follows: vial A (1000 µg mL^{-1}) consists of 40 µL of BSA stock solution; vial B (500
224 µg mL^{-1}), 20 µL of vial A plus 20 µL 2-DE rehydration solution; vial C (250 µg mL^{-1}), 20
225 µL vial B plus 20 µL 2-DE rehydration solution; vial D (125 µg mL^{-1}), 20 µL vial C plus 20
226 µL 2-DE rehydration solution; vial E (62.5 µg mL^{-1}), 20 µL vial D plus 20 µL 2-DE
227 rehydration solution; vial F (31.25 µg mL^{-1}), 20 µL vial E plus 20 µL 2-DE rehydration
228 solution; and vial G (0.00 µg mL^{-1}), 20 µL 2-DE rehydration solution. These solutions,
229 except solution in vial F, from which 20 µL were pipetted and diluted**,** were mixed with 600
230 µL of Coomassie Brilliant Blue G-250 (final volume of 620 µL) which offer BSA
231 concentrations of 0.0, 1.0, 2.0, 4.03, 8.06, 16.1, and 32.26 µg mL^{-1}. Samples (re-dissolved
232 protein pellets) were prepared by mixing 1 µL with 19 µL of 2-DE rehydration solution
233 before addition of 600 µL of chromogenic reagent. Finally, both samples and standards were
234 kept at room temperature for at least 5 min before spectrophotometric quantification at 595
235 nm.

236 **2.6. Separation of proteins by 2D PAGE**

237 Aliquots of the re-dissolved protein pellet from plankton samples (300 µg) were applied to
238 IPG strips, previously rehydrated for 12 h, for IEF. The IPG strips (17 cm, pH 3-10) were
239 then recovered with DryStrip Cover Fluid, and IEF was performed using a Protean-IEF and
240 applying the following program: voltage at 250V for 15min, 2h linear gradient until 4000V,
241 4h linear gradient until 8000V, 1h at 8000V, and, stop and hold at 500V. After IEF, the IPG
242 strips were covered with a buffer solution (equilibration buffer described in section 2.2) for
243 15 min. SDS-PAGE was performed using 15% polyacrylamide gel by applying an electric
244 current of 15mA at 10ºC for 6 h. A molecular weight marker containing proteins with the 2 -
245 250 kDa molecular weight range was used for assessing molecular weights of the separated

246 proteins (Dual Xtra Precision Protein Prestained Standards, BioRad). Finally, the 2D-PAGE
247 gels were silver nitrate stained following standard procedures.
248 IEF (IPG strips pH 3-10, 7 cm) for dissolved proteins from seawater sample (50 µg of re-
249 dissolved protein pellet were loaded) was performed applying the following program: voltage
250 at 250V for 15min, 2h linear gradient until 4000V, 1h at 4000V, and, stop and hold at 500V.
251 SDS-PAGE when fractionating dissolved proteins was also performed using 15%
252 polyacrylamide gel under the same operating conditions than those listed above (15mA,
253 10ºC) but for 1.5 h.
254 Before LA-ICP-MS analysis, gels were vacuum dried at 80ºC for 2 h by placing them on
255 chromatography paper and covering with a porous cellophane sheet.
256 **2.7. LA-ICP-MS measurements and sample preparation**
257 LA was performed with an Nd-YAG LA system operating at 213 nm. Before LA
258 experiments, the ICP-MS instrument was set for routine multi-element analysis in accordance
259 with the manufacturer´s instructions: the nebulizer gas flow rate, lens voltage, and daily
260 performance of the instrument were optimized by aspirating a solution containing Rh, Mg,
261 Pb, Ba, and Ce (10 µg L^{-1} of each one), while autolens calibration was performed by
262 aspirating a solution of Be, Co, In and U (10 µg L^{-1} of each one). The plasma was
263 extinguished and the spray chamber and nebulizer assembly were replaced with LA transfer
264 line and its adapter. Argon was used to transport sample aerosol to the ICP-MS. The final
265 optimization of lens voltage and Ar carrier gas flow for dry plasma conditions was performed
266 by monitoring the ^{63}Cu intensity of a NIST sample. Dried gels sections corresponding to the
267 protein migration zone were cut with a size to fit in the ablation chamber for LA-ICP-MS
268 analyses. The gels sections were fixed to the support using double-sided adhesive tape. This
269 operation was required to prevent the gel can shrivel and to maintain a flat surface during the
270 laser ablation process. Each protein spot was ablated in raster mode, and different mass-to-

charge ratios for each metal were monitored. Signal drift correction was performed by using ^{13}C as an internal standard. Matrix-matched standards with known metals concentrations were used for metal quantification in gels.

3. Results and discussion

3.1. 2D-SDS-PAGE

The assessment of total protein contents in protein pellets from surface seawater and plankton samples was firstly performed by the Bradford assay (section 2.5). Regarding plankton samples, determinations in duplicate showed total protein concentrations of 9.5±0.5 and 10.4±0.5 μg μL^{-1}. However, very low total protein content was determined in protein pellets from surface seawater, a value of 2.9±0.2 μg μL^{-1} was found (pre-concentration factor of 3000). Therefore, a mass of 300 μg of protein pellet from plankton samples was used for performing the 2D-SDS-PAGE; whereas, the total amount of the isolated protein pellet from seawater (50 μg) was loaded for 2D-SDS-PAGE experiments.

3.1.1. Proteins from plankton samples

Examination of silver-stained 2DE gels obtained when loading pellets from plankton demonstrated the existence of several proteins, around 80 – 100 different protein spots. Some of them offer a low intensity, which means that proteins occur at low concentrations; and also, some of the protein bands are not properly resolved. As an example, 2DE gel obtained from plankton sample coded as P1 is shown in Figure 1. Well-resolved and intense protein bands are highlighted and coded in different rectangles. These protein spots were further cut and subjected to LA-ICP-MS for assessing metal bound to them. In general, proteins exhibit molecular weights ranging from 5 to 50 kDa with pI between 3 and 10. Table 1 lists the approximate molecular weights and isoelectric points calculated for each protein band (protein spots in rectangles 1 to 18 in Figure 1 for plankton sample P1), and also for protein spots from plankton sample P2 (2D-PAGE gel not given). The occurrence of single protein

296 spots was detected at Mw of 31 kDa for pIs around 6.5 and 7.5 (rectangles 3 and 4 in Figure
297 1) in both plankton samples. In addition, other single protein spots were also observed for
298 proteins of high Mw (18 kDa) and alkaline pI (9.7), as protein spot in rectangle 9; for proteins
299 of low Mw (6 kDa) and acid pI (5.0), rectangle 18; and for proteins of variable Mw (10, 16,
300 20 and 22 kDa) and neutral pIs (within the 6.5 – 7.5 range), single protein spots in rectangles
301 5, 6, 13 and 14. Other proteins were found to exhibit similar Mws and pIs. This is the case of
302 proteins in rectangle 1 (Mw of 23 kDa and pIs from 4.0 to 4.6), proteins in rectangle 2 (Mw
303 of 25.5 and 22 kDa, and pIs from 5.3 to 6.0), proteins in rectangle 7 (Mw/pI ratio of 20-
304 21/7.5-8.0), proteins in rectangle 8 (Mw/pI ratio of 22/9.1-9.4), proteins in rectangle 10
305 (Mw/pI ratio of 10.5/3.9-4.1), proteins in rectangles 11 and 12 (Mw/pI ratio of 11-13/4.5-
306 6.0), proteins in rectangle 15 (Mw/pI ratio of 11-12/8.0-8.2), and proteins in rectangles 16
307 and 17 (Mw/pI ratio of 8.0/9.4-10).
308 Most of the results agree with those obtained when analyzing similar plankton samples by
309 OFFGEL-lab-on-chip (LOC) electrophoresis [17,32]. Although the higher resolution of the
310 latter technique allowed the identification of more protein bands, coincidences for several
311 Mw/pI ratios, mainly for single protein spots, are observed.
312 **3.1.2. Proteins from surface seawater**
313 Examination of silver-stained 2D-PAGE gel from surface seawater (Figure 2) demonstrated
314 the occurrence of few protein spots (denoted as *a* to *e* in Figure 2). Proteins exhibit similar
315 Mws, within the 10 – 14 kDa range; whereas, pIs vary from 5.8 to 7.3 (Table 2).Results agree
316 with those previously reported for surface seawater by 2D-PAGE, Mw from 16 to 48 kDa
317 [14,15], and also after OFFGEL–LOC electrophoresis, Mws within the 15 – 63 kDa range
318 [24]. In addition, proteins pIs are also quite similar (from 4.8 to 8.4) [14,15], and most
319 abundant proteins in surface seawater from Ría de Arousa estuary offered pI from 5.7 to 8.1
320 [24].

3.2. Detection of metals in gels with LA-ICP-MS

Matrix-matched standards with known metal concentrations were prepared and used for metal quantification by LA-ICP-MS. Standard solutions containing metals at concentrations of 100, 10, 1.0, and 0.1 μg mL^{-1} (20μL) were loaded on 50 mg portions of gels (15% polyacrylamide). Similarly, a blank was also prepared by loading 20μL of a blank solution, which will be used as standard of concentration 0.0 μg mL^{-1}. Once dried, the gel portions were weighed (weights approximately 10 mg), and the metal concentrations were re-calculated and refereed to mass of metal to mass of gel: final metal concentrations of 200, 20, 2.0, 0.2 and 0.0 μg g^{-1}. The different gel portions were fixed to the support using double-sided adhesive tape and were ablated. Carbon-13 was used as the internal standard for correcting plasma instabilities. Table 3 lists the equations for the calibrations curves obtained. Regression coefficients were higher than 0.995 for several targets, although values within the 0.977 – 0.9850 range were obtained for some metals.

The limit of detection (LOD), based on 3Sd criterion (3Sd/m, where *Sd* is the standard deviation of 5 measurements of blank gels, and *m* is the slope of calibration graph) was established. Table 3 also lists the LODs obtained, which range from 0.0266 μg g^{-1} (Mn detection) to 4.96 μg g^{-1} (Cr detection).

3.3. Detection of metals bound to proteins with LA-ICP-MS

As previously mentioned intense and resolved protein spots, highlighted inside rectangles in Figures 1 and 2, were cut and further analyzed by LA-ICP-MS. In addition, region number BLK in the gel from surface seawater (Figure 2) was also analyzed as a blank to assess the presence background trace metals in the gels and reagents used for performing 2DE.

Regarding isolated proteins from plankton, bioactive elements such as Cu and Zn, as well as Cr and Cd, were identified in the protein spot coded as 11a (~13 kDa, pI of ~4.5) in plankton sample P1. This sample also showed the presence of Zn in protein spot coded as 13 (~16

346 kDa, pI of ~6.7), and Cd in protein spots coded as 2f (~22 kDa, pI of ~5.5) and 17b (~9.0
347 kDa, pI of ~10). The relative concentration of these elements, expressed as µg g^{-1}, are listed
348 in Table 4, and accounted for low values for Cd, Cu and Zn (7.5, 4.9, and within the 10.7 –
349 19.2 µg g^{-1} range, respectively), and high levels for Cr (74.4 µg g^{-1}). The presence of Cd, Cr
350 and Zn was also observed in some protein spots from plankton sample P2 (Table 4), although
351 at lower concentrations than in plankton sample P1. Studies based on OFFGEL-LOC
352 electrophoresis when analyzing similar plankton samples [32] showed the association of Zn
353 and Cd with proteins of acid pIs (within the 5.9 – 6.5 range), result similar to that found when
354 analyzing plankton sample P1. In this technique, proteins are separated according to their
355 isoelectric point (pI) by OFFGEL electrophoresis, using immobilized pH-gradient gels (IPG),
356 and the separated components are recovered in liquid fractions to be separated according to
357 their molecular weight using a microfluidic Lab-on-chip electrophoresis. In general, the
358 number of elements bound to the isolated proteins, as well as the concentrations, is lower
359 than that obtained when analyzing similar plankton samples by OFFGEL-LOC
360 electrophoresis [32]. Under OFFGEL conditions, the association between certain proteins and
361 other bioactive trace metals such as Fe and Mn is guaranteed. This fact can be attributed to
362 the more drastic conditions inherent to conventional 2D-PAGE which imply metal-protein
363 breakdown during isoelectric focusing [31,32]. However, parameters affecting the protein
364 extraction process, such as the low temperature and the presence of proteinase inhibitors, can
365 also contribute to minimize metal-protein complexes degradation.
366 The assessment of trace metal associated to proteins from surface seawater showed the
367 presence of Cd, Cu, Cr and Zn in some proteins spots (Figure 2). Table 4 lists the relative
368 concentrations of these metals in the ablated spots. Proteins in spots coded as *c* (Mw/pI of
369 ~14 kDa/~6.3) and *d* (Mw/pI of ~13 kDa/~7.0) are associated to Cd, Cu, Cr and Zn; whereas,
370 the occurrence of Cd, Cr and Zn was also found in the protein spot coded as *a* (Mw/pI of ~10

kDa/~5.8). Protein spots coded as *b* (Mw/pI of ~13 kDa/~5.8) and *e* (Mw/pI of ~12 kDa/~7.3) only showed association with Cd (spot e) and Zn (spot b). The determination of metals associated to protein fractions of similar pIs (OFFGEL conditions) when analyzing dissolved proteins [24] showed the occurrence of Cd and Zn in proteins of acid pIs (within the 5.2 – 6.0 range), which agree to the current results for protein spots *a* and *d* (Figure 2). As previously mentioned for proteins from plankton, OFFGEL conditions led to the assessment of other bioactive metals such as Fe and Mn bound to certain dissolved proteins [24]. These metal-proteins association were not observed when performing 2D-PAGE and can be attributed to the used 2D-PAGE conditions [31], as well as to conditions inherent to the proteins extraction itself.

Conclusions

LA-ICP-MS has effectively been used to assess trace metals bound to dissolved proteins and proteins from marine plankton after effective protein isolation methods and conventional 2D-PAGE. The presence of certain metals bound to dissolved proteins in seawater after two-dimensional pI and MW separation has been demonstrated by the first time. These findings confirm results from earlier studies which reported the existence of trace metal – protein complexes in seawater after isolating groups of dissolved proteins of similar pIs [24]. Results showed that bioactive metals such as Cu and Zn are mainly associated to dissolved proteins of neutral pIs (approximately pIs of 6.3 and 7.0), although Zn was also found to be associated to proteins of pIs 6.0 and 7.6. Other metals such Cd and Cr were also detected in some isolated proteins exhibiting variable pIs (from 5.8 to 7.0). The presence of metals in some protein spots from fresh marine plankton was also proved. Elements such as Cd, Cr, Cu and Zn were found to be associated to certain proteins of Mw ranging from 9 to 22 kDa and pIs from 5.5 and 10, results which also agree to earlier findings for fresh marine plankton when

assessing metals bound to protein fractions of similar pIs by OFFGEL electrophoresis [32]. In general, it can be concluded that the used 2D-PAGE conditions appear to be more drastic than those involved when using OFFGEL electrophoresis. This fact explains certain breaking metal-protein complexes dissociations, mainly Fe- and Mn-proteins, which were found in fresh marine plankton after OFFGEL [17,32], and which were not observed in the current application. Finally, findings regarding dissolved proteins can not be used to prove correlation between the MW and the pI of the isolated proteins and the type of metal bound to them. This is because the isolated proteins exhibits very close MW (from 10 to 14 kDa) and pI (from 5.8 to 7.3) values. Regarding plankton proteins, no correlation between the type of metal and the protein' pIs have been shown. However, certain essential elements such as Cr, Cu and Zn, appears to be mainly bound to proteins of low MW.

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415 **Table 1**. MW range and pI range of protein spots on 2-DE for plankton.

Plankton P1			Plankton P2		
Protein spot	MW (kDa)	pI	Protein spot	MW (kDa)	pI
1a	23	4.0			
1b	23	4.3			
1c	23	4.6			
2a	25	5.3	2a	25	6.0
2b	25	5.5	2b	25	6.1
2c	25	5.7			
2d	25	5.7			
2e	22	5.8			
2f	22	5.5			
3	31	6.5	3	31	6.7
4	31	7.4	4	31	7.5
5	20	6.2			
6	22	6.8	6	23	6.9
7a	20	7.5	7a	21	7.6
7b	20	8.0	7b	21	8.0
8a	22	9.1	8a	22	9.0
8b	22	9.4	8b	21	9.1
			8c	21	9.2
			8d	21	9.3
9	18	9.7	9	18	9.7
10a	10	3.9	10a	11	4.1
10b	10	4.1	10b	10	4.3
11a	13	4.5	11a	13	5.2
11b	13	5.0	11b	12	5.3
11c	12				
12a	13	5.7	12a	14	6.0
12b	13	6.0	12b	13	6.0
12c	11	5.7			
12d	11	6.0			
13	16	6.7	13	16	6.9
14	10	7.5			
15a	11	8.0	15a	12	7.7
15b	12	8.2	15b	11	8.0
16a	9	9.0	16a	10	8.8
16b	9	9.4	16b	10	9.1
			16c	9	9.3
17a	9	9.8	17	9	9.8
17b	9	10			
18	6	5.0	18	6	5.3

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418 **Table 2**. MW range and pI range of protein spots on 2-DE for surface seawater

Protein spot	MW (kDa)	pI
a	10	5.8
b	13	5.8
c	14	6.3
d	13	7.0
e	12	7.3

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420 **Table 3.** Calibration curves and limit of detection of the method

	Calibration curve	Correlation (R^2)	LOD ($\mu g\ g^{-1}$)
^{111}Cd	y = 0.0003 [ ] + 0.0008	0.9999	0.96
^{114}Cd	y = 0.0009 [ ] + 0.0011	0.9988	0.43
^{59}Co	y = 0.0272 [ ] + 0.169	0.9834	0.047
^{63}Cu	y = 0.0127 [ ] + 0.0902	0.9783	0.060
^{65}Cu	y = 0.0062 [ ] + 0.0605	0.9776	1.4
^{57}Fe	y = 0.0006 [ ] + 0.0091	0.9787	2.6
^{55}Mn	y = 0.0391 [ ] + 0.2735	0.9815	0.027
^{60}Ni	y = 0.0053 [ ] + 0.0322	0.9843	0.15
^{62}Ni	y = 0.0008 [ ] + 0.0051	0.9850	0.34
^{206}Pb	y = 0.0276 [ ] + 0.2184	0.9775	0.034
^{207}Pb	y = 0.021 [ ] + 0.1673	0.9772	0.045
^{208}Pb	y = 0.0524 [ ] + 0.4146	0.9774	0.052
^{68}Zn	y = 0.001 [ ] + 0.0204	0.9802	1.6
^{64}Zn	y = 0.0026 [ ] + 0.0341	0.9955	2.3
^{66}Zn	y = 0.0015 [ ] + 0.0143	0.9958	0.94
^{50}Cr	y = 0.0012 [ ] + 0.0361	0.9819	4.9
^{53}Cr	y = 0.0027 [ ] + 0.0274	0.9795	0.21

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Table 4. Trace metal concentrations in protein spots from plankton and surface seawater samples.

Trace metal	Protein spot code	Relative concentration (μg g^{-1})
Plankton P1		
Cd	2f	0.5
	11a	3.0
	17b	0.6
Cu	11a	4.9
Cr	11a	74.4
Zn	11a	19.2
	13	10.7
Plankton P2		
Cd	8b	0.9
	8c	0.6
	8d	0.7
	15b	3.2
Cr	15b	5.8
Zn	8a	11.0
	8b	4.0
	8d	4.5
	13	3.8
	15a	9.5
	15b	2.9
	16a	14.5
Surface seawater		
Cd	a	4.8
	c	11.7
	d	5.5
	e	1.0
Cu	c	6.7
	d	2.0
Cr	a	16.9
	c	60.0
	d	22.0
Zn	a	6.0
	b	9.4
	c	12.7
	d	7.6

Figures' captions

Figure 1. 2DE gel of proteins extracted from marine plankton (plankton sample P1)

Figure 2. 2DE gel of proteins extracted from surface seawater

437 **References**

[1] P. Falkowski, Nature 483 (2012) S17-S20.

[2] F. M. M. Morel, N. M. Price, Science 300 (2003) 944-947.

[3] E. C. Minor, J. Simjouw, J. J. Boon, A. E. Kerkhoff, J. van der Horst, Mar. Chem. 78 (2002) 75-102.

[4] M. Gonzalez-Davila, Mar. Chem. 48 (1995) 215-236.

[5] K. Hirose, Appl. Geochem. 22 (2007) 1636-1645.

[6] N. Rossi, J. Jamet, Mar. Pollut. Bull. 56 (2008) 1862-1870.

[7] J. S. Garcia, C. Schmidt de Magalhaes, M.A.Z. Arruda, Talanta. 69 (2006) 1-15.

[8] P. P. Kulkarni, Y. M. She, S. D. Smith, E. A. Roberts, B. Sarkar, Chem. Eur. J. 12 (2006) 2410-2422.

[9] Y. Wang, Y.-L. Guo, H.-L- Yuan, Y.-F. Wei, H.-T. Yan, H.-H. Chen, Spectrosc. Spect. Anal., 32 (2012), 223-228.

[10] N. Yamada, E. Tanoue, Limnol. Oceanogr. 48 (2003) 1037-1048.

[11] E. Tanoue, Mar. Chem. 51 (1995) 239-252.

[12] E. Tanoue, M. Ishii, T. Midorikawa, Limnol. Oceanogr. 41 (1996) 1334-1343.

[13] S. Saijo, E. Tanoue, Limnol. Oceanogr. 49 (2004) 953-963.

[14] N. Yamada, E. Tanoue, Prog. Oceanogr. 69 (2006) 1-18.

[15] N. Yamada, E. Tanoue, J. Oceanogr. 65 (2009) 223-233.

[16] M.S. Jimenez, L. Rodriguez, J.R. Bertolin, M.T. Gomez, J.R. Castillo, Anal. Bioanal. Chem. 405 (2013) 359-368.

[17] N. García-Otero, M. C. Barciela-Alonso, A. Moreda-Piñeiro, P. Bermejo-Barrera, Talanta (2013) 631-641.

[18] E. Barbarino, S.O. Lourenco, J. Appl. Phycol. 17 (2005) 447-460.

[19] D. Wang, L. Lin, L.L. Chan, H. Hong, Harmful Algae. 8 (2009) 685-691

[20] M.J. Powell, J.N. Sutton, C.E. Del Castillo, A.T. Timperman, Mar. Chem. 95 (2005) 183-198.

[21] N. García-Otero, P. Bermejo-Barrera, A. Moreda-Piñeiro, Anal. Chim. Acta 760 (2013) 83–92.

[22] K. Mopper, A. Stubbins, J.D. Ritchie, H.M. Bialk, P.G. Hatcher, Chem. Rev. 107 (2007) 419-442

[23]. W. Wang, R. Vignani, M. Scali, M. Cresti, Electrophoresis. 27 (2006) 2782-2786.

[24] N. García-Otero, E. Peña-Vázquez, M. C. Barciela-Alonso, P. Bermejo-Barrera, A. Moreda-Piñeiro, Anal. Chem. (2013) 5909-5916.

[25] A. Venkatachalam, C.U. Koehler, I. Feldmann, P. Lampen, A. Manz, P.H. Roos, N. Jakubowski, J. Anal. At. Spectrom. 22 (2007) 1023-1032

[26] .S. Becker, M. Zoriy, C. Pickhardt, M. Przybylski, J.S. Becker, Int. J. Mass Spectrom. 242 (2005) 135-144.

[27] J.S. Becker, D. Pozebon, A. Matusch, V.L. Dressler, J.S. Becker, Int. J. Mass Spectrom. 307 (2011) 66-69.

[28] M.A.O. Silva, M.A.Z. Arruda, Microchim. Acta. 176 (2012) 131-136.

[29] A. Polatajko, M. Azzolini, I. Feldmann, T. Stuezel, N. Jakubowski, J. Anal. At. Spectrom. 22 (2007) 878-887.

[30] J.S. Becker, R. Lobinski, J.S. Becker, Metallomics. 1 (2009) 312-316.

[31] M.S. Jimenez, M.T. Gomez, L. Rodriguez, R. Velarte, J.R. Castillo, Anal. Chim. Acta. 676 (2010) 9-14.

[32] N. García-Otero, M. C. Barciela-Alonso, A. Moreda-Piñeiro, R. Domínguez-González, P. Herbello-Hermelo, P. Bermejo-Barrera, Electrophoresis (2013) Submitted for publication.