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Natacha Jean, Estelle Dumont, Faouzi Herzi, Thierry Balliau, Mohamed Laabir, et al.. Modifications of the soluble proteome of a mediterranean strain of the invasive neurotoxic dinoflagellate *Alexandrium catenella* under metal stress conditions. *Aquatic Toxicology*, 2017, 188, pp.80-91. 10.1016/j.aquatox.2017.04.011 . hal-01569507v2

HAL Id: hal-01569507

<https://hal.science/hal-01569507v2>

Submitted on 23 Sep 2018

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1 **Modifications of the soluble proteome of a mediterranean strain of the invasive**
2 **neurotoxic dinoflagellate *Alexandrium catenella* under metallic stress conditions**

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7 4 Natacha JEAN¹ (*), Estelle DUMONT¹, Faouzi HERZI¹, Thierry BALLIAU², Mohamed
8
9 LAABIR³, Estelle MASSERET³, Stéphane MOUNIER¹

10 5
11 6
12 7 ¹ Université de Toulon, PROTEE, EA 3819, 83957 La Garde, France

13 8
14 9 ² Plate-forme d'Analyse Protéomique Paris Sud-Ouest (PAPPSO), INRA, Unité Mixte de
15 10 Recherche en Génétique Végétale (UMR 320), Ferme du Moulon, 91190 Gif-sur-Yvette,
16 11 France

17 12
18 13 ³ MARBEC UMR 9190 IRD-Ifremer-CNRS-Université de Montpellier, Place Eugène
19 14 Bataillon, Case 093, 34095 Montpellier Cedex 5, France

20 15
21 16 E-mail addresses: jean@univ-tln.fr (N. JEAN); estelle.dumont1@gmail.com (E. DUMONT);
22 17 herzi_faouzi@yahoo.fr (F. HERZI); balliau@moulon.inra.fr (T. BALLIAU);
23 18 Mohamed.Laabir@umontpellier.fr (M. LAABIR); estelle.masseret@umontpellier.fr (E.
24 19 MASSERET); mounier@univ-tln.fr (S. MOUNIER)

25 20
26 21 * Corresponding author. Tel.: +33 4 94 14 25 29; E-mail address: jean@univ-tln.fr (N. JEAN)

31 **Abstract**

32 The soluble proteome of the mediterranean strain ACT03 of the invasive neurotoxic
33 dinoflagellate *Alexandrium catenella* **exposed to** lead or zinc at 6, 12 or 18 M (total
34 concentrations), or grown **under** control conditions, has been characterized by two-
35 dimensional gel electrophoresis (2-DE). **Concentrations of zinc increasingly reduced** (P <
36 0.05) the total number of protein spots constituting the soluble proteome (-41%, -52% and -
37 60%, at 6, 12 or 18 M, respectively) of *A. catenella* ACT03. **Besides, most of the proteins**
38 **constituting the soluble proteome were down-regulated in response to lead or zinc stresses.**
39 **These proteins** were involved mainly in photosynthesis (20-37% for lead; 36-50% for zinc)
40 (ribulose-1,5-bisphosphate carboxylase/oxygenase: RUBISCO; ferredoxin-NADP⁺ reductase:
41 FNR; peridinin-chlorophyll *a*-protein: PCP) and in the oxidative stress response (29-34% for
42 lead; 17-36% for zinc) (superoxide dismutase: SOD; proteasome α/β subunits). **These**
43 **harmful effects could be partly compensated by the up-regulation of specific proteins as** ATP-
44 **synthase β subunit (+16.3 fold after exposure to lead at 12 M). Indeed, increase in**
45 **abundance of ATP-synthase could enrich the ATP pool and provide more energy available for**
46 **the cells to survive under metallic stress conditions, and make more efficient the ATP-**
47 **synthase transport of metal cations out of the cells. Finally, this study shows that exposure to**
48 **lead or zinc have a harmful effect** on the soluble proteome of *A. catenella* ACT03, **but** also
49 **suggests a proteomic adaptative response to metallic stresses**, which could contribute to
50 **sustain this dinoflagellate development in some trace metal-contaminated ecosystems.**

51
52 **Keywords:** *Alexandrium catenella*; harmful algal bloom; proteomics; stress proteins;
53 trace metals; two-dimensional electrophoresis

64 Abbreviations

1	65	BiP	Binding immunoglobulin protein
2	66	CALR	Calreticulin
3	67	CaM	Calmodulin
4	68	CHAPS	3-[3-(cholamidopropyl)-dimethylammonio]1-propanesulfonate
5	69	2-DE	Two-dimensional gel electrophoresis
6	70	DTT	Dithiothreitol
7	71	DHAP	Dihydroxyacetone phosphate
8	72	FNR	Ferredoxin-NADP ⁺ reductase
9	73	G3P	Glyceraldehyde 3-phosphate
10	74	HAB	Harmful algal bloom
11	75	HSP	Heat shock protein
12	76	IEF	Isoelectric focusing
13	77	IPG	Immobilized pH gradient
14	78	LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
15	79	MS	Mass spectrometry
16	80	MS/MS	Tandem mass spectrometry
17	81	MW	Molecular weight
18	82	NCBI	National centre for biotechnology information
19	83	NADPH	Nicotinamide adenine dinucleotide phosphate
20	84	PCP	Peridinin-chlorophyll <i>a</i> -protein
21	85	pI	Isoelectric point
22	86	PSP	Paralytic shellfish poisoning
23	87	RBP	Rubisco binding protein
24	88	ROS	Reactive oxygen species
25	89	RPI	Ribose-5-phosphate isomerase
26	90	R5P	Ribose-5-phosphate
27	91	RUBISCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
28	92	RuBP	Ribulose-1,5-bisphosphate
29	93	Ru5P	Ribose-5-phosphate isomerase
30	94	SDS	Sodium dodecylsulfate
31	95	SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
32	96	SOD	Superoxide dismutase
33	97		
34	98		
35	99		
36	100		
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38	102		
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116 1. Introduction

117 **Harmful Algal Blooms (HABs) are proliferations of noxious phytoplankton**
118 **including toxic species, able to (i) threaten human health after the consumption of**
119 **seafood contaminated by toxins (ii) affect negatively many components of the marine life**
120 **(shellfish, fishes, seabirds and mammals) (iii) impact socioeconomic activity due to the**
121 **closure of aquaculture areas during HAB events (Anderson et al., 2012).**

122 Since the last decades, frequency and distribution of HABs have increased in coastal
123 mediterranean ecosystems subject to growing anthropisation (Ferrante et al., 2013).
124 **Alexandrium genus, belonging the dinoflagellates**, is involved in severe, diverse and widely
125 distributed HABs (Anderson et al., 2012). Indeed, the toxic *Alexandrium* species (*A.*
126 *catenella*, *A. fundyense*, *A. minutum*, *A. tamarense*, *A. taylori*) can produce saxitoxins **and**
127 **congeners, inducing** the Paralytic Shellfish Poisoning (PSP) syndrome.

128 Occurrences of the *Alexandrium* genus have been reported in several mediterranean
129 areas receiving trace metal contaminations, as Catalan and Balearic coasts (Bravo et al., 2006;
130 Penna et al., 2005), Tyrrhenian coast (Italy), Thau lagoon (France) (Laabir et al., 2013; Péna
131 and Picot, 1991), **Bizerte lagoon (Tunisia) (Fertouna et al., 2015)** and Toulon Bay (western
132 Mediterranean, France) (Abadie, personal communication; Jean et al., 2005, 2006). This latter
133 is a severely trace metal contaminated ecosystem, both in the water column and the sediments
134 (Jean et al., 2012; Tessier et al., 2011). Thus, occurrence of *Alexandrium* in such **area**,
135 suggests that it could be potentially tolerant to trace metal contaminations, **although** this has
136 not been demonstrated so far. Consequently, studies should be carried out to characterize the
137 potential tolerance of *Alexandrium* to metallic stresses, which could contribute to explain its
138 development in metal polluted anthropized environments.

139 **In response to changes in the environment, an organism can modify its proteome**
140 **by increasing or decreasing the expression of some proteins, making proteomics a**
141 **dynamic field of investigation. These modifications, activating or slowing down some**
142 **metabolic pathways, allow the organism to adapt to environmental changes. Therefore,**
143 **modifications of the proteome are at the basis of the adaptive organism response to**
144 **environmental stresses, as suggested in studies reporting higher protein expression (for**
145 **actin and calreticulin) in zooplankton communities sampled in the most metal polluted**
146 **stations of Toulon Bay (Jean et al., 2012).**

147 **Many studies have characterized the proteomic responses of aquatic organisms**
148 **exposed to metal stresses, whether these are marine pluricellulars, as *Chlamys farreri***

149 (scallop) (Gao et al., 2007), *Eriocheir sinensis* (crab) (Silvestre et al., 2006), *Montastraea*
150 *franki* (coral) (Venn et al., 2009), *Mytilus edulis* (mussel) (Sanders and Martin, 1991), *Perna*
151 *veridis* (bivalve) (Leung et al., 2011), *Saccostrea glomerata* and *Crassostrea gigas* (oyster)
152 (Choi et al., 2008; Thompson et al., 2011, 2012), *Ectocarpus siliculosus*, *Fucus serratus*,
153 *Lessonia nigrescens*, *Sargassum fusiforme* and *Scytosiphon gracilis* (brown algae)
154 (Contreras et al., 2010; Ireland et al., 2004, **Lovazzano et al., 2013; Ritter et al., 2010; Zou**
155 **et al., 2015**), or **aquatic unicellulars, as** *Anabaena* sp. (Pandey et al., 2012), *Klebsiella*
156 *pneumoniae*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* (Daware et al., 2012;
157 Kiliç et al., 2010; Poirier et al., 2008) or *Tetrahymena pyriformis* (Zhang et al., 2012).
158 **However, only one study has been devoted to the characterization of the proteomic**
159 **response of a dinoflagellate, *Karenia brevis*, notably submitted to lead contamination,**
160 **and in which some proteins (heat shock 60 kDa protein and superoxide dismutases) have**
161 **been induced by the metal stress (Miller-Morey and Van Dolah, 2004).**

162 **Under** an ecotoxicoproteomic approach, the purpose of the present study is to
163 characterize the proteomic response to metallic stresses, of the invasive neurotoxic
164 dinoflagellate *Alexandrium catenella*. **A monoclonal mediterranean strain of *A. catenella***
165 **exposed to metallic contaminations, either by lead (toxic for the cells whatever its**
166 **concentration), or by zinc (first required by the cells as a trace element, then harmful at**
167 **higher concentrations), has been used. Then, the soluble proteomes of this strain, grown**
168 **respectively under control and metallic stress conditions, have been characterized by**
169 **two-dimensional gel electrophoresis (2-DE), and compared. This comparison** has
170 generated a list of proteins of interest whose expression **was** significantly modified in
171 response to the metallic stresses. The proteins of interest can be considered as potential
172 biomarkers of **metallic** stresses in the *A. catenella* cells. Besides, they provide informations
173 about the **metabolic pathways which were modified in *A. catenella* under metallic stress**
174 **conditions**. Some of these proteins could be candidates **as potential** detoxification and
175 protection processes in *A. catenella*, thus contributing to its adaptive response and tolerance
176 capacity towards metallic stresses. Consequently, the overall results obtained could **allow** to
177 understand the biochemical mechanisms **making** *A. catenella* **able** to develop in anthropized
178 coastal marine ecosystems contaminated by trace metals.

183 2. Materials and Methods

184 2.1. Cultures of *Alexandrium catenella* ACT03

185 *A. catenella* strain **ACT03** was obtained after isolation of a single vegetative cell from
186 seawater sample **collected** during a toxic bloom event in October 2003, in Thau lagoon
187 (Laabir et al., 2013). It was maintained **in** enriched f/2 culture medium (Guillard and Ryther,
188 1962), at 20°C in sterile 250 mL flasks (75 cm² Greiner, Dominique Dutscher SAS), with a
189 12h: 12h photoperiod and under a light intensity of 135 mol photons m⁻² s⁻¹ (Herzi et al.,
190 2013, 2014). Natural seawater filtered through a GF/F glass fibre filter (Ø = 47 mm,
191 Whatman) and then sterilized **was** used as a basis for the culture medium.

192 To study **modifications of the soluble proteome** of *A. catenella* ACT03 under
193 **metallic** stress, the culture medium was **supplemented with** sterile stock solutions of trace
194 metals prepared with ZnSO₄ · 7H₂O, or with Pb(CH₃COO)₂ · 3H₂O salts dissolved **in**
195 **ultrapure water**. For each **metal, three total concentrations** (6, 12 and 18 M) were **tested**.
196 **A metal-free culture medium was considered as the control**. Based on the MINEQL-
197 program and on the known composition of the f/2 culture medium, the corresponding free
198 metal concentrations (Zn²⁺ or Pb²⁺) bioavailable for toxicity towards cells, were calculated
199 (Table I) (Herzi et al., 2014).

200 **Table I.** Total (M²⁺_T) and free (M²⁺_F) trace metal concentrations (expressed in M), used for lead and zinc
201 contaminations of the *Alexandrium catenella* ACT03 cultures
202
203

[M ²⁺] _T (M)	[Pb ²⁺] _F (M)	[Zn ²⁺] _F (M)
6 × 10 ⁻⁶	1.1 × 10 ⁻⁸	2.8 × 10 ⁻⁷
12 × 10 ⁻⁶	2.6 × 10 ⁻⁸	6.2 × 10 ⁻⁷
18 × 10 ⁻⁶	4.4 × 10 ⁻⁸	1.0 × 10 ⁻⁶

205 2.2. Preparation of the protein extracts

206 Control and metal-contaminated cultures were **studied** at the end of their exponential
207 phase-early stationary phase of growth. Cell density of these cultures was estimated by
208 counting (in triplicates) all the cells in 50 L **sub-sample** of a 1 mL Lugol fixed **culture**, with
209 an inverted microscope (magnification: 100 ×). Taking the obtained density into account, a
210 volume of culture containing 8 × 10⁶ cells **was** used for proteomic **analysis**.

211 **The sample** was centrifuged for 15 min at 1 500 g, **at** 15°C. The obtained cell pellet
212 was washed twice in natural seawater (prepared as described in 2.1.), for 10 min at 15 000 g,
213 to be immediately **used**. As recommended in Wang et al. (2008), the washed pellet **was then**
214 re-suspended in an extraction solution, containing 2 mL 40 mM Tris **at** pH 8.7, 2.4 L

215 benzonase nuclease (Sigma-Aldrich) and 10 L of protease inhibitor cocktail (Sigma-
216 Aldrich). Then, protein extraction **was** achieved by sonication of the obtained suspension in
217 an ice-water bath, using a microtip Vibra Cell 734 24 (Bioblock Scientific) during 3 min at 50
218 W and 25 kHz, with 10 s on/ 10 s off cycles. Then, the solution was centrifuged for 30 min at
219 15 000 g, to obtain a supernatant which **contains** the soluble protein extract. This **fraction**
220 **was further** concentrated by ultrafiltration at 1 500 g, **at** 15°C, through Vivaspin concentrator
221 tubes with molecular weight cutoff membranes of 15 kDa (15R Hydrosart 10 kDa, Thermo
222 Fisher Scientific), until reaching a volume of 150 L. To obtain the protein extract ready **to**
223 **be used** for proteomics, the concentrate had to be finally mixed with 400 L of a sample
224 solution consisting of urea (7 M), thiourea (2 M), CHAPS (1% w/v), Triton X-100 (3% v/v),
225 DTT (1% w/v), carrier ampholytes (0.2% v/v) and **bromophenol blue** (0.002% v/v).

2.3. Protein determination

228 Protein determination of the extracts was achieved according to Lowry et al. (1951),
229 using the Reagent Compatible Detergent Compatible Protein Assay (RC DC Protein Assay,
230 Bio-Rad), and bovine serum albumin (BSA) as standard.

2.4. Two-dimensional electrophoresis

233 **A pre-prepared immobilized pH gradient (IPG) strip (17 cm length, linear**
234 **gradient, pH 3-10, Bio-Rad) was used, on which the extract sample (350 L containing**
235 **200 g proteins) was transferred (Linares et al., 2016).** The rehydration of the IPG strip in
236 presence of the sample, and **then isoelectric focusing** (IEF), were performed in the horizontal
237 electrophoresis system PROTEAN IEF Cell (Bio-Rad), at 20°C, in the following manner: 18
238 h at 50 V (active rehydration), 2 h at 100 V, 2 h at 250 V, 2 h at 500 V, 2 h at 1 000 V, 2 h at
239 4 000 V and 5 h at 10 000 V, so as to reach a total minimal value of 60 000 Vh for each
240 loaded IPG strip. After IEF, the IPG strip was successively equilibrated for 10 min at room
241 temperature in equilibration buffers 1 (6 M urea, 2% w/v SDS, 0.375 M Tris pH 8.8, 20% v/v
242 glycerol, 2% w/v DTT) and 2 (6 M urea, 2% w/v SDS, 0.375 M Tris pH 8.8, 20% v/v
243 glycerol, 2.5% w/v iodoacetamide).

244 Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was
245 performed according to Laemmli (1970). The equilibrated IPG strip was placed in dyed
246 (BPB) melted agarose, across a handmade polyacrylamide gel (18 cm × 18 cm × 1 mm;
247 stacking gel: 5%, resolving gel: 11%). A volume of 20 L of molecular weight marker
248 solution (10-250 kDa range, Precision Plus Protein Standards Dual Color, Bio-Rad) was

249 loaded at the left top of the gel. The gels were run at 4°C in a Protean II XL (Bio-Rad), with a
250 constant current of 20 mA per gel for 1 h, and then, with a constant current of 30 mA per gel
251 until the dye reached the bottom of the gel. After SDS-PAGE, gels were washed three times
252 for 5 min in ultrapure water, then stained with the Imperial Protein Stain (Thermo Fisher
253 Scientific) under orbital shaking for 1 h 30 min, to be destained in ultrapure water until
254 visualization of separated protein spots. Each gel presented (Figures 1 & 2) is representative
255 of three different biological gel replicates. The protein spots on the gels were analysed using
256 the PD Quest 2-D Analysis Software 8.0.1 version (Bio-Rad). Abundance of a given protein
257 spot has been obtained after normalization, as the *ratio* (in %) of its individual abundance on
258 the abundance of all the marker bands.

2.5. Tracking of protein of interest in the soluble proteome of *A. catenella* ACT03

261 Among the proteins making the soluble proteome of *A. catenella* ACT03, the proteins
262 of interest are those whose expression was significantly affected by the metallic stresses,
263 through up- or down-regulation, or through appearance or disappearance. The up- or down-
264 regulated proteins were respectively tracked thanks to the *ratio* of the abundance of each
265 protein spot on the 2D-gel after metallic stress, on the abundance on the 2D-gel under control
266 conditions (for up-regulation), and the *ratio* of the abundance of each protein spot on the 2D-
267 gel under control conditions, on the abundance on the 2D-gel after metallic stress (for down-
268 regulation). **First**, to be considered as a protein of interest, a protein had a *ratio* greater than
269 or equal to 2 (Bae et al., 2003). Then, a statistical Student test was applied between the
270 abundances of this protein on the 2D-gel replicates obtained under metallic stress conditions,
271 and the abundances of this same protein on the 2D-gel replicates under control condition. This
272 statistical test allowed to determine if the fold difference in up-regulation or down-regulation
273 was significant (very significant: $P < 0.01$; significant: $0.01 < P < 0.05$; rather significant:
274 $0.05 < P < 0.10$), in which case, the concerned protein was significantly up-regulated or
275 down-regulated, and considered as a protein of interest. The other proteins of interest were
276 those appearing or disappearing on at least two 2D-gels among the three obtained replicates.

283 2.6. Protein identification by liquid chromatography tandem mass spectrometry

284 Proteins of interest were picked on gel in order to be identified by mass spectrometry.
285 In-gel digestion of the picked 2-D spots was performed with the Progest system (Genomic
286 Solution) according to a standard trypsin protocol (Page et al., 2010). HPLC was performed
287 on a NanoLC-Ultra system (Eksigent). A 4 μ L sample of the peptide solution was loaded at
288 7.5 μ L min⁻¹ on a precolumn cartridge (stationary phase: C18 Biosphere, 5 μ m; column: 100
289 μ m inner diameter, 2 cm; Nanoseparations) and desalted with 0.1% HCOOH. After 3 min, the
290 precolumn cartridge was connected to the separating PepMap C18 column (stationary phase:
291 C18 Biosphere, 3 μ m; column: 75 μ m inner diameter, 150 mm; Nanoseparations). Buffers A
292 and B respectively were prepared with 0.1% HCOOH in water, and with 0.1% HCOOH in
293 acetonitrile. The peptide separation was achieved with a linear gradient from 5 to 30% B for
294 28 min at 0.3 μ L min⁻¹. Including the regeneration step at 95% B and the equilibration step at
295 95% A, one run took 45 min.

296 Eluted peptides were on-line analyzed with an LTQ XL ion trap (Thermo Electron)
297 using a nanoelectrospray interface. Ionization (1.5 kV ionization potential) was performed
298 with liquid junction and a non-coated capillary probe (10 μ m inner diameter; New Objective).
299 Peptide ions were analysed using Xcalibur 2.07 with the following data-dependent acquisition
300 steps: (1) full MS scan (mass to-charge ratio (*m/z*) 300-1 400, centroid mode) and (2) MS/MS
301 (*qz* = 0.25, activation time = 30 ms, and collision energy = 35%; centroid mode). Step 2 was
302 repeated for the three major ions detected in step 1. Dynamic exclusion was set to 30 s.

303 A database search was performed with XTandem (version 2010.12.01.1)
304 (<http://www.thegpm.org/TANDEM/>). Enzymatic cleavage was declared as a trypsin digestion
305 with one possible miscleavage. Cys carboxyamidomethylation and Met oxidation were set to
306 static and possible modifications, respectively. Precursor mass and fragment mass tolerance
307 were 2.0 and 0.8, respectively. A refinement search was added with similar parameters except
308 that semi-trypsinic peptide, and possible N-ter proteins acetylation were searched. The
309 UniprotKB database (<http://www.uniprot.org/>) restricted to Metazoa excepted Mammalia
310 (1755330 entries, version 201201), and a contaminant database (trypsin, keratins.) were used.
311 Only peptides with an *E* value smaller than 0.1 were reported.

312 Identified proteins were filtered and grouped using XTandem Pipeline
313 (<http://pappso.inra.fr/bioinfo/xtandempipeline/>) according to: (1) A minimum of two different
314 peptides required with an *E* value smaller than 0.05, (2) a protein *E* value (calculated as the
315 product of unique peptide *E* values) smaller than 10⁻⁴. In the case of identification with only
316 two or three MS/MS spectra, similarity between the experimental and the theoretical MS/MS

317 spectra was visually checked. Identification by database searching was completed by a *de*
1 318 *nov* approach. Peptides sequences were determined by automatic *de novo* interpretation from
2 319 MS/MS spectra, using PepNovo software (version 2010225). This analysis was performed on
3 320 MS/MS spectra with a quality score smaller than 0.1. Trypsin digestion, Cys
4 321 carboxyamidomethylation and Met oxidation were set to enzymatic cleavage, static and
5 322 possible modifications, respectively. Only sequences with a score greater than 70 were
6 323 selected.

324 Homology searches were performed by Fasts software (version 36.06) using the
12 325 MD20-MS matrix. Sequences corresponding to keratins or trypsin were firstly removed by
13 326 interrogating a homemade contaminant database. Secondly, the search computing process was
14 327 carried out on the same database. Homologies with a minimum of two independent peptides
15 328 and an *E* value smaller than 0.001, were only selected. In **all** cases, the automatic *de novo*
16 329 interpretation of MS/MS spectra was visually confirmed. Since the genome of *A. catenella*
17 330 has not been fully **sequenced**, protein identification has been performed by homology with
18 331 the known peptidic sequences from the National Centre for Biotechnology Information
19 332 (NCBI) protein database (<http://www.ncbi.nlm.nih.gov/>), preferentially coming from, in this
20 333 order: (i) other *Alexandrium* species (*A. fundyense*, *A. minutum*, *A. ostenfeldii*) (ii) other
21 334 dinoflagellates (*Amphidinium carterae*, *Gonyaulax polyedra*, *Heterocapsa triquetra*,
22 335 *Karlodinium micrum*, *Oxyrrhis marina*) (iii) other microalgae (*Cryptocodinium cohnii*,
23 336 *Symbiodinium* sp.) (iv) Protozoans (*Neospora caninum*, *Paramecium tetraurelia*,
24 337 *Plasmodinium berghei*, *Tetrahymena thermophila*, *Toxoplasma gondii*).

3. Results

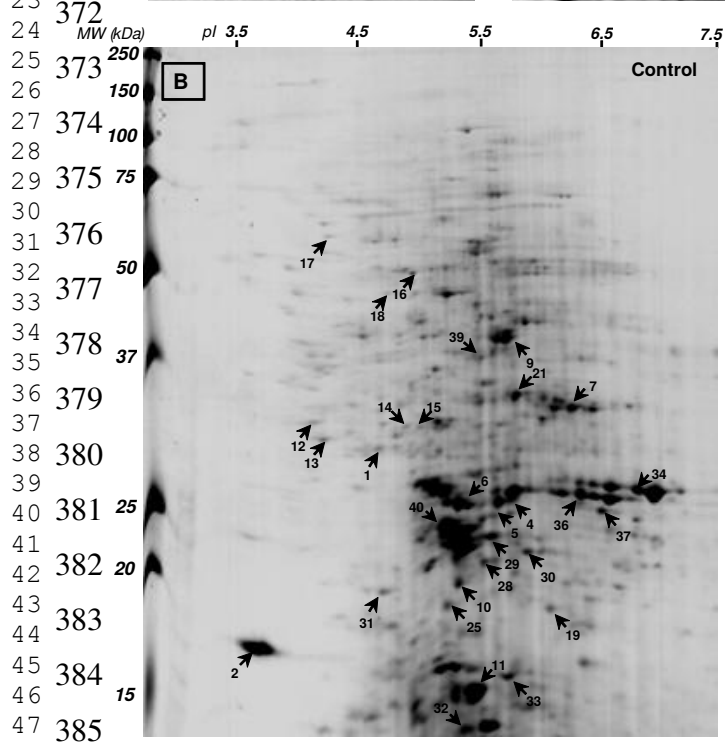
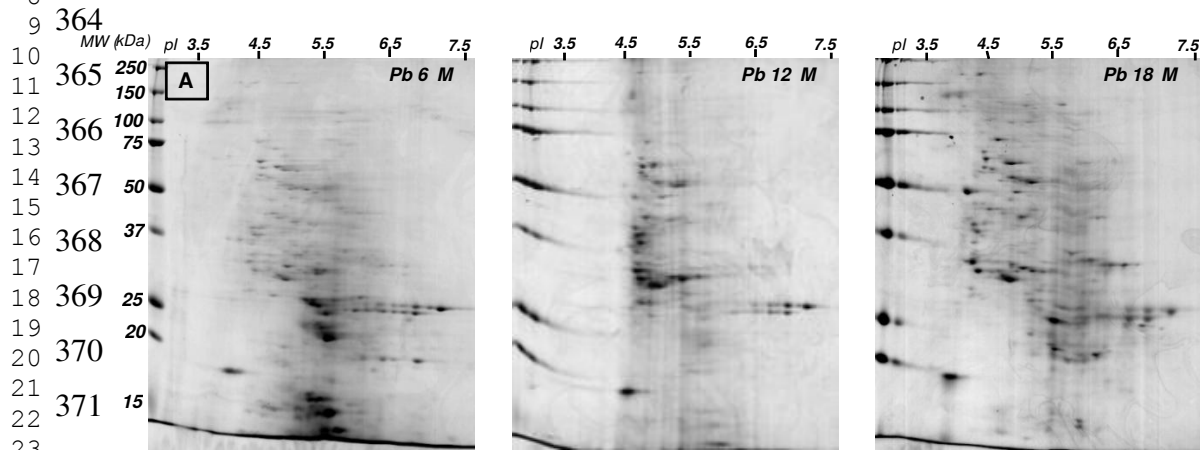
The results of mass spectrometry protein identification are shown in Table II (see also SuppData 1).

Table II. LC-MS/MS identification of the proteins up- or down-regulated in the soluble proteome of *Alexandrium catenella* ACT03 under metallic stresses (lead or zinc at 6, 12 or 18 M)

Spot	Peptide number	MW (kDa) / pI		Protein name	Species Accession number	Putative function
		Exp	Theo			
28	13	19.8/5.6	33.2/5.9	Ribose-5-phosphate isomerase	<i>Heterocapsa triquetra</i> Q5ENN9	Pentose phosphate pathway (02.07)
9	25	38.8/5.9	59.5/5.4	Ribulose-1,5-bisphosphate carboxylase	<i>Gonyaulax polyedra</i> Q42813	Photosynthesis (02.30)
29	16	21.6/5.7	79.1/5.8	Ribulose-1,5-bisphosphate carboxylase	<i>Heterocapsa triquetra</i> Q5ENN5	Photosynthesis (02.30)
4	15	25.0/5.9	20.6/8.8	Peridinin-chlorophyll <i>a</i> -binding protein	<i>Heterocapsa pygmaea</i> Q9FEY3	Light absorption (02.45.03)
34	17	25.7/6.9	37.8/6.6	Peridinin-chlorophyll <i>a</i> -binding protein	<i>Symbiodinium sp.</i> P51874	Light absorption (02.45.03)
36	3	24.8/6.4	38.0/9.1	Peridinin-chlorophyll <i>a</i> -binding protein	<i>Symbiodinium sp.</i> AFH88375	Light absorption (02.45.03)
41	18	24.6/6.7	38.9/9.0	Peridinin-chlorophyll <i>a</i> -binding protein	<i>Gonyaulax polyedra</i> O00941	Light absorption (02.45.03)
43	17	25.7/6.7	38.2/8.7	Peridinin-chlorophyll <i>a</i> -binding protein	<i>Amphidinium carterae</i> P80484	Light absorption (02.45.03)
44	20	25.8/6.5	37.8/6.6	Peridinin-chlorophyll <i>a</i> -binding protein	<i>Symbiodinium sp.</i> P51874	Light absorption (02.45.03)
46	7	25.8/6.2	38.0/9.1	Peridinin-chlorophyll <i>a</i> -binding protein	<i>Symbiodinium sp.</i> AFH88375	Light absorption (02.45.03)
16	3	49.4/5.1	60.0/6.4	ATP beta synthase subunit	<i>Neospora caninum</i> F0VGD5	Energy generation (02.45.15)
6	11	24.3/5.3	74.3/5.1	BiP	<i>Cryptocodinium cohnii</i> Q8S4R0	Chaperone (14.01)
47	5	25.0/6.2	74.3/5.1	BiP	<i>Cryptocodinium cohnii</i> Q8S4R0	Chaperone (14.01)
10	2	18.9/5.4	70.6/5.1	HSP70	<i>Cryptocodinium cohnii</i> Q8S4Q8	Chaperone (14.01)
40	7	22.9/5.3	70.6/5.1	HSP70	<i>Cryptocodinium cohnii</i> Q8S4Q8	Chaperone (14.01)
5	2	24.5/5.8	29.0/5.4	Proteasome alpha subunit	<i>Perkinsus marinus</i> XP_002775560	Proteasomal degradation (14.13.01.01)
30	4	20.4/6.0	22.6/6.1	Proteasome beta subunit	<i>Daphnia magna</i> JAN48661	Proteasomal degradation (14.13.01.01)
33	3	15.6/5.8	27.9/5.8	Proteasome alpha subunit	<i>Perkinsus marinus</i> XP_002764909	Proteasomal degradation (14.13.01.01)
35	4	24.8/6.5	27.9/5.8	Proteasome alpha subunit	<i>Perkinsus marinus</i> XP_002764909	Proteasomal degradation (14.13.01.01)
37	7	23.8/6.6	27.9/5.8	Proteasome alpha subunit	<i>Perkinsus marinus</i> XP_002764909	Proteasomal degradation (14.13.01.01)
45	8	25.8/6.4	27.9/5.8	Proteasome alpha subunit	<i>Alexandrium fundyense</i> A4UHA5	Proteasomal degradation (14.13.01.01)
19	10	18.1/6.2	27.2/5.0	Calreticulin	<i>Heterocapsa triquetra</i> Q5ENL5	Calcium binding (16.17.01)
26	3	14.1/4.5	16.8/4.1	Calmodulin	<i>Prorocentrum minimum</i> AAL61535	Calcium binding (16.17.01)
7	17	31.7/6.4	45.0/7.0	Ferredoxin-NADP ⁺ - reductase	<i>Cryptocodinium cohnii</i> Q5ENS9	Electron transport (20.01.15)
8	19	31.7/6.3	45.0/7.0	Ferredoxin-NADP ⁺ - reductase	<i>Heterocapsa triquetra</i> Q5ENS9	Electron transport (20.01.15)
21	23	32.8/5.9	45.0/7.0	Ferredoxin-NADP ⁺ - reductase	<i>Heterocapsa triquetra</i> Q5ENS9	Electron transport (20.01.15)
39	2	34.1/5.7	45.0/7.0	Ferredoxin-NADP ⁺ - reductase	<i>Heterocapsa triquetra</i> Q5ENS9	Electron transport (20.01.15)
25	11	18.2/5.3	17.9/5.9	Superoxide dismutase [Cu-Zn]	<i>Tetrahymena thermophila</i> Q22H00	Detoxification (32.07)
1	22	29.2/4.8		Chromosome undetermined scaffold_2	<i>Paramecium tetraurelia</i> AOCJD5	Unclassified protein (99)
12	16	31.7/4.3	50.3/5.1	Hypothetical protein	<i>Plasmodium berghei</i> Q4YAV6	Unclassified protein (99)
31	3	19.4/4.8		Unknown	<i>Alexandrium minutum</i> GW796469	Unclassified protein (99)
32	2	14.1/5.4		Unknown	<i>Oxyrrhis marina</i> EG736358	Unclassified protein (99)

359 3.1. Modifications of the soluble proteome under lead exposure

360 Whatever its concentration, lead did not induce a significant ($P > 0.05$) decrease in the
 361 total number of protein spots constituting the soluble proteome of *A. catenella* ACT03 (6 M:
 362 240 ± 74 spots; 12 M: 202 ± 98 spots; 18 M: 239 ± 92 spots), compared to the control
 363 (339 ± 89 spots) (Figures 1A & 1B).



Spot n°	Protein name	6 M	12 M	18 M
1	Unknown protein	+2.0**	+8.2***	
2	Unidentified protein	-4.1**		
4	Peridinin -chlorophyll <i>a</i> -protein	4.0***		-3.0**
5	Proteasome alpha subunit	D	D	D
6	Binding immunoglobulin protein		D	
7	Ferredoxin -NADP ⁺ reductase	-7.3*		
9	RUBISCO	D	D	D
10	HSP 70	-2.9*	D	
11	Unidentified protein			-3.9*
12	Unknown protein		+18.5***	
13	Unidentified protein		+13.5**	
14	Unidentified protein		+17.7***	+2.6*
15	Unidentified protein		+16.4**	+1.7*
16	ATP synthase beta subunit		+16.3***	
17	Unidentified protein		+19.2***	
18	Unidentified protein		+16.7***	
19	Calreticulin		D	D
21	Ferredoxin -NADP ⁺ reductase	-7.3***	D	
25	Superoxide dismutase	-6.5*	D	D
28	Ribose -5-phosphate isomerase		D	
29	RUBISCO	-2.8**	D	D
30	Proteasome beta subunit		D	
31	Unknown protein		D	D
32	Unknown protein		D	
33	Proteasome beta subunit	-2.3***	D	
34	Peridinin -chlorophyll <i>a</i> -protein	-2.2*	-2.3*	
36	Peridinin -chlorophyll <i>a</i> -protein	-3.2**		-1.5*
37	Proteasome alpha subunit		D	
39	Ferredoxin-NADP ⁺ reductase	-6.0**	D	
40	HSP 70	-2.4**	D	

392 **Figure 1.** Representative 2-DE maps comparing the soluble proteomes of *Alexandrium catenella* ACT03 grown
393 under **exposure** to lead 6, 12 or 18 M ($[Pb^{2+}]_F = 11, 26$ and 44 nM respectively) **and control** conditions. (A):
394 Protein profiles of *Alexandrium catenella* ACT03 grown under **exposure** to lead 6, 12 or 18 M (B): Protein
395 profile of *Alexandrium catenella* ACT03 grown **under control condition**. Proteins of interest are identified by
396 their spot numbers (C): Names of the proteins of interest are shown with the fold differences in their expression,
397 according to the level of **lead exposure**. In black, up-regulated proteins; in grey, down-regulated proteins; D,
398 disappeared proteins. *, ** and ***: proteins whose expression was different under lead **exposure**, with $0.05 < P <$
399 0.10 ; $0.01 < P < 0.05$ and $P < 0.01$, respectively

3.1.a. **Exposure to lead 6 M**

401 **Exposure to lead 6 M led to** 46 proteins of interest (*i.e.* whose expression was
402 significantly modified in comparison with **control**: $P < 0.10$) (**Figures 1B & 1C**). Among
403 these differentially expressed proteins, 12 proteins could be identified, which were
404 significantly **down-regulated**, whereas only one protein, not recognized in the protein
405 database, was significantly up-regulated. **The down-regulated proteins** are: (i) involved in
406 photosynthesis, **for** RUBISCO (spots 9 and 29), PCP (spots 4, 34 and 36) and FNR (spots 7,
407 21 and 39) (ii) involved in **the** response to oxidative stress, as proteasome α subunit (spots 5
408 and 33) and SOD (spot 25) (iii) the chaperone HSP 70 (spots 10 and 40). The fold differences
409 in expression of these **proteins ranged** from -7.3 to +2.0 fold, with the highest harmful **effect**
410 on FNR (-7.3 fold) (spots 7 and 21) (**Figure 1C**).

3.1.b. **Exposure to lead 12 M**

414 **Exposure to lead 12 M led to** 82 proteins of interest ($P < 0.10$). Eight proteins were
415 significantly up-regulated and 17 proteins were significantly **down-regulated** (Figures 1B &
416 1C). Only one protein **up-regulated could be** identified: it was the ATP-synthase β subunit
417 (spot 16), involved in energy metabolism. Fold differences in expression of the up-regulated
418 proteins reached the highest values observed in this study, *i.e.* exceeding +16.0 fold (for spots
419 12 and 15-18) (**Figure 1C**). These results showed the **strong effect** of lead 12 M on this
420 protein up-regulation. This **effect** was opposite regarding the **down-regulated proteins**: (i)
421 PCP (spot 34), RUBISCO (spots 9 and 29) and FNR (spots 21 and 39) involved in
422 photosynthesis (ii) proteasome α/β subunits (spots 5, 30, 33 and 37) and SOD (spot 25)
423 implied in the response to oxidative stress (iii) CALR (spot 19) chaperone **also** involved in
424 Ca^{2+} binding (iv) RPI (spot 28) taking part in carbohydrate metabolism (v) HSP 70 (spots 10
425 and 40) and BiP (spot 6), two chaperones.

430 3.1.c. *Exposure to lead 18 M*

431 A number of 59 proteins of interest ($P < 0.10$) have been detected in response to
432 **exposure to lead 18 M (Figures 1B & 1C). These proteins of interest were mainly down-**
433 **regulated. Among them, we have** identified: (i) RUBISCO (spots 9 and 29), enzyme at the
434 root of photosynthesis (ii) proteasome α subunit (spot 5) and SOD (spot 25), involved in the
435 response to oxidative stress (iii) CALR (spot 19), chaperone **also** involved in Ca^{2+} binding (iv)
436 PCP (spots 4 and 36), implied in photosynthetic activity. Finally, two proteins have been
437 significantly up-regulated (spots 14 and 15), with a moderate fold difference around +2.0 fold,
438 but their identification has not been successful (Figure 1C).

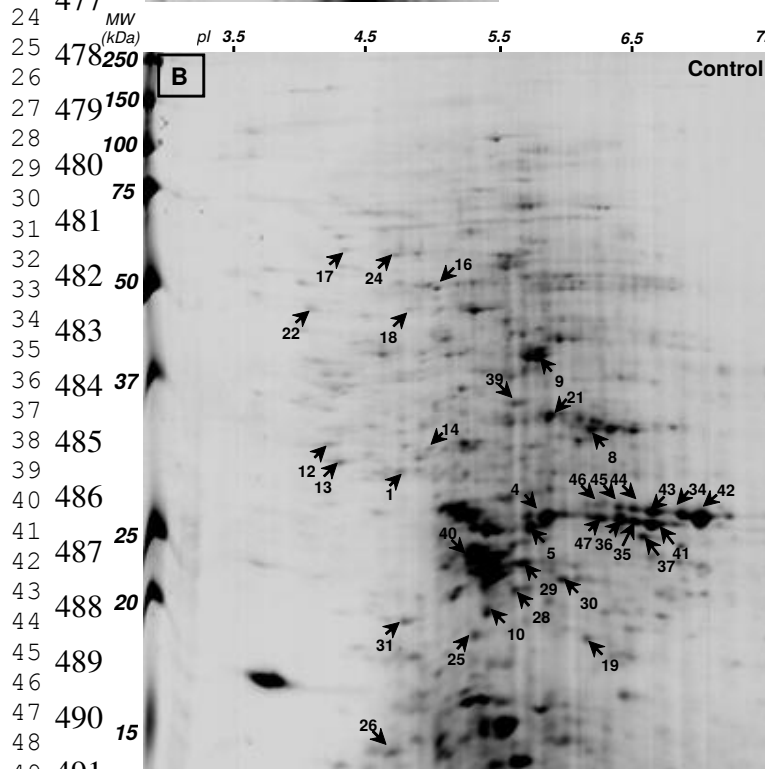
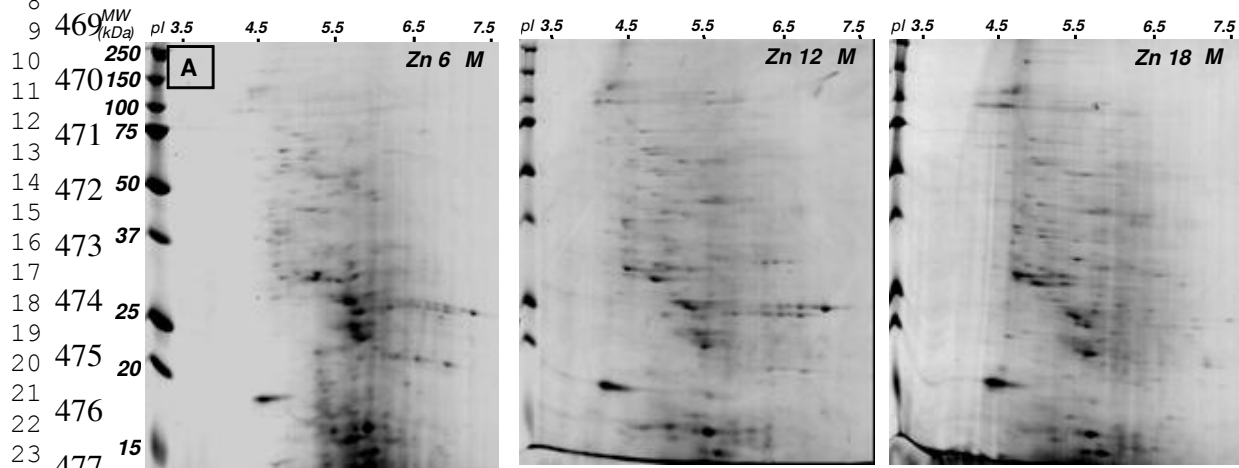
439 3.1.d. *Intercomparison of the proteomic modifications under lead stresses*

440 No significant ($P > 0.05$) difference was observed between the total numbers of proteins
441 **constituting** the soluble proteomes **of *A. catenella* respectively exposed to lead 6, 12 or 18**
442 **M** (Figure 1A). However, **in comparison with the other concentrations, lead at 12 M**
443 **induced the greatest number of modifications in protein expression** (82 proteins of interest),
444 **with** the highest numbers of up-regulated and **down-regulated** proteins (Figures 1B & 1C).
445 Besides, **lead at 12 M** induced the maximal fold differences in up-regulation, reaching
446 between +13.5 (spot 13) and +19.2 (spot 17) fold (Figures 1B & 1C).

447 **Whatever the lead concentration**, the proteins RUBISCO (spot 9) and proteasome α
448 subunit (spot 5) disappeared (Figures 1B & 1C, Figure 1S in SuppData 2). **On the other**
449 **hand**, SOD (spot 25) ended up disappearing **after exposure to lead at 12 M** and 18 M.
450 Such a harmful effect was also observed for HSP 70 (spots 10 and 40), FNR (spots 21 and
451 39), RUBISCO (spot 29) and proteasome α subunit (spot 33). In contrast, **only** ATP-synthase
452 β subunit (spot 16) was up-regulated **after exposure to lead at 12 M**.

3.2. Modifications of the soluble proteome under zinc exposure

In comparison with control (339 ± 89 spots), **systematical significant** (P < 0.05) decreases in the total numbers of protein spots **were observed in the soluble proteomes** of *A. catenella* ACT03 exposed to: zinc 6 M: 200 ± 11 spots (-41%); zinc 12 M: 162 ± 90 spots (-52%); zinc 18 M: 136 ± 37 spots (-60%) (Figures 2A & 2B).



Spot n°	Protein name	6 M	12 M	18 M
1	Unknown protein		+2.0**	+6.0***
4	Peridinin-chlorophyll <i>a</i> -protein	-3.2**	-3.4**	D
5	Proteasome alpha subunit	-3.9***	D	D
8	Ferredoxin -NADP ⁺ reductase	D	D	
9	RUBISCO	D	D	D
10	HSP 70		D	D
12	Unknown protein		D	
13	Unidentified protein			+6.8***
14	Unidentified protein	+2.1*		+2.3*
16	ATP synthase beta subunit			D
17	Unidentified protein		D	D
18	Unidentified protein	-2.7*	D	D
19	Calreticulin		D	
21	Ferredoxin -NADP ⁺ reductase	D	-4.4**	D
22	Unidentified protein		D	
24	Unidentified protein			D
25	Superoxide dismutase	-28.0**	D	D
26	Calmodulin		-21.8*	D
28	Ribose -5-phosphate isomerase			D
29	RUBISCO		-13.2**	D
30	Proteasome beta subunit	D		D
31	Unknown protein		-3.1**	D
34	Peridinin-chlorophyll <i>a</i> -protein	-2.7*		D
35	Proteasome alpha subunit	-6.5**	-2.7*	D
36	Peridinin-chlorophyll <i>a</i> -protein	-3.3**	-2.1*	-2.0*
37	Proteasome alpha subunit			D
39	Ferredoxin -NADP ⁺ reductase		-8.0**	D
40	Heat shock protein 70			D
41	Peridinin-chlorophyll <i>a</i> -protein	-2.4**	-2.2*	-4.4**
43	Peridinin-chlorophyll <i>a</i> -protein		-2.0*	-2.0*
44	Proteasome alpha subunit			D
45	Proteasome alpha subunit			D
46	Peridinin-chlorophyll <i>a</i> -protein			D
47	Binding immunoglobulin protein			D

498 **Figure 2.** Representative 2-DE maps comparing the soluble proteomes of *Alexandrium catenella* ACT03 grown
499 under **exposure to** zinc 6, 12 **or** 18 M ($[Zn^{2+}]_F = 280, 620$ and $1\ 000$ nM respectively) **and control** conditions.
500 (A): Protein profiles of *Alexandrium catenella* ACT03 grown under **exposure to** zinc 6, 12 **or** 18 M (B):
501 Protein profile of *Alexandrium catenella* ACT03 grown **in** control conditions. Proteins of interest are identified
502 by their spot numbers (C): Names of the proteins of interest are shown with the fold differences in their
503 expression, according **to** the level of **zinc exposure**. In black, up-regulated proteins; in grey, down-regulated
504 proteins; D, disappeared proteins. *, ** and ***: proteins whose expression was different under zinc **exposure**, with
505 $0.05 < P < 0.10$; $0.01 < P < 0.05$ and $P < 0.01$, respectively

3.2.a. *Exposure to zinc 6 M*

507
508
509 **With 6 M,** 54 proteins of interest **were detected** ($P < 0.10$). Among these
510 differentially expressed proteins, only one protein (spot 14) was significantly up-regulated (its
511 identification failed), whereas 12 proteins were significantly **down-regulated** (Figures 2B &
512 2C). The proteins **down-regulated** were **involved**: (i) in photosynthesis for RUBISCO (spot
513 9), PCP (spots 4, 34, 36 and 41) and FNR (spot 8) (ii) **in the** response to oxidative stress for
514 proteasome α/β subunits (spots 5, 30 and 35) and SOD (spot 25). Regarding the fold
515 differences in expression of these proteins in comparison with control, the main result was the
516 very strong **down-regulation** of SOD (spot 25) (-28.0 fold) (Figure 2C).

3.2.b. *Exposure to zinc 12 M*

517
518
519 **With 12 M,** 68 proteins of interest **were detected** ($P < 0.10$), among which 20 proteins
520 were significantly down-regulated, whereas one protein was significantly up-regulated (not
521 recognized in the protein database) (Figures 2B & 2C). The proteins down-regulated were
522 **involved**: (i) in photosynthesis for RUBISCO (spots 9 and 29), PCP (spots 4, 36, 41 and 43)
523 and FNR (spots 8, 21 and 39) (ii) **in the** response to oxidative stress for proteasome α subunit
524 (spots 5 and 35) and SOD (spot 25) (iii) in cell signaling, activation of protein kinases and
525 Ca^{2+} binding for CaM (spot 26) (iv) **with** the chaperones HSP 70 (spot 10), and CALR (spot
526 19) **which is** also involved in Ca^{2+} binding. **Effect** of zinc 12 M was particularly harmful on
527 the protein CaM, as indicated by the maximal fold difference in its **down-regulation** (-21.8
528 fold) with regards to control (Figure 2C).

536 3.2.c. *Exposure to zinc (18 M)*

537 **With 18 M**, 111 proteins of interest **were detected** ($P < 0.10$), among which 25
538 proteins **were** identified (Figures 2B & 2C). As observed **for lead at the same**
539 **concentration**, zinc induced the disappearance of many proteins. These proteins **are involved**
540 (i) in photosynthesis for RUBISCO (spots 9 and 29), FNR (spots 21 and 39) and PCP (spots
541 4, 34, 36, 41, 43 and 46) (ii) **in the** response to oxidative stress for proteasome α/β subunits
542 (spots 5, 30, 35, 37, 44 and 45, 46) and SOD (spot 25) (iii) in energy metabolism for ATP-
543 synthase β subunit (spot 16) (iv) take part in cell signaling, activation of protein kinases and
544 Ca^{2+} binding for CaM (spot 26) (v) is the chaperone HSP 70 (spots 10 and 40) (vi) in
545 carbohydrate metabolism for RPI (spot 28). Three proteins were significantly up-regulated
546 (spots 1, 13 and 14), but their identification failed, or led to unknown peptide sequences. The
547 fold differences in expression of the up- and down-regulated proteins in comparison with
548 control, **ranged** between -4.4 (PCP: spot 41) and +6.8 (spot 13) fold (Figure 2C).

549 3.2.d. *Intercomparison of the proteomic modifications under zinc stresses*

551 The total number of proteins **constituting** the soluble proteome of *A. catenella* ACT03
552 **exposed to 18 M of zinc** was significantly lower (of 32%) **than with 6 M** ($P = 0.04$),
553 **confirming the harmful effect of zinc** (Figure 2A). **In comparison with the other**
554 **concentrations**, zinc 18 M **induced** the most drastic modifications in the **protein**
555 **expression**, with the highest numbers of up- and **down-regulated** proteins (Figures 2B &
556 **2C**). Besides, **zinc at 18 M led to** the strongest **up-regulation of some proteins**, reaching
557 +6.0 (spot 1: unknown protein) and +6.8 (spot 13: unidentified protein) fold (Figure 2C). **Zinc**
558 at 12 M and 18 M resulted in the up-regulation of a same unknown protein (spot 1) (at 18
559 M: +6.0 fold; at 12 M: +2.0 fold), whereas an unidentified protein (spot 14) was up-
560 regulated in common for zinc at 6 M and 18 M (+2.0 fold) (Figure 2C). Whatever the zinc
561 **concentration**, RUBISCO (spot 9) disappeared and PCP (spots 36 and 41) was **down-**
562 **regulated** (Figures 2B & 2C, Figure 2S in SuppData 2). Finally, **zinc concentration had**
563 **an increasing effect** on the down-regulation of proteasome α subunit (spots 5 and 35), FNR
564 (spots 21 and 39), SOD (spot 25), CaM (spot 26) and PCP (spots 4, 34, 41 and 43).

3.3. Comparison of the proteomic modifications under lead or zinc stresses

Exposure to lead or zinc led to the up-regulation of three proteins (spots 1, 13 and 14) (Figures 1C & 2C). However, this up-regulation, observed at a lower concentration for lead than for zinc, was stronger for lead (**higher values of the fold differences**). Only lead resulted in the up-regulation of five supplementary proteins (spots 12, 15, 16, 17 and 18), among which ATP-synthase β subunit has been identified. Whatever the trace **metal and its concentration, the** disappearance of RUBISCO (spot 9) **was observed**. Similarly, zinc or lead at 12 M and 18 M, induced disappearance of SOD (spot 25). **However**, higher concentrations **of zinc were** required to induce the disappearance of RUBISCO (spot 29). **Only lead** had a harmful effect on BiP (spot 6) and on proteasome β subunit (spot 33), **and** HSP 70 (spot 40) was **perturbed in expression** from 6 M for lead, against 18 M for zinc.

3.4. Biological functions assigned to the proteins of interest

Biological functions have been assigned to the identified proteins of interest, **in order** to characterize the metabolic pathways implied in the proteomic response of *A. catenella* ACT03 exposed to metallic stresses (Figure 3). These biological functions **are**: calcium binding (CALR, CaM), carbohydrate metabolism (RPI); cell signaling and activation of protein kinases (CaM), chaperone activity (BiP, CALR, HSP 70), energy metabolism (ATP-synthase β subunit; FNR), oxidative stress response (BiP, CALR, HSP 70, proteasome α/β subunits, SOD), photorespiration (RUBISCO) and photosynthesis (ATP-synthase β subunit, FNR, PCP, RPI, RUBISCO). **The most affected proteins were** involved in the photosynthesis (from 20 to 37% of the proteins of interest identified, for lead **at** 6, 12 or 18 M and between 36 and 50% of the proteins of interest identified for zinc **at** 6, 12 or 18 M) and in the oxidative stress response (from 29 to 34% for lead **at** 6, 12 or 18 M, and between 17 and 36% for zinc **at** 6, 12 or 18 M). **The** metabolic pathways potentially **concerned** by modifications in protein expression were (i) energy metabolism (8-9% for lead **at** 6 and 12 M 6-9% for zinc **at** 6 and 18 M) (ii) photorespiration (5-13% for lead **at** 6, 12 or 18 M ; 4-6% for zinc **at** 6, 12 or 18 M) (iii) chaperone activity (4-8% for lead **at** 6 and 12 M ; 6% for zinc at 12 and 18 M) (iv) calcium binding (2-4% for lead **at** 12 and 18 M ; 2-5% for zinc **at** 12 and 18 M) **and** (v) carbohydrate metabolism (3% for lead **at** 12 M and 2% for zinc 18 M), whereas cell signaling and activation of protein kinases (CaM) could be only **perturbed** in response to zinc stresses (2-3% for zinc **at** 12 and 18 M).

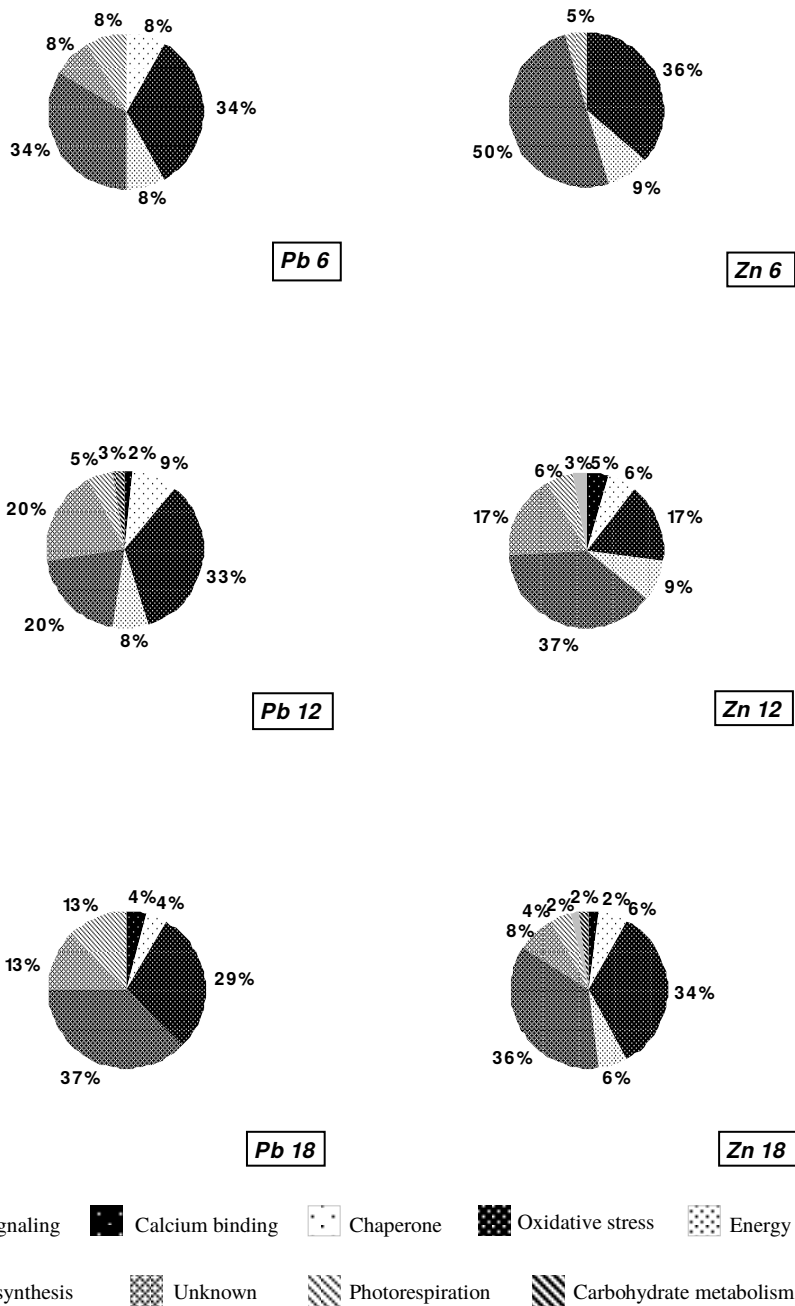


Figure 3. Biological functions assigned to the proteins of interest identified in the soluble proteome of *Alexandrium catenella* ACT03 exposed to lead or zinc at 6, 12 or 18 M

613 4. Discussion

614 4.1. Proteomic modifications and oxidative stress response

615 Through **down-regulation** of key proteins such as SOD and proteasome α/β subunits,
616 the oxidative stress response of *A. catenella* ACT03 **could be a metabolic pathway**
617 **particularly perturbed** in response to **the** metallic stresses.

618 **The** induction of SOD is known to minimize oxidative cell damages among organisms
619 living in polluted environments (Okamoto and Colepicolo, 1998). Indeed, SOD participates to
620 the first antioxidant response to the ROS generated by metal stress (Bareen et al., 2012). In
621 the dinoflagellate *Karenia brevis*, a fast induction of [Mn] SOD has been observed in
622 response to contamination by lead at 18 M (Miller-Morey and Van Dolah, 2004). Similarly,
623 in the marine bivalve *Perna viridis*, a significant expression of [Cu-Zn] SOD has been
624 recorded, following a contamination by cadmium (Leung et al., 2011). In the dinoflagellate
625 *Gonyaulax polyedra* exposed to mercury, cadmium, lead and copper, activity of SOD
626 increased during the first day of exposure to stress, with induction of [Fe] SOD and [Mn]
627 SOD, which was not observed for [Cu-Zn] SOD (Okamoto and Colepicolo, 1998). In the
628 **chlorophyte** *Chlamydomonas reinhardtii* exposed to cadmium, increase **in** [Mn] SOD **co-**
629 **occurred with a** decrease **in** [Fe] SOD (Gillet et al., 2006). A strong stimulation in **the**
630 expression of [Mn] SOD was recorded at the same time as a slight decrease in expression of
631 [Cu-Zn] SOD, in response to cadmium stress in *Saccharomyces cerevisiae* (Vido et al., 2001).
632 However, **lead or zinc at 12 and 18 M**, systematically **induced the disappearance of SOD**
633 **from** the soluble proteome of *A. catenella* ACT03, **whereas** zinc **at 6 M led to a** fold
634 difference in down-regulation **reaching** the very low value of -28.0 (Figures 1C & 2C). Trace
635 metals are known to generate oxidative stress producing ROS **which** damages polypeptide
636 sequences (Stohs and Bagchi, 1995). Then, **these** damaged proteins are targeted for the
637 proteasomal degradation (Delattre et al., 2005), which could **explain** the decreased SOD
638 abundances measured in the soluble proteome of *A. catenella* ACT03, **induced by** metallic
639 stresses.

640 **The** proteasome contributes to cell proteolytic activity, and thus, plays a decisive role
641 in the oxidative stress response, by eliminating oxidized proteins, which have been
642 beforehand targeted by ubiquitination (Flick and Kaiser, 2012). Hence, some studies reported
643 a significant increase in abundance of ubiquitinated proteins, in cells exposed to metal stress
644 conditions (Marzano et al., 2012). Often, increases in proteasomal degradation observed
645 under oxidative stress conditions, correspond to prior increases in abundance of the

646 proteasome subunits (Rodrigues et al., 2012). For instance, an over-expression of the
647 proteasome α subunits, **with a difference of +2.1 fold**, was observed in the crab *Eriocheir*
648 *sinensis* exposed to chronic stress by cadmium (Silvestre et al., 2006). Similarly, proteasome
649 α subunits were over-expressed in the macroalgae *Scytosiphon gracilis* and in the fish *Oryzias*
650 *melanostigma*, under copper and mercury contaminated conditions, respectively (Contreras et
651 al., 2010; Wang et al., 2011). Nevertheless, other studies also mentioned that oxidative stress
652 targets the proteasome itself, during reactions of glutathionylation or nitrosylation, which
653 leads to a dysfunctioning of the proteasomal activity (Dasuri et al., 2013). In the present
654 study, zinc or lead often induced **the decrease** in abundance of proteasome subunits (Figures
655 1C & 2C), which could indicate such a proteasomal degradation **in** oxidative stress
656 conditions.

657 **4.2. Proteomic modifications and photosynthesis**

658 Important decreases in abundances of key **photosynthetic** proteins (RUBISCO, PCP
659 and FNR) have been recorded, making photosynthesis **a metabolic pathway potentially and**
660 **severely perturbed in response to the metallic stresses tested**. These results are in
661 agreement with many studies reporting **harmful effects of trace metals on photosynthesis,**
662 **as** chlorophyll degradation, destruction of chloroplast ultrastructure and down-regulation of
663 many proteins involved in the Calvin cycle (Ahsan et al., 2009; Führs et al. 2008; Gillet et al.,
664 2006; Kieffer et al., 2008).

665 Concerning RUBISCO, which catalyses carboxylation of the substrate ribulose-1,5-
666 bisphosphate (RuBP) during the second step of photosynthesis (Reuman and Weber, 2006),
667 trace metals generally decrease its abundance in the chloroplasts, as observed in the
668 microalgae *Pseudokirchneriella subcapitata* exposed to chromium and palladium (Vannini et
669 al., 2009), or in the **chlorophyte** *Chlamydomonas reinhardtii* stressed by cadmium (Gillet et
670 al., 2006). This could result from high sensitivity of RUBISCO methionine amino acids to the
671 metal-induced oxidative stress, which leads to methionine sulfoxide residues responsible for
672 the targeting of proteins intended to the proteolytic degradation (Brot and Weissbach, 2000;
673 Desimone et al., 1996; Ge et al., 2009; Stadtman et al., 2005). The oxidative degradation of
674 the RUBISCO Binding Protein (RBP), which contributes to the RUBISCO assembly (Roy et
675 al., 1988), as suggested in a study on *P. subcapitata* exposed to stress by chromium (Vannini
676 et al., 2009), **could** also explain this result. Other harmful effects of metals on photosynthesis
677 have been reported, as a significant decrease in RUBISCO activity (around 50%) in the
678 aquatic plant *Salvinia natans* submitted to metal stresses by cobalt, zinc or copper (Dhir et al.,
679

680 2011). This could be due, either to the harmful substitution of cation Mg^{2+} (organizing centre
681 of the active sites of RUBISCO) by the metallic contaminant cations (Van Assche and
682 Clijsters, 1990), or to the irreversible bonds likely to form between metal contaminant cations
683 and thiol groups in RUBISCO (Pankovic et al., 2000). The degradation, in metal stress
684 conditions, of the RUBISCO activase involved in the RUBISCO activation (Salvucci et al.,
685 1987), for example observed in *P. subcapitata* after a stress by chromium (Vannini et al.,
686 2009), also could explain decrease in RUBISCO activity. Similarly, in the present study,
687 RUBISCO in *A. catenella* ACT03 either disappeared whatever the metal tested and its
688 concentration, or was less abundant in the soluble proteome as the metallic concentrations
689 increased, with a stronger harmful effect of lead **compared to** zinc (Figures 1C & 2C).
690 Among the diverse consequences of this decrease in RUBISCO, a lower CO_2 assimilation by
691 the cells (Dhir et al., 2011) and a loss in efficiency of the RuBP recycling which is required
692 by the Calvin cycle (Kieffer et al., 2008), could be **expected** in the cells of *A. catenella*
693 ACT03.

694 **Light energy input for photosynthesis in dinoflagellates is linked to the PCP**
695 **complex** (Hofmann et al., 1996). **This PCP** contains a carotenoid pigment, called peridinin,
696 **which** is probably used as a quencher for the cells, deactivating by energy transfer, the
697 excited state of a chemical species, and thus, acting as an antioxidant (Sigaud-Kutner et al.,
698 2002). For instance, in the dinoflagellate *Lingulodinium polyedrum*, **production** of peridinin
699 increased **up to** 60% in response to **an** oxidative stress (Pinto et al., 2000). However, opposite
700 results have been obtained **with** the dinoflagellate *Gonyaulax polyedra* for which peridinin
701 abundance decreased after cell **exposure** to mercury, cadmium or copper (Okamoto et al.,
702 2001). Even though this **process** reflects decrease in photosynthetic activity, the reduced
703 peridinin **concentration** could also contribute to a regulation of the electron flux between the
704 photosystems I and II, avoiding O_2^- superoxide anion formation, and consequently reducing
705 oxidative damages (Okamoto et al., 2001). Here, PCP abundances decreased in response to
706 most of the metallic **levels** (Figures 1C & 2C), **suggesting** such a form of antioxidant
707 response towards metal stress.

708 **Similarly, the FNR enzyme**, involved in the photophosphorylation step during
709 photosynthesis, was down-regulated in the soluble proteome of *A. catenella* ACT03 exposed
710 to zinc or lead. **Gillet et al. (2006)** have mentioned a toxic effect of cadmium **at** 150 μM on
711 **the** expression of FNR by *C. reinhardtii* (fold difference: -1.7), **showing the** extreme FNR
712 sensitivity to trace metals. **As shown in higher plants**, the FNR degradation by solubilisation
713 can take place under oxidative conditions (Palatnik et al., 1997), which **could also** occur in

714 the present study. Globally, these results confirm the harmful **effects** of metallic stresses on
715 **the** photosynthesis, suggesting *in fine* a potential loss of energy available for the *A. catenella*
716 ACT03 cells. This could be compensated by the up-regulation of some proteins, as **the** ATP-
717 synthase β subunit, as suggested below.

719 **4.3. Proteomic modifications and energy metabolism**

720 **Exposure to** lead at 12 M **induced the** up-regulation (+16.3 fold) of ATP-synthase
721 β subunit (Figure 1C). Such a metal-induced up-regulation of ATP-synthase was also
722 observed in the autotrophs: *Typha angustifolia* exposed to millimolar concentrations of
723 chromium (+1.5 fold) (Bah et al., 2010), *Anabaena* sp. and *Pteris vittata* submitted to arsenic
724 contaminations (Bona et al., 2010; Pandey et al., 2012), *C. reinhardtii* and lichens in response
725 to mercury and cadmium stresses (Gillet et al., 2006; Nicolardi et al., 2012). Associated with
726 the up-regulation of ATP-synthase, the potential increase in the ATP pool could contribute to
727 an adaptive response of these organisms, by bringing more energy to survive under metal
728 stress conditions (Kosova et al., 2011) and sustaining their perturbed photosynthetic activity
729 (Bona et al., 2010). This energy could be used for detoxication processes, for example making
730 more efficient the ATP-synthase active transport of contaminant metal cations out of the cells
731 (Gillet et al., 2006). Moreover, ATP-synthase is known to assume peptidase function and
732 chaperone activity, degrading abnormal and damaged proteins, or repairing them, so as to
733 ensure the survival of the cells (Suzuki et al., 1997).

735 **4.4. Proteomic modifications and photorespiration**

736 Despite its essential contribution to photosynthesis, RUBISCO also catalyses
737 oxygenation of the RuBP substrate during the **first** step of the photorespiratory pathway in
738 autotrophs (Reumann and Weber, 2006). As a result, **down-regulation** of RUBISCO
739 recorded **in the present study** could also suggest a weakening of photorespiration in the cells
740 of *A. catenella* ACT03. Since photorespiration produces CO₂ and NH₃, and consumes O₂ and
741 ATP (Zhang et al., 2012), it is *a priori* considered as an energy counter-productive pathway
742 for autotrophs, **contrarily** to photosynthesis. However, some studies highlight that
743 consumption of O₂ and ATP associated with photorespiration could contribute to the
744 protection of photosynthetic organisms living in oxidative conditions (Wingler et al., 2000).
745 As a result, in regards to photosynthesis, the photorespiratory pathway would be used by
746 stressed autotrophs as wells of energy avoiding the hyper-reduction of the photosynthetic
747 chain of electron transport (Wingler et al., 2000). In addition, the photorespiratory pathway

748 leads to metabolites as glycine amino acid which is used in the biosynthesis of glutathion
749 involved in the cellular response to oxidative stress (Noctor et al., 1999).

751 **4.5. Proteomic modifications and chaperone activity**

752 In *A. catenella* ACT03, **lead at 6 and 12 M, and zinc at 12 and 18 M, perturbed**
753 **expression of** diverse chaperones belonging to the HSPs 70 family, for example **BiP**, also
754 called “heat shock 70 kDa protein 5 (HSPA5)” (Figures 1C & 2C). These results are in
755 accordance with those reported for *Phanerochaete chrysosporium* exposed to cadmium or
756 copper at 50 M, in which abundances in HSP 20 were reduced from 70 to 80% (Ozcan et al.,
757 2007). Similarly, in the Sydney Rock oysters, HSP 70 was the most **affected** protein after
758 contamination by cadmium (50 g L⁻¹), lead (5 g L⁻¹) or zinc (5 g L⁻¹) (Thompson et al.,
759 2012), whereas in the soya beans, HSP 70 abundances were four times lower under cadmium
760 stress (Hossain et al., 2012). Besides, it is known that regulation in HSP expression is metal
761 and concentration depending, as observed for HSP 90 in human lung cells, which was down-
762 regulated by cadmium, nickel and chromium, and up-regulated by arsenic (Andrew et al.,
763 2003), and for HSP 22 in *Argopectens irradians* which decreased in response to stress by
764 cadmium at 200 g L⁻¹, while it was over-expressed in this bivalve exposed to cadmium at
765 100 g L⁻¹ (Leung et al., 2011).

766 Heat shock proteins (HSPs) represent **up to** 2% of the cellular proteins (Gao et al.,
767 2007). These chaperones are known to (i) help protein maturation and folding, optimizing
768 their metabolic functionalities (Beere, 2005) (ii) avoid protein aggregation and ensuring
769 protein membrane transport (Hossain et al., 2012). On the other hand, HSPs take part to the
770 response to many environmental stresses, which explains increases in their abundances
771 observed after exposure to extreme temperatures, UV, trace metals, and as a result, their use
772 as general biomarkers under stress conditions (Timperio et al., 2008; Venn et al., 2009).
773 During stresses, HSPs are required to (i) target the damaged polypeptides so as to allow their
774 subsequent proteasomal degradation (ii) maintain the less damaged polypeptides in their
775 folded conformation so as to avoid their proteasomal degradation, and then, to repair and
776 reactivate them (Miller-Morey and Van Dolah, 2004). Consequently, recovery of the cellular
777 metabolic processes after stress is generally associated with an increase in HSP abundances
778 (Thompson et al., 2012). For example, studies have mentioned that **exposure** of the
779 dinoflagellate *Karenia brevis* to lead at 18 M, induced the up-regulation of HSP 60 (Miller-
780 Morey and Van Dolah, 2004), whereas HSP 70 was up-regulated in marine macroalgae and
781 aquatic plants exposed to cadmium (Ireland et al., 2004).

782 In the present study, **the chaperone calreticulin was down-regulated** by the metallic
1 783 stresses tested (Figures 1C & 2C). CALR is known as an endoplasmic reticulum (ER)
2 784 molecular chaperone, and an ubiquitous multifunctional calcium-binding protein (Ryu et al.,
3 785 2012). Calreticulin is needed in Ca²⁺ buffering and in quality control processes during protein
4 786 synthesis and folding, in particular during refolding of misfolded proteins and ER stress
5 787 responses (Leung et al., 2011). In addition to bind Ca²⁺, CALR **would also** be able to bind
6 788 Zn²⁺ (Michalak et al., 1992), and requires zinc for its interaction with the ERp57 thiol
7 789 oxidoreductase (Leach et al., 2002). Studies showed evidence that cadmium stress induced a
8 790 calreticulin up-regulation in hepatopancreas of *Perna viridis* (Leung et al., 2011), and in leaf
9 791 of *Phytolacca americana* (Zhao et al., 2011). Thus, it has been suggested that elevated levels
10 792 of CALR could serve to protect the cells against cadmium cytotoxicity (Zhao et al., 2011). The
11 793 metal stress inducing ER stress, the resulting misfolded proteins would need the up-regulation
12 794 of ER chaperone, like calreticulin, to be refolded (Leung et al., 2011).
13 795

25 796 **4.6. Proteomic modifications and carbohydrate metabolism**

27 797 Carbohydrate metabolism **could also be perturbed in *A. catenella* ACT03 exposed**
28 798 **to metal stresses**, due to **down-regulation** of the ribose-5-phosphate isomerase (RPI), which
29 799 catalyses the interconversion of Ru5P (or R5P) into R5P (or Ru5P) during (*i*) the Calvin cycle
30 800 (photosynthesis), in the penultimate step leading to final regeneration of RuBP (*ii*) the pentose
31 801 phosphate pathway, in its non oxidative second phase (Zhang et al., 2003). This modification
32 802 in RPI expression in the soluble proteome of *A. catenella* ACT03 was observed in response to
33 803 contaminations by lead at 12 M and by zinc at 18 M. This result agrees with the decrease in
34 804 abundance also reported for RPI in: poplar leaves exposed at cadmium 20 M, *C. reinhardtii*
35 805 stressed by cadmium at 150 M (fold difference: -1.9), tomato roots in response to
36 806 contamination by aluminium at 20 M (Gillet et al., 2006; Kieffer et al., 2008; Zhou et al.,
37 807 2009), confirming the **harmful effects** of trace metals.
38 808

49 809 **4.7. Proteomic modifications and cellular signalling/activation of protein kinases**

51 810 In the present study, calmodulin was **down-regulated after exposure to** zinc at 12
52 811 M (-21.8 fold), to finally disappear at zinc 18 M. Thanks to the formation of a complex
53 812 with Ca²⁺, CaM is activated to take part in cellular signaling and regulate activity of proteins
54 813 called “CaM kinases”, with which it interacts (Alves et al., 2010; Stefan et al., 2008). Indeed,
55 814 kinases are known to regulate activity of transcription factors, which trigger or repress the
56 815 ADN transcription rate into proteins (Mittler, 2002). More generally, kinases stimulate
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816 metabolic pathways *via* activation of many enzymes (ex: phosphorylase kinase involved in
817 glycogenolysis) (Maksymiec et al., 2007). It is known that stress conditions induce a series of
818 reactions increasing the Ca²⁺ levels, which induce CaM kinase activation. Yet, as observed
819 here, a CaM decrease (-3.8) was observed in the liver of sea-bream exposed to chronic stress
820 (Alves et al., 2010). Some studies have reported a possible competition between Ca²⁺ and
821 metal cations on the CaM binding site, as observed during germination of radish plants
822 contaminated by Cd²⁺ (Rivetta et al., 1997). The trace metal binding to CaM could lead to
823 perturbations of the cellular signaling (Clemens, 2006), through inhibition, by the Ca²⁺-
824 calmodulin complex, of the CaM kinase phosphodiesterase. Taking these informations into
825 account, the **down-regulated** CaM **could mean** that **the** synthesis of some proteins would be
826 inhibited **after exposure to** zinc *via* the repression of transcription factors, which **could also**
827 explain the decreases in abundance (**rather than down-regulation**) of many proteins of the
828 soluble proteome of *A. catenella* ACT03 **exposed to metallic stress** conditions.

830 Conclusion

831 **This** study showed the harmful effects of lead or zinc on the soluble proteome of *A.*
832 *catenella* ACT03, and thus potentially, on key metabolic pathways **in** this dinoflagellate,
833 through down-regulation of many proteins to be considered as potential biomarkers of metal
834 stress. However, an adaptive proteomic response could also take place in the cells, **at the**
835 **same time**, thanks to the up-regulation of other proteins as ATP-synthase implied in
836 production of energy, which could be used to sustain detoxification processes and cell
837 survival in presence of the metals. This suggests that, despite the clear harmful **effect** of trace
838 metals, differences in the expression of some proteins **could also** help to maintain the
839 development of *A. catenella* ACT03 in metal-contaminated ecosystems.

841 Acknowledgments

842 This research **was supported** by the GdR « PHYCOTOX: Des micro-algues aux
843 risques pour l'Homme et l'écosystème », the Conseil Général du Var (CG 83), Toulon
844 Provence Méditerranée (TPM) and the research programme ARCUS CERES (PACA-MAE).
845 **It also benefited from the financial help of LAGUNOTOX project funded by Fondation**
846 **TOTAL.**

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Highlight 1:

Soluble proteome expressed by the invasive neurotoxic dinoflagellate *Alexandrium catenella* strain ACT03 grown under control and trace metal conditions (lead or zinc at 6, 12 and 18 M) was studied thanks to two-dimensional electrophoresis (2-DE).

Highlight 2:

Zinc (6, 12 and 18 M) significantly decreased the total number of proteins constituting the soluble proteome.

Highlight 3:

Exposures to trace metal led to down-regulation of most proteins constituting the soluble proteome.

Highlight 4:

The down-regulated proteins of the soluble proteome were above all involved in the photosynthesis (RUBISCO; FNR; PCP) and in the oxidative stress response (SOD; proteasome α/β subunits).

Highlight 5:

The up-regulation of some proteins like ATP-synthase β subunit (+16.3 fold in response to contamination by lead at 12 M) suggests a proteomic adaptive response, may be contributing to the tolerance of *A. catenella* ACT03 in trace metal-contaminated ecosystems.