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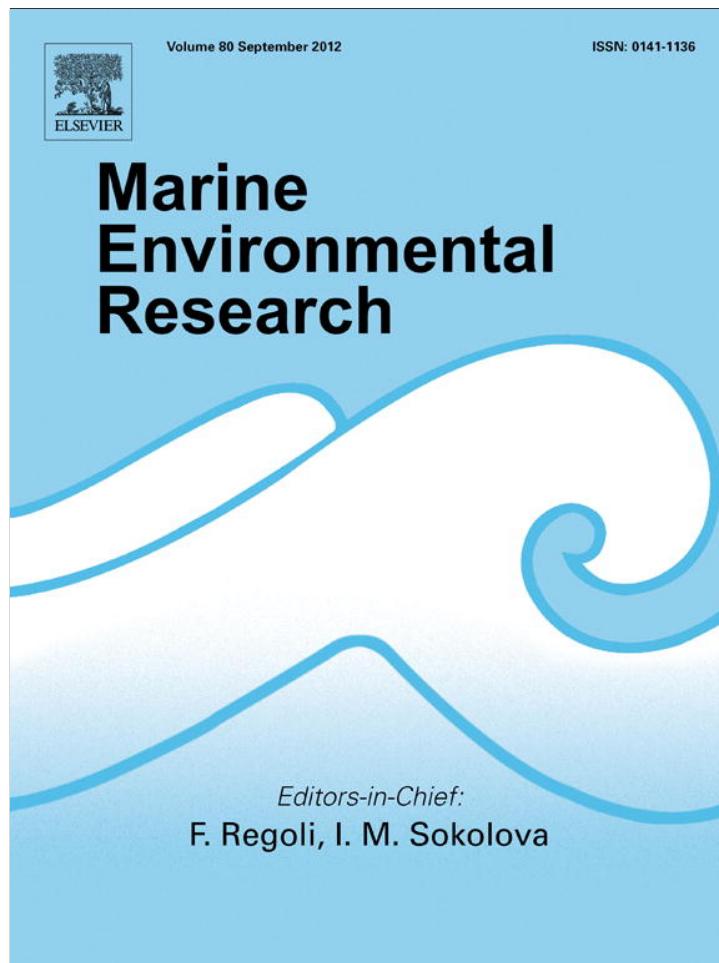
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## Protein expression from zooplankton communities in a metal contaminated NW mediterranean coastal ecosystem

Natacha Jean<sup>a,\*</sup>, Estelle Dumont<sup>a</sup>, Gael Durrieu<sup>a</sup>, Thierry Balliau<sup>b</sup>, Jean-Louis Jamet<sup>a</sup>, Sébastien Personnic<sup>a</sup>, Cédric Garnier<sup>a</sup>

<sup>a</sup>Laboratoire Processus de Transfert et d'Echanges dans l'Environnement (EA 3819), Université du Sud Toulon – Var, BP 20132, 83 957 La Garde Cedex, France

<sup>b</sup>Plate-forme d'Analyse Protéomique Paris Sud-Ouest, INRA, Unité Mixte de Recherche en Génétique Végétale (UMR 320), Ferme du Moulon, 91 190 Gif-sur-Yvette, France

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## ABSTRACT

Bidimensional and monodimensional polyacrylamide gel electrophoresis were used to study protein expression from zooplankton collected in thirteen stations of Toulon Bay (NW Mediterranean). In this ecosystem, Little Bay showed higher trace metal concentrations (13.5–23.8 nM for Cu, 0.73–1.24 nM for Pb, 27.8–58.7 nM for Zn) than Large Bay (Cu 2.2–15.6 nM; Pb 0.19–0.78 nM; Zn 9.0–38.8 nM). Trace metals positively correlated ( $p < 0.05$ ) with expression of four zooplankton proteins (MW in kDa/pI: 25.0/5.6; 48.8/4.1; 38.2/4.4; 38.3/5.8) and with biomass of *Oithona nana*, predominant copepod in Little Bay. Sequencing by LC–MS/MS putatively provided zooplankton identity of these proteins: they were cytoskeleton actin, except one protein that was the chaperone calreticulin. We suggest that actin and calreticulin could be regarded as zooplankton markers of metal stress and be involved in a possible tolerance of *O. nana* to contamination, contributing to its development in a marine perturbed ecosystem.

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### 1. Introduction

Recently, many authors have mentioned the relevance of investigating the new field of marine proteomics (Johnson and Browman, 2007; Lopez, 2007; Nunn and Timperman, 2007). This field presents two coexisting approaches: the first involves analysing proteins from microorganisms cultured under specific

*Abbreviations:* AFDW, ash-free dry weight; BSA, bovine serum albumin; 2-DE, bidimensional polyacrylamide gel electrophoresis; DOC, dissolved organic carbon; DOM, dissolved organic matter; DW, dry weight; ER, endoplasmic reticulum; HSP, heat shock protein; HPLC, high performance liquid chromatography; IPG, immobilized pH gradient; IEF, isoelectric focusing; pI, isoelectric point; LC–MS/MS, liquid chromatography tandem mass spectrometry; MW, molecular weight; 1-DE, monodimensional polyacrylamide gel electrophoresis; NCBI, National Centre for Biotechnology Information; POM, particulate organic matter; ROS, reactive oxygen species; SDS–PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

\* Corresponding author. Tel.: +33 4 94 14 25 29; fax: +33 4 94 14 21 68.

*E-mail addresses:* [jean@univ-tln.fr](mailto:jean@univ-tln.fr) (N. Jean), [estelle.dumont@univ-tln.fr](mailto:estelle.dumont@univ-tln.fr) (E. Dumont), [durrieu@univ-tln.fr](mailto:durrieu@univ-tln.fr) (G. Durrieu), [balliau@moulon.inra.fr](mailto:balliau@moulon.inra.fr) (T. Balliau), [jamet@univ-tln.fr](mailto:jamet@univ-tln.fr) (J.-L. Jamet), [sebastien.personnic@univ-tln.fr](mailto:sebastien.personnic@univ-tln.fr) (S. Personnic), [cgarnier@univ-tln.fr](mailto:cgarnier@univ-tln.fr) (C. Garnier).

environmental conditions, the second involves the recovery and analysis of proteins directly sampled from the marine environment (Nunn and Timperman, 2007). According to the first approach, some studies have been carried out on pure cultures of marine microorganisms such as the bacterium *Pseudomonas fluorescens* (Poirier et al., 2008), the cyanobacterium *Prochlorococcus marinus* (Pandhal et al., 2007), the phytoplankton *Alexandrium* (Chan et al., 2005, 2006; Lee and Lo, 2008; Wang et al., 2008), *Prorocentrum* (Chan et al., 2002, 2004), *Lingulodinium polyedrum* (Akimoto et al., 2004), and the zooplankton *Calanus finmarchicus* (Hansen et al., 2007). According to the second approach, proteomic studies have taken place in marine environments to sample targeted organisms, such as the zooplankton species *Acartia tonsa* (Tartarotti and Torres, 2009) and *Eurytemora affinis* (Kimmel and Bradley, 2001), as the fish *Paralichthys olivaceus* (Ling et al., 2009; Zhu et al., 2006) or the bivalve *Mytilus* (Manduzio et al., 2005; Mosquera et al., 2003; Ronzitti et al., 2008).

However, it may be assumed that the many species composing a high-complexity community constitute a metaorganism, in which metaproteome shifts could be regarded as a functional response to the dynamic changes affecting the environment (Lacerda et al.,

2007; Wilmes and Bond, 2004). In this way, very recent studies have focused on the metaproteomics characterization of the dissolved organic matter (DOM) and the particulate organic matter (POM) sampled in the South China Sea (Dong et al., 2010; Wang et al., 2011).

Proteomics provides an excellent tool to analyse changes in protein expression in response to contaminant exposure (Andacht and Winn, 2006). Now, coastal marine ecosystems are increasingly exposed to contamination, among which pollution by metals is a serious threat due to their persistence and toxicity towards marine organisms (Huang et al., 2005). Although zooplankton represent a point of entry of contaminants into the food web, through grazing on phytoplankton, and being themselves an important prey for fish (Hansen et al., 2007), few studies have assessed effects of contaminants on zooplankton communities.

Here, we present the first metaproteomics study characterising protein expression from zooplankton communities in a coastal marine ecosystem contaminated by trace metals. We used monodimensional gel electrophoresis (1-DE) and bidimensional polyacrylamide gel electrophoresis (2-DE) to detect shifts in protein expression, from zooplankton sampled in thirteen stations of Toulon Bay (France). We then related the protein shifts to the structure of the zooplankton communities, and to the trace metal concentrations measured in this ecosystem. Our goal was to understand how the zooplankton protein expression changed according to the metal contamination of a marine natural environment. This was to (i) define zooplankton protein markers of metal stresses, (ii) identify stress proteins potentially involved in the tolerance of some zooplankton species to contamination, (iii) understand the potential contribution of some proteins in the changes in zooplankton diversity recurrently observed in the perturbed Toulon Bay (Jamet et al., 2001, 2005; Richard and Jamet, 2001).

## 2. Material and methods

### 2.1. Sampling site

On June 11th 2009, seawater and zooplankton have been sampled in thirteen stations –  $S_1$  to  $S_{13}$  – of Toulon Bay, on the NW Mediterranean French coast (Fig. 1). Thanks to a sea wall, Toulon Bay is divided in two smaller bays: Large Bay (LaB) in the south-east (maximal depth: 17 m), with sampling stations  $S_1$ – $S_9$ , and Little

Bay (LiB) in the north-west (maximal depth: 12 m), with sampling stations  $S_{10}$ – $S_{13}$ .

LiB, semi-closed with a surface area of 11 km<sup>2</sup>, harbours major commercial traffic as well as a military port (French Navy). Through the Las river, the first river crossing Toulon city, anthropogenic inputs from the urbanized Toulon area (population density = approximately 600 000 inhabitants) flow into LiB. This ecosystem is significantly polluted, as shown by the high trace metal concentrations measured in *Mytilus* during the 'Mussel Watch Programme' carried out by the Réseau d'Observation de la Contamination Chimique: 7.26 mg Pb per kg of dry weight (4.4 times the national media of 1.65 mg per kg of dry weight) and 0.54 mg Hg per kg of dry weight (3.6 times the national media of 0.15 mg per kg of dry weight) (Ifremer, 2010). In LiB, the macrophyte *Posidonia oceanica* has disappeared for thirty years (Bernard et al., 2001). The proliferation of harmful phytoplankton species *Dinophysis* and *Pseudo-nitzschia* occurs at some periods of the year (Ifremer, 2010). The dominance and high abundance of the Cyclopoid Copepod *Oithona nana* have been previously reported there, resulting in lower zooplankton diversity (Jamet et al., 2005).

Unlike LiB, LaB is connected to the open-sea, the renewal of its water masses being provided by the deep northwest to southeast current, and then by the Liguro-Provençal drift. LaB receives anthropogenic inputs carried by the Eygoutier, the second river passing through Toulon. Compared to the Las, the Eygoutier is regularly dry in summer, suggesting that it contributes less to the pollution of LaB. A recent study reported that sediments were less impacted by metals in LaB than in LiB (Tessier et al., 2011). Great meadows of *P. oceanica* (surface = 325 ha) and low occurrences of harmful phytoplankton species confirm that LaB is less affected by anthropogenic activities than LiB.

### 2.2. Sampling procedures

All samplings were carried out in LaB ( $S_1$ – $S_9$ ) and in LiB ( $S_{10}$ – $S_{13}$ ) (Fig. 1) on June 11th 2009.

Zooplankton samples were collected using a nylon net (Hydro-Bios, model Apstein) with 90  $\mu$ m mesh (0.5 m mouth diameter, 2.5 m length), equipped with a flowmeter (Hydro-Bios, model 438 110). Each zooplankton sample had a volume ranging from 1.0 to 1.6 L and came from a filtration of volumes comprised between 7.5 and 10.7 m<sup>3</sup> of seawater.

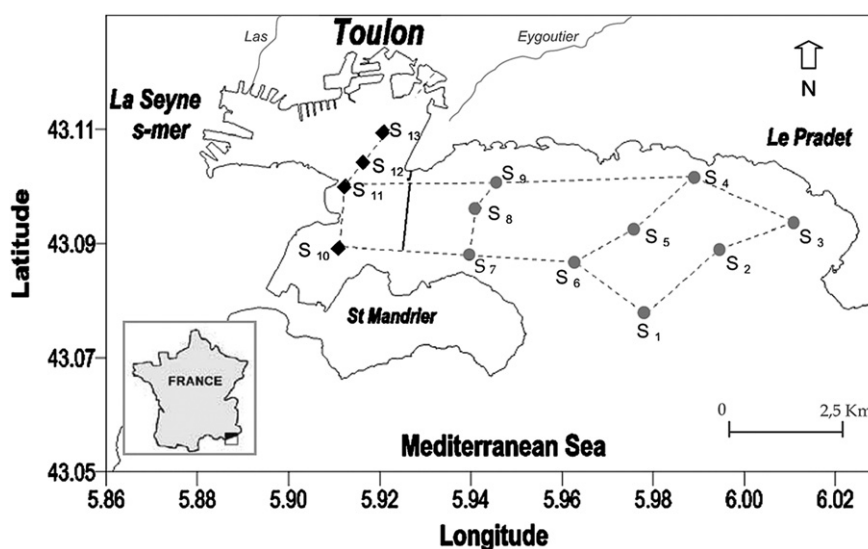


Fig. 1. Location of the  $S_1$ – $S_9$  sampling stations in the Large Bay of Toulon (●), and the  $S_{10}$ – $S_{13}$  stations in the Little Bay of Toulon (◆).

Seawater samples for total dissolved trace metal analysis were taken at 3 m depth by scuba diving in pre-cleaned 1 L Fluorinated Ethylene Propylene (FEP) bottles (Nalgene) and stored in a coolbox until the filtration. Then, samples were filtered through 0.45  $\mu\text{m}$  pre-cleaned cellulose nitrate filters (Sartorius), and stored at 4 °C in the dark until analysis in pre-cleaned 60 mL FEP (Nalgene) for trace metal measurements (preservation by acidification at  $\text{pH} < 2$ ). For logistic reasons, no seawater sampling for dissolved trace metal analysis was carried out in  $S_{12}$ .

### 2.3. Preparation of protein extracts

At the laboratory, zooplankton samples were immediately filtered through Whatman GF/C glass fibre filters ( $\varnothing = 47$  mm) to collect the zooplankton individuals. The filters with zooplankton were kept frozen at  $-30$  °C overnight and then, lyophilized with a Heto Power Dry LL 1500 Lyophilizator (Thermo Electro Corporation), as recommended for studying proteins in plankton (Tanoue, 1996). Then, surface of the filter was carefully peeled off together with a small amount of glass fibre and finely powdered using a mortar and pestle. The powdered zooplankton samples were immediately extracted, or kept frozen at  $-30$  °C until extraction.

#### 2.3.1. 1-DE extraction

Proteins for 1-DE were extracted according to Tanoue et al. (1996), by homogenizing 0.1 g of zooplankton powder in 1 mL of a sample solution containing tris-HCl (62.5 mM  $\text{pH}$  6.8), sodium dodecylsulfate (SDS, 2% w/v), bromophenol blue (BPB, 0.1% w/v) and 2-mercaptoethanol (5% v/v), and then heated at 100 °C for 3 min. After cooling, urea was added (8 M, final concentration) to facilitate the solubilization of zooplankton proteins. After centrifugation at  $2000 \times g$  for 5 min, the supernatant was adjusted to  $\text{pH}$  6.8 with HCl, to obtain the definitive protein extract from which protein determination and 1-DE were achieved.

#### 2.3.2. 2-DE extraction

Proteins for 2-DE were differently extracted, according to a modified method of Saijo and Tanoue (2004), more intensive than that used for 1-DE. Indeed, a fraction of 0.1 g of zooplankton powder was homogenized with 1 mL of a sample solution consisting of urea (7 M), thiourea (2 M), 3-[3-(cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS, 1% w/v), polyethyleneglycol p-ter-octylphenylether (Triton X-100, 3% v/v), dithiothreitol (DTT, 1% w/v), carrier ampholytes (0.2% v/v), BPB (0.002% v/v). The mixture was incubated for 1 h with stirring for 30 s every 15 min, and was then submitted to centrifugation at  $3000 \times g$  for 30 min. Two freezing-thawing cycles ( $-30$  °C/ $+20$  °C) were applied to the obtained supernatant. To complete cell disruption, this was sonicated in an ice-water bath, using a microtip Vibra cell 734 24 (Biorblock Scientific) during 5 min, at 20 kHz and 50 W, with 10 s on/10 s off cycles. The final protein extract was centrifuged at  $3000 \times g$  for 30 min. Protein determination and 2-DE were achieved from the supernatant.

### 2.4. Protein determination

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

### 2.5. 1-DE

1-DE was performed as described by Laemmli (1970), using handmade polyacrylamide mini-gels (10 cm  $\times$  7 cm  $\times$  1.5 mm,

stacking gel: 5%, resolving gel: 11%). Proteins of a same extract were resolved in triplicate (or in duplicate for  $S_1$ ,  $S_2$ ,  $S_3$  extracts), by loading three sample volumes containing 100  $\mu\text{g}$  zooplankton protein each, in three different wells. A volume of 10  $\mu\text{L}$  of a molecular weight marker solution (15–250 kDa range, Precision Plus Protein Standards Dual Color, Bio-Rad) was loaded at the left top of the gel. The gels were run in a Mini-PROTEAN 3 Cell (Bio-Rad) at  $+4$  °C, under 100 V and 20 mA per gel for 10 min, and then, under 150 V and 40 mA per gel, until the dye reached the bottom of the gel. Then, gels were stained with Coomassie Brilliant Blue (CBB) R-250 (Bio-Rad), and destained with an acetic acid (7% v/v)–ethanol (5% v/v) solution, until visualization of separated protein bands.

The protein bands on the gels were analysed using the Quantity One 1D-Analysis software 4.6.7. version (Bio-Rad). All  $R_f$  (relative migration distance run by a protein band) values have been converted into  $\text{Log}(\text{MW})$  to calculate the MW in each lane of the gels. Intensity of each zooplankton protein band has been calculated as a percentage of the intensity of the 250 kDa marker band.

### 2.6. 2-DE

Isoelectric focusing (IEF) was carried out according to O'Farrell (1975). The IEF needed pre-prepared immobilized pH gradient (IPG) strip (17 cm length, linear gradient,  $\text{pH}$  3–10, Bio-Rad), under which a sample (350  $\mu\text{L}$ ) containing 300  $\mu\text{g}$  zooplankton protein, was transferred. Rehydration and subsequent IEF of the sample were performed in the horizontal electrophoresis system PROTEAN IEF Cell (Bio-Rad), in the following manner: 18 h at 50 V (active rehydration), 2 h at 100 V, 2 h at 250 V, 2 h at 500 V, 2 h at 1000 V, 2 h at 4000 V and 5 h at 10 000 V, so as to reach a total minimal value of 60 000 V h for each loaded IPG strip. All steps were run at  $+20$  °C. After focusing, the IPG strip was successively equilibrated for 10 min at room temperature in equilibration buffers 1 (urea 6 M, SDS 2% w/v, tris-HCl 0.375 M  $\text{pH}$  8.8, glycerol 20% v/v, DTT, 2% w/v) and 2 (urea 6 M, SDS 2% w/v, tris-HCl 0.375 M  $\text{pH}$  8.8, glycerol 20% v/v, iodoacetamide 2.5% w/v).

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). The equilibrated IPG strip was placed in dyed (BPB) melted agarose, across a handmade polyacrylamide gel (18 cm  $\times$  18 cm  $\times$  1 mm, stacking gel: 5%, resolving gel: 11%). A volume of 20  $\mu\text{L}$  of molecular weight marker solution (10–250 kDa range, Precision Plus Protein Standards Dual Color, Bio-Rad) was loaded at the left top of the gel. The gels were run at  $+4$  °C in a Protean II XL (Bio-Rad), with a constant current of 20 mA per gel for 1 h and then, with a constant current of 30 mA per gel until the dye reached the bottom of the gel. After electrophoresis, the gels were stained as described in Section 2.5. The 2D-gels presented in this study were representative of two gels performed with the same sample, using an identical extraction procedure but independent 2-DE experiments.

The protein spots on the gels were analysed using the PD-Quest 2D-Analysis software 8.0.1. version (Bio-Rad). Intensity of each zooplankton protein spot has been calculated as a percentage of the total intensity from the marker bands.

### 2.7. Protein identification by liquid chromatography tandem mass spectrometry

In-gel digestion of the picked 2-D spots was performed with the Progest system (Genomic Solution) according to a standard trypsin protocol (Page et al., 2010).

HPLC was performed on a NanoLC-Ultra system (Eksigent). A 4  $\mu\text{L}$  sample of the peptide solution was loaded at 7.5  $\mu\text{L min}^{-1}$  on



a precolumn cartridge (stationary phase: C18 Biosphere, 5  $\mu\text{m}$ ; column: 100  $\mu\text{m}$  inner diameter, 2 cm; Nanoseparations) and desalted with 0.1% HCOOH. After 3 min, the precolumn cartridge was connected to the separating PepMap C18 column (stationary phase: C18 Biosphere, 3  $\mu\text{m}$ ; column: 75  $\mu\text{m}$  inner diameter, 150 mm; Nanoseparations). Buffers A and B respectively were prepared with 0.1% HCOOH in water, and with 0.1% HCOOH in acetonitrile. The peptide separation was achieved with a linear gradient from 5 to 30% B for 28 min at 300 nL min<sup>-1</sup>. Including the regeneration step at 95% B and the equilibration step at 95% A, one run took 45 min.

Eluted peptides were on-line analysed with an LTQ XL ion trap (Thermo Electron) using a nano electrospray interface. Ionization (1.5 kV ionization potential) was performed with liquid junction and a non-coated capillary probe (10  $\mu\text{m}$  inner diameter; New Objective). Peptide ions were analysed using Xcalibur 2.07 with the following data-dependent acquisition steps: (1) full MS scan (mass-to-charge ratio ( $m/z$ ) 300–1400, centroid mode) and (2) MS/MS ( $q_z = 0.25$ , activation time = 30 ms, and collision energy = 35%; centroid mode). Step 2 was repeated for the three major ions detected in step 1. Dynamic exclusion was set to 30 s.

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

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

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

Homology searches were performed by Fasts software (version 36.06) using the MD20-MS matrix (2). Sequences corresponding to keratins or trypsin were firstly removed by interrogating a home-made contaminant database. Secondly, the search computing process was carried out on the same database. Only homologies with a minimum of 2 independent peptides and an  $E$  value smaller than 0.001 were selected. In any case, the automatic *de novo* interpretation of MS/MS spectra was visually confirmed. After searching in the National Centre for Biotechnology Information (NCBI) protein database (<http://www.ncbi.nlm.nih.gov/>) for sequence comparison and theoretical MW/pI, identified proteins were classified according to their biological functions with the Funcat automatic classification (<http://www.helmholtz-muenchen.de/en/mips/projects/funcat/>).

## 2.8. Zooplankton counts and biomasses

In the laboratory, 250 mL from each zooplankton sample collected with the net was preserved in seawater buffered with 5% formaldehyde and studied for counts from 1 mL aliquots taken with a Hensen pipette. A minimum of 250 organisms per each aliquot was identified at the species level, if possible. All zooplankton organisms were enumerated using an inverted microscope at a magnification of 100 $\times$ . Biomasses were calculated as dry weight (DW, in  $\mu\text{g}$ ) for each zooplankton species, using abundances and length–weight regression equations (Table S1 of Appendix). For the Cladoceran *Evadne nordmanii* and the Cirripede nauplii, biomasses were calculated from the individual carbon weights ( $W$ , in  $\mu\text{g C}$ ) of 2 and 2.5  $\mu\text{g C}$ , respectively (Rodhouse and Roden, 1987). The  $W$  values were converted to DW assuming a carbon content of 0.5 (Omori, 1969). Ash-free dry weights (AFDW, in  $\mu\text{g}$ ) reported in the literature were converted to DW assuming an ash content of 6.4% AFDW (Chisholm and Roff, 1990). Diversity of the zooplankton communities has been estimated thanks to the Shannon Index ( $H'$ ) (Shannon and Weaver, 1949), calculated using the following formula:

$$H' = - \sum_{i=1}^S f_i \times \log_2 f_i$$

where  $f_i$  is the frequency of the species  $i$ .

## 2.9. Total dissolved trace metals

Total dissolved metal concentrations were determined in acidified and UV-digested samples (2 h in quartz tubes on the Metrohm 705 UV Digester) by Differential Pulse Anodic Stripping Voltammetry (DPASV). Measurements were carried out with a voltammetric analyser  $\mu$ Autolab III (Eco Chemie) controlled by GPES 4.9 software (Eco Chemie) coupled with a three-electrode cell of 663 VA Stand (Metrohm) (Louis et al., 2009a). Parameters of the DPASV procedures were adapted from Omanović et al. (2006). Briefly, a first procedure (DPASV1) was adapted for the simultaneous measurement of Cd, Cu and Pb with the following parameters: 300 s of deposition at  $-1.1$  V followed by 10 s of equilibrium time before the anodic scan from  $-0.75$  to 0 V, all others parameters similar to Omanović et al. (2006). A second procedure (DPASV2) was adapted for Zn measurement, with 120 s of deposition at  $-1.2$  V followed by 10 s of equilibrium time before the anodic scan from  $-1.05$  to  $-0.75$  V. Total dissolved metal concentrations were determined by a fully automated standard addition method. Cd, Cu and Pb concentrations were determined by duplicate DPASV1 measurements on the unspiked sample followed by four additions (using Cavo XL 3000 Syringe Pump) of a 50 nM Cd, 200 nM Pb and 5  $\mu\text{M}$  Cu standard solution. Then, 200  $\mu\text{L}$  of a 4 M sodium acetate solution are added to increase the pH to 4. Finally, Zn concentration is determined by duplicate DPASV2 measurements followed by four additions of a 5  $\mu\text{M}$  Zn standard solution.

This analytical procedure was applied to the analysis of Cd, Cu, and Zn total dissolved concentrations in certified seawater (CASS-5, NRC-CNRC, Cd:  $0.19 \pm 0.02$  nM, Cu:  $5.98 \pm 0.44$  nM, Zn:  $11.0 \pm 1.0$  nM), leading to results within the certified limits. In this certified seawater, Pb determination was not achieved with the 300 s of deposition time used, due to the extremely low certified value ( $0.053 \pm 0.010$  nM), at least 10 times lower than values measured in our samples.

## 2.10. Statistical analysis

The non-parametric Spearman's rank correlation test (Statistica 7.1. version) was used to compare the data series (protein spot and

band intensities, zooplankton biomasses, trace metal concentrations). Correlations and relations, characterized by the Spearman's rank correlation coefficient (Spearman's rho, here noted  $r$ ), and by  $p$  values, were considered as significant if  $p < 0.05$  (for correlations) or  $0.05 < p < 0.10$  (for relations).

### 3. Results

#### 3.1. Protein expression

Despite the strong background staining each lane of the 1D-gels, few distinct protein bands were visible, varying from 12 ( $S_{12}$ ) to 25 bands ( $S_1, S_2, S_3$ ) according to the sampling stations (Fig. 2A and Fig. S1 of Appendix). Among these,  $53 \pm 11\%$  ( $n = 13$ ) characterized protein groups with  $50 \text{ kDa} < \text{MW} < 242 \text{ kDa}$ , corresponding to high MW proteins. The low number of protein bands associated with the high MW that they presented suggest that the 1D extraction protocol here used may be was not enough efficient to dissociate some zooplankton protein complexes, which therefore remained combined with high MW. However, we distinguished 25 bands with similar MW that were common between the stations  $S_1$  and  $S_{13}$  of Toulon Bay, indicating same protein species in these stations. These proteins are presented with their respective band numbers and intensities (Fig. 2B and Fig. S1 of Appendix).

On the 2D-gels, the total number of zooplankton protein spots ranged from 16 ( $S_9$ ) to 98 spots ( $S_3$ ). Among these,  $97 \pm 3\%$  ( $n = 13$ ) corresponded to small proteins with  $13 \text{ kDa} < \text{MW} < 50 \text{ kDa}$ , and pI between 4.1 and 6.7 (Fig. 3).

This proportion, greatly differing from that obtained for the 1D-gels, indicated a discrepancy between our 1-DE and 2-DE results. This suggests that the 1D and 2D extraction protocols may induce a different solubilisation of the zooplankton proteins that would lead to a better dissociation of protein complexes by the 2-DE extraction protocol. Thirteen spots with similar MW/pI were common between the stations  $S_1$  and  $S_{13}$  of Toulon Bay. These proteins, named a, b, c, d, e, A, B, C, D, 1, 2, 3 and 4, are presented with their respective MW/pI and intensities (Table 1). Of the 13 spots, those named a, d, e, A, B, C, D, 1 and 4 were putatively identified by comparison to sequences in the protein database at the NCBI (Table S2 of Appendix). Most of the protein spots (a, d, e, A, C, D, 1) were identified as actin (entire or fragments) (Table 2). Two spots were differently assigned, to tubulin (spot 4) and to calreticulin (spot B). Actin and tubulin are involved into cytoskeletal activities, whereas calreticulin is a protein chaperone.

#### 3.2. Zooplankton communities

Total zooplankton biomass (from  $4 \text{ mg DW m}^{-3}$  in  $S_3$  to  $396 \text{ mg DW m}^{-3}$  in  $S_7$ ), was slightly higher in LiB (mean =  $141 \text{ mg DW m}^{-3}$ ) than in LaB (mean =  $125 \text{ mg DW m}^{-3}$ ) (Table 3). However, total zooplankton abundance was twice as high in LiB (mean =  $32\,730 \text{ ind m}^{-3}$ ) than in LaB (mean =  $16\,859 \text{ ind m}^{-3}$ ). Save for the Appendicularian *Oikopleura* spp., zooplankton biomass was principally due to Copepods, which also represented most of the total zooplankton abundance (71% on average). Copepod biomass and abundance were twice as high in LiB ( $15 \text{ mg DW m}^{-3}$  and  $26\,029 \text{ ind m}^{-3}$ ) than in LaB ( $8 \text{ mg DW m}^{-3}$  and  $11\,693 \text{ ind m}^{-3}$ ).

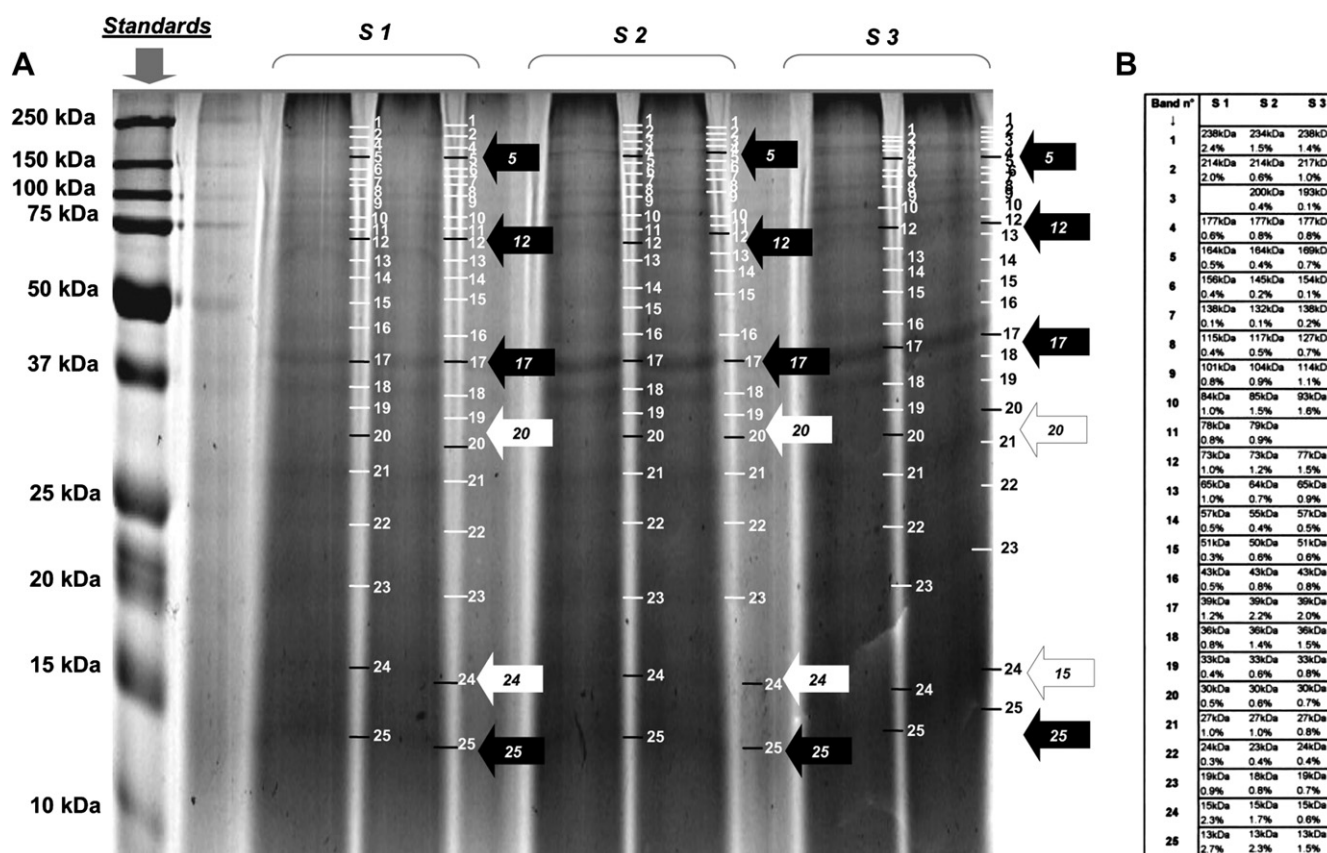
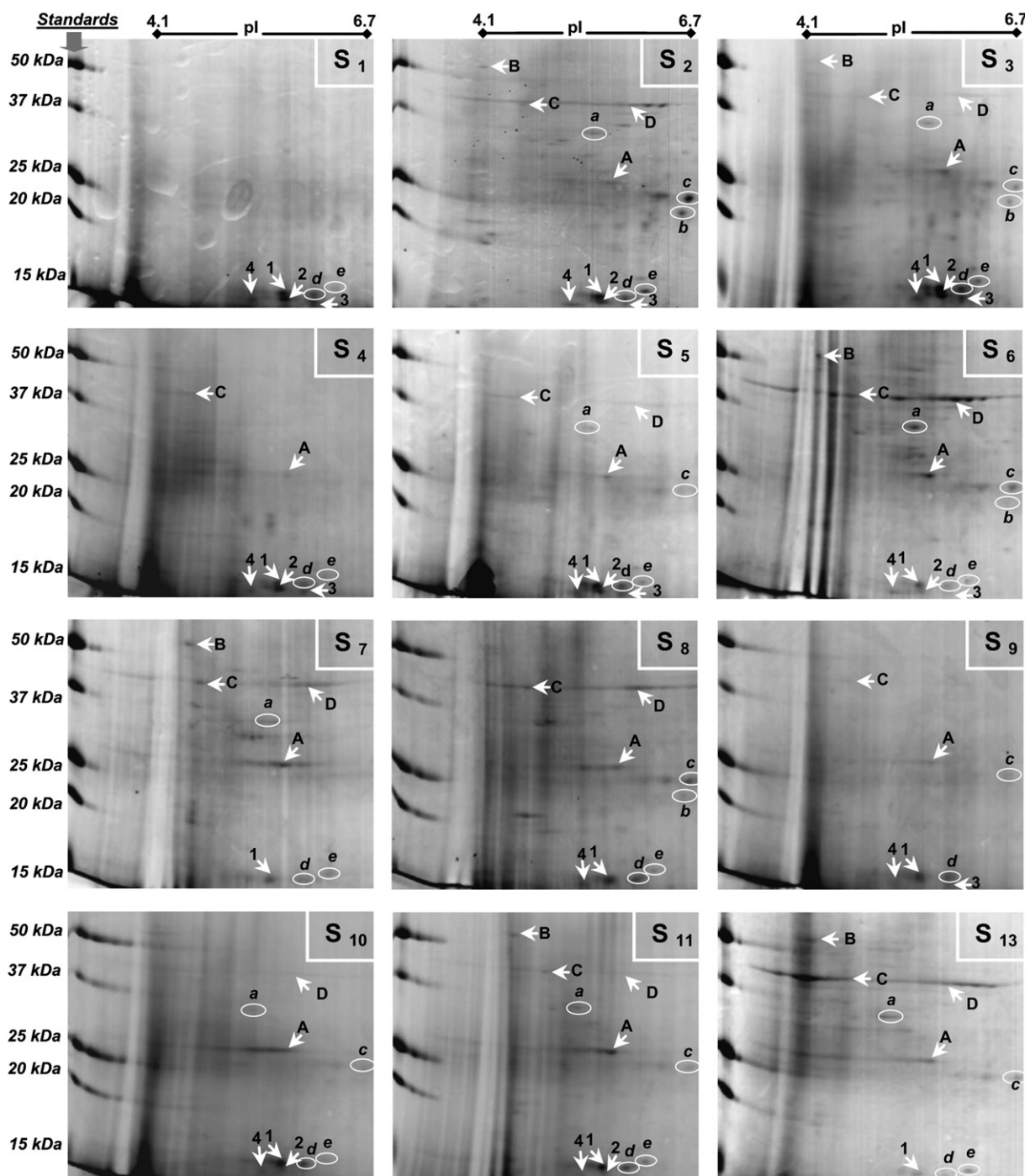


Fig. 2. A: 1D-polyacrylamide gel of total zooplankton proteins from the sampling stations  $S_1, S_2, S_3$  of Toulon Bay. Protein bands present in most of the sampling stations are marked with white lines whereas black lines mark proteins showing significant correlations with trace metal concentrations (Cu, Pb and Zn). Black and white arrows show protein bands respectively correlating negatively or positively with the trace metals. B: Molecular weight and relative intensity of each protein band detected.



**Fig. 3.** 2D-polyacrylamide gels of total zooplankton proteins for the sampling stations S<sub>1</sub>–S<sub>13</sub> of Toulon Bay.\* Protein spots a, b, c, d, e and f, are proteins showing no correlation with trace metal concentrations, but correlations ( $p < 0.05$ ) and relations ( $0.05 < p < 0.10$ ) with biomasses of some zooplankton species, genera, or groups. Protein spots A, B, C and D, are proteins showing positive correlations ( $p < 0.05$ ) with trace metal concentrations (Cu, Pb and Zn), and correlations ( $p < 0.05$ ) with biomasses of some zooplankton species, genera, or groups. Protein spots 1, 2, 3 and 4, are proteins showing negative correlations ( $p < 0.05$ ) with trace metal concentrations (Cu, Pb and Zn), and correlations ( $p < 0.05$ ) with biomasses of some zooplankton species, genera, or groups. \*2D-gel was not shown for S<sub>12</sub>, due to the incomplete data obtained in this station.

In LiB, Copepods were mainly composed of Cyclopoids (mean biomass = 8 mg DW m<sup>-3</sup> [48% of Copepod biomass]; mean abundance = 13 384 ind m<sup>-3</sup> [50% of Copepod abundance]), although Calanoids were also present (mean biomass = 6 mg DW m<sup>-3</sup> [37% of Copepod biomass]; mean abundance = 2282 ind m<sup>-3</sup> [9% of Copepod abundance]). Among the Cyclopoids in LiB, the species *O. nana* was clearly predominant in S<sub>11</sub> and in S<sub>12</sub> (80% of the Cyclopoid biomass and 74% of the Cyclopoid abundance in S<sub>11</sub>; 76% of the Cyclopoid

biomass and 74% of the Cyclopoid abundance in S<sub>12</sub>). This result was confirmed by the strong contributions of the *Oithona* genus to the whole zooplankton biomass in S<sub>11</sub> (43%) and in S<sub>12</sub> (36%) (Fig. 4). The *O. nana* females were generally more present than the male ones, except in S<sub>13</sub> where equal proportions in biomass prevailed.

In LaB, although Cyclopoids were more numerous than Calanoids (mean abundances = 2429 ind m<sup>-3</sup> for Cyclopoids vs 1216 ind m<sup>-3</sup> for Calanoids, mean contributions to Copepod



**Table 1**  
Molecular weights (MW in kDa), isoelectric points (pI) and intensities (in %) of zooplankton protein spots detected on the 2D-gels, for the sampling stations  $S_1$ – $S_{13}$  in Toulon Bay. MW, pI and intensities values were not shown for  $S_{12}$ , due to the incomplete data obtained in this station.

Spot n°		$S_1$	$S_2$	$S_3$	$S_4$	$S_5$	$S_6$	$S_7$	$S_8$	$S_9$	$S_{10}$	$S_{11}$	$S_{13}$
a	MW 31.2 pI 5.3	–	0.6	1.2	–	0.8	5.2	1.2	–	–	0.3	0.7	1.6
b	MW 20.1 pI 6.6	–	5.7	1.7	–	–	0.1	–	0.3	–	–	–	–
c	MW 22.6 pI 6.6	–	17.8	1.5	–	1.0	1.8	–	7.4	0.3	0.7	3.3	1.0
d	MW 14.2 pI 6.0	4.5	0.5	4.3	1.5	10.0	1.0	1.3	7.8	1.7	3.2	3.0	0.1
e	MW 14.5 pI 6.2	0.5	6.2	2.0	0.3	2.5	0.9	0.5	1.4	–	0.7	2.4	2.0
A	MW 25.0 pI 5.6	–	0.5	3.8	0.3	3.7	5.9	9.6	8.8	1.9	5.9	6.8	2.9
B	MW 48.8 pI 4.1	–	1.1	0.8	–	–	4.4	1.8	–	–	0.3	1.2	0.3
C	MW 38.2 pI 4.4	–	0.3	0.2	0.6	0.1	1.9	1.7	1.3	–	0.8	1.1	0.9
D	MW 38.3 pI 5.8	–	1.1	0.1	–	0.6	21.7	4.7	3.6	–	1.1	0.3	5.8
1	MW 14.2 pI 5.6	14.9	19.6	16.3	6.6	17.7	3.3	4.2	24.5	12.2	9.4	13.2	0.8
2	MW 13.9 pI 5.6	7.8	7.8	7.5	5.3	9.3	2.3	–	–	–	0.2	5.1	–
3	MW 13.7 pI 6.0	6.8	1.5	2.8	1.1	2.4	0.2	–	–	0.1	–	0.7	–
4	MW 13.8 pI 5.2	3.1	3.1	3.0	0.7	8.1	1.8	–	3.3	0.4	0.8	1.0	–

abundance = 22% for Cyclopoids vs 8% for Calanoids), Copepod biomass resulted from Calanoids (mean biomasses = 4 mg DW  $m^{-3}$  for Calanoids vs 1 mg DW  $m^{-3}$  for Cyclopoids, mean contributions to Copepod biomass = 44% for Calanoids vs 24% for Cyclopoids). Regarding minority zooplankton groups, Mollusca larvae were greater contributors to the whole zooplankton biomass in LiB (mean contribution = 8%) than in LaB (mean contribution = 3%). In contrast, Cladocerans and Harpacticoids were less represented in LiB (8% and 1%, respectively) than in LaB (16% and 10%, respectively). The Shannon index of diversity indicated a drastic decrease in LiB, from 3.62 bits  $ind^{-1}$  in  $S_{10}$  (the nearest station to LaB) to 1.91 bits  $ind^{-1}$  in  $S_{13}$  (the station nearest to the port).

### 3.3. Total dissolved trace metal concentrations

Very low levels of total dissolved Cd concentrations were recorded in all stations of Toulon Bay (mean = 0.05 ± 0.01 nM), without clear trend (Fig. 5). In contrast, clear decreasing gradients were observed for the other three trace metals, from the north to the south of LiB (i.e. from  $S_{13}$  to  $S_9$ ), and from the LiB/LaB interface to the open sea (i.e. from  $S_7$  to  $S_1$ ). Except for Cd, distributions of trace metals in Toulon Bay strongly correlated between them ( $p < 0.05$ ),

with  $r$  values of 0.96 (Zn vs Cu), 0.80 (Pb vs Cu), and 0.79 (Pb vs Zn). In LiB, minimal and maximal concentrations were respectively (expressed in nM): 13.5 ± 0.2 and 23.8 ± 0.5 for Cu, 0.73 ± 0.02 and 1.24 ± 0.02 for Pb, and 27.8 ± 1.3 and 58.7 ± 2.2 for Zn. These levels were clearly lower in LaB, reaching: 2.2 ± 0.4 and 15.6 ± 0.2 for Cu, 0.19 ± 0.02 and 0.78 ± 0.02 for Pb, and 9.0 ± 0.1 and 38.8 ± 0.7 for Zn.

### 3.4. Relationships between zooplankton and total dissolved trace metals

The changes of some zooplankton species, genera or group (expressed in % of the total biomass) were closely related to those of total dissolved trace metals (Table 4). Biomasses of *Euterpina acutifrons*, *Microsetella norvegica*, *Acartia* spp. and *Evadne nordmanni*, members of a zooplankton group that we defined as zooplankton group 1, composed of Cladocerans, Calanoids, Harpacticoids and Cirripede nauplii, negatively correlated ( $p < 0.05$ ) with total dissolved Cu, Pb and Zn concentrations. By contrast, biomasses of Mollusca larvae and *O. nana*, members of a zooplankton group that we defined as zooplankton group 2, composed of Mollusca larvae and Cyclopoids, positively correlated ( $p < 0.05$ ) with total dissolved

**Table 2**  
LC–MS/MS identification of zooplankton protein spots present on the 2D-gels.

Spot n°	Number of peptides	MW (kDa)/pI		Putative protein identification	Species name/NCBI accession number	Annotated biological function
		Exp.	Theo.			
a	4	31.2/5.3	41.8/5.5	Actin	<i>Aplysia californica</i> P17304	Cytoskeleton structure/function (42.04.03)
d	18	14.2/6.0	41.8/5.5	Actin	<i>Artemia</i> sp. P18601	Cytoskeleton structure/function (42.04.03)
e	9	14.5/6.2	41.7/5.7	Actin	<i>Mytilus galloprovincialis</i> Q9Y0D6	Cytoskeleton structure/function (42.04.03)
A	23	25.0/5.6	41.8/5.4	Actin	<i>Strongylocentrotus purpuratus</i> P18499	Cytoskeleton structure/function (42.04.03)
B	4	48.8/4.1	47.3/4.7	Calreticulin	<i>Amblyomma rotundatum</i> Q64K96	Chaperone (14.01)
C	11	38.2/4.4	41.8/5.5	Actin	<i>Spodoptera littoralis</i> Q11212 (fragment)	Cytoskeleton structure/function (42.04.03)
D	27	38.3/5.8	41.7/5.7	Actin	<i>Mytilus galloprovincialis</i> Q9Y0D6	Cytoskeleton structure/function (42.04.03)
1	12	14.2/5.6	42.0/5.4	Actin	<i>Molgula oculata</i> P53467	Cytoskeleton structure/function (42.04.03)
4	6	13.8/5.2	49.6/4.9	Tubulin	<i>Danio rerio</i> Q6PE34	Cytoskeleton structure/function (42.04.05)

**Table 3**

Biomasses (in mg DW m<sup>-3</sup>) of zooplankton groups and species, and zooplankton diversity expressed as Shannon index ( $H'$  in bits ind<sup>-1</sup>), for the sampling stations S<sub>1</sub>–S<sub>13</sub> in Toulon Bay.

Zooplankton biomass	S <sub>1</sub>	S <sub>2</sub>	S <sub>3</sub>	S <sub>4</sub>	S <sub>5</sub>	S <sub>6</sub>	S <sub>7</sub>	S <sub>8</sub>	S <sub>9</sub>	S <sub>10</sub>	S <sub>11</sub>	S <sub>12</sub>	S <sub>13</sub>
<i>Polydora</i> spp.	–	–	–	–	–	–	0.61	–	–	–	–	–	–
<i>Parasagitta</i> spp.	–	–	–	–	–	–	12.63	–	–	–	–	–	–
Gasteropoda larvae	0.24	–	–	–	0.19	–	1.29	0.38	0.32	1.39	2.13	1.17	0.43
Bivalvia larvae	–	0.02	–	–	–	0.02	0.06	–	–	0.18	0.18	0.08	–
<i>Evadne nordmanni</i>	–	–	–	0.58	1.28	–	–	–	–	–	–	–	–
<i>Evadne spinifera</i>	–	–	–	–	–	1.16	1.46	–	3.30	–	–	–	–
<i>Penilia avirostris</i>	0.60	1.51	0.41	0.43	–	–	–	0.48	–	2.61	1.95	1.46	–
Cirripedia nauplii	–	–	0.70	1.44	–	–	2.74	3.25	–	–	1.64	–	–
Calanoida copepodites	0.84	4.81	–	2.36	0.26	0.48	0.90	0.27	2.70	2.17	4.05	2.43	2.38
<i>Acartia</i> spp.	2.47	3.08	–	1.74	–	–	–	–	3.31	–	–	–	–
<i>Acartia clausi</i>	–	3.08	–	–	–	–	2.20	–	–	–	–	–	–
<i>Clausocalanus parapergens</i>	–	–	–	–	–	–	0.86	–	–	–	–	–	–
<i>Clausocalanus pergens</i>	–	0.61	–	1.36	–	0.69	–	0.77	2.60	3.48	–	1.17	–
<i>Paracalanus</i> spp.	–	–	–	–	–	–	–	–	–	–	3.12	2.92	–
<i>Paracalanus parvus</i>	–	0.61	–	–	–	–	1.73	–	–	0.70	–	–	–
<i>Corycaeus latus</i>	–	–	–	–	0.54	–	–	–	–	0.50	–	0.42	–
Cyclopoida copepodites	0.41	1.76	0.34	0.52	0.39	0.24	0.59	0.26	0.78	1.91	2.20	1.25	2.73
<i>Oithona nana</i> copepodites	–	0.21	–	–	–	–	0.07	–	0.22	1.07	3.87	2.26	–
<i>Oithona nana</i> females	–	–	–	–	–	0.13	0.17	–	–	0.55	6.10	3.21	0.51
<i>Oithona nana</i> males	–	–	–	–	–	–	–	–	–	0.27	1.22	0.57	0.51
<i>Oithona setigera</i>	–	–	–	0.18	–	–	–	–	–	–	–	–	–
<i>Oithona similis</i>	0.16	1.20	1.52	0.56	0.25	0.11	0.57	0.51	0.43	0.58	0.52	0.19	–
<i>Harpacticoida</i> spp.	–	–	0.05	0.05	0.34	–	–	–	–	–	–	–	0.06
<i>Euterpina acutifrons</i>	0.08	0.10	0.21	0.22	0.24	0.16	0.21	–	0.10	0.22	0.06	–	–
<i>Microsetella rosea</i>	–	0.74	–	0.28	–	–	–	–	–	–	–	–	–
<i>Microsetella norvegica</i>	0.39	2.70	0.27	0.55	0.62	–	0.35	–	–	–	0.32	–	–
Nauplii of Copepoda	1.28	2.17	0.32	1.47	0.74	1.52	1.42	0.72	1.80	0.99	1.86	1.88	2.41
<i>Parasagitta</i> spp.	–	–	–	–	–	–	12.63	–	–	–	–	–	–
<i>Oikopleura</i> spp.	68.54	21.50	–	169.01	107.69	73.25	367.92	27.22	184.63	197.64	221.05	41.49	30.51
Shannon index ( $H'$ )	2.65	2.75	2.28	2.09	3.08	2.31	3.36	2.72	2.64	3.62	3.29	3.07	1.91
Total biomass	75.02	44.10	3.81	180.75	112.54	77.76	395.78	33.85	200.20	214.25	250.27	60.50	39.53

Cu, Pb and Zn concentrations. Finally, total dissolved Cd concentrations showed some correlations ( $p < 0.05$ ) with zooplankton; these were positive with Cyclopoida copepodites, *Oithona* genus, Cyclopoids and Copepods, and negative with *Oikopleura* spp.

### 3.5. Relationships between protein expression and zooplankton

Among the protein bands detected, 17 presented positive correlations ( $p < 0.05$ ) with biomasses (in %) of zooplankton species, genera, or groups (Table 5). Two groups of positive correlations were distinguished: correlations between bands n°1 (237 kDa), 4 (176 kDa), 6 (151 kDa), 7 (135 kDa), 11 (77 kDa), 12 (72 kDa), 14 (58 kDa), 15 (51 kDa), 16 (43 kDa), 17 (40 kDa), 18 (36 kDa), 19 (34 kDa), 23 (20 kDa) and 25 (12 kDa) and members of zooplankton group 1 (in grey in Table 5), and then, correlations between bands n°8 (118 kDa), 10 (85 kDa) and 24 (15 kDa) and members of zooplankton group 2 (in black in Table 5). However, correlations with the highest MW protein groups have to be cautiously regarded, to take into account the potential incomplete dissociation of protein complexes due to the 1D extraction protocol.

Among the spots detected, 13 presented positive correlations ( $p < 0.05$ ) or relations ( $0.05 < p < 0.10$ ) with biomasses (in %) of zooplankton species, genera, or groups. Positive correlations or relations were found between spots 1, 2, 3, 4, b and some zooplankton members of group 1 (Cladocerans, Harpacticoids) (in grey in Table 6), whereas positive correlations or relations were obtained between spots B, C, D, a, c, e and some zooplankton members of group 2 (Mollusca larvae, Cyclopoids) (in black in Table 6). In addition, spots A and d negatively correlated ( $p < 0.05$ ) respectively with biomass of a zooplankton member of group 1 (*Acartia* spp.:  $r = -0.80$ ) and with biomass of a zooplankton member of group 2 (*O. nana*:  $r = -0.60$ ).

### 3.6. Relationships between zooplankton protein expression and total dissolved trace metals

Protein bands n°20 (30 kDa) and 24 (15 kDa) showed positive correlations ( $p < 0.05$ ) with the total dissolved Cu, Pb and Zn concentrations in seawater (Fig. 6).

No correlation or relation was found between spots a, b, c, d, e, f and Cu, Pb, Zn. By contrast, spots A, C and D assigned to actin, positively correlated ( $p < 0.05$ ) or related ( $0.05 < p < 0.10$ ) with these trace metals, whereas spot B, assigned to calreticulin, positively related ( $0.05 < p < 0.10$ ) with Zn (Fig. 6). However, spots 1, 2, 3, 4, identified as actin for 1, and as tubulin for 4, showed negative correlations ( $p < 0.05$ ) with Cu and Zn (spot 1:  $r = -0.60$  with Cu and  $-0.59$  with Zn; spot 2:  $r = -0.84$  with Cu and  $-0.80$  with Zn; spot 3:  $r = -0.85$  with Cu and  $-0.85$  with Zn; spot 4:  $r = -0.70$  with Cu and  $-0.65$  with Zn). Spots 1, 2, 3 negatively related ( $0.05 < p < 0.10$ ) with Pb (spot 1:  $r = -0.55$ ; spot 2:  $r = -0.53$ ; spot 3:  $r = -0.55$ ).

## 4. Discussion

### 4.1. Zooplankton proteins

Unexpected different patterns in protein distribution were observed according to the method used to extract and separate proteins. Zooplankton extracts submitted to 1-DE contained similar proportions of small ( $13 \text{ kDa} < \text{MW} < 50 \text{ kDa}$ ) and large proteins ( $50 \text{ kDa} < \text{MW} < 242 \text{ kDa}$ ). This was not observed for the zooplankton extracts analysed by 2-DE, which contained almost exclusively small proteins ( $13 \text{ kDa} < \text{MW} < 50 \text{ kDa}$ ). This discrepancy could result from the different extraction protocols used: 1-DE extraction used SDS, 2-mercaptoethanol and urea, whereas 2-DE extraction needed urea, thiourea, CHAPS, Triton X-100 and DTT. Similarly to us, Jones et al. (2004) observed a discrepancy between

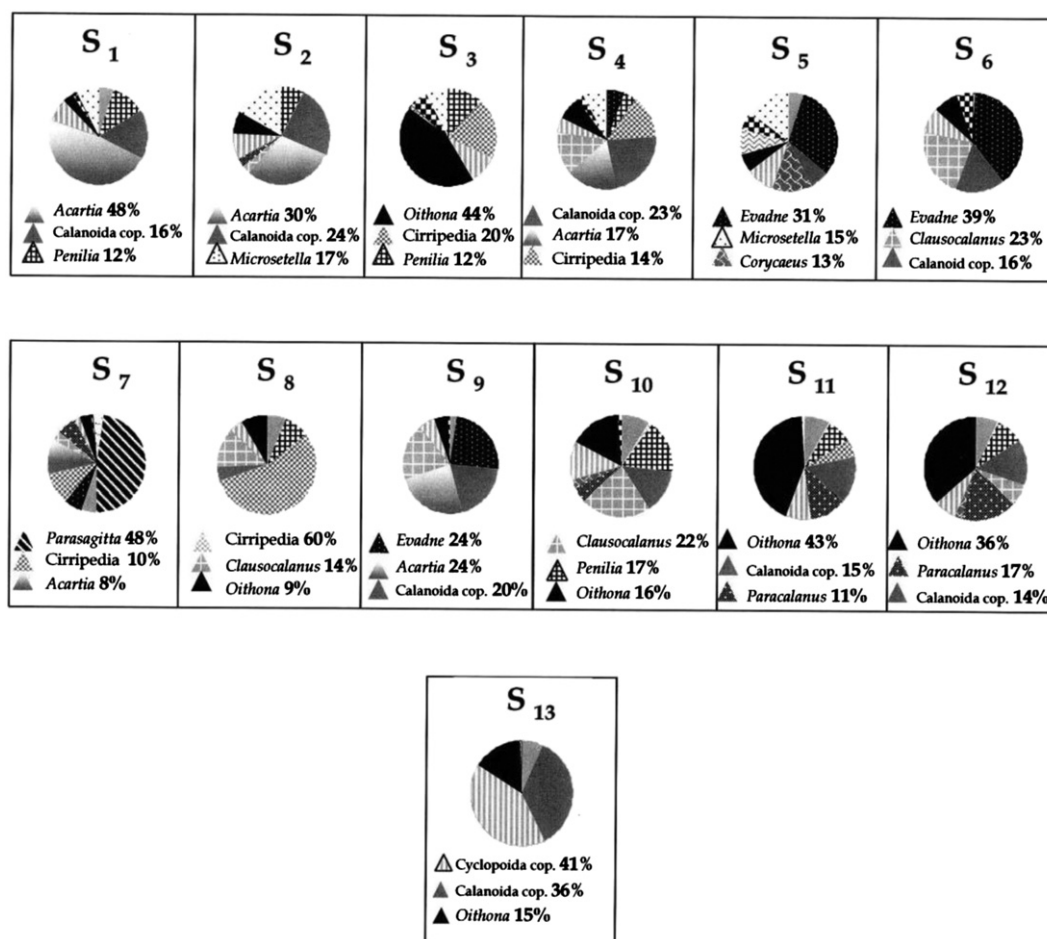


Fig. 4. Contributions (in %) of zooplankton genera and groups to zooplankton biomass (without *Oikopleura* spp. and Nauplii of Copepoda) for the sampling stations S<sub>1</sub>–S<sub>13</sub> in Toulon Bay. For each station, the three predominant groups are indicated with their respective contributions to biomass.

1-DE and 2-DE results about the protein characterisation in aquatic samples. These authors interpret this result by the different potential to solubilize proteins of the solutions used in SDS-PAGE compared with 2D-PAGE. They induce a different solvation of proteins and non-proteinaceous contaminants, and thus lead to visualisation of different subsets of compounds on SDS-PAGE and 2D-PAGE. According to Jones et al. (2004), both methods would provide different informations and thus, could be used together as a tool for protein characterization: 1-DE to provide a sample-specific fingerprint, and 2-DE to characterize individual protein molecules. Here, 2-DE extraction was completed by freezing–thawing cycles and sonication, which enhanced efficiency of the protein extraction. As a result, membrane proteins could be better extracted, involving more numerous small proteins on 2D-gels, whereas protein groups with high MW on SDS–polyacrylamide gels could result from membrane protein complexes combining several proteins (Daley, 2008), more difficult to dissociate by the 1-DE extraction protocol.

#### 4.2. Zooplankton in Toulon Bay

Previous annual cycles taking place in Toulon Bay showed peak in late spring–early summer (Jamet et al., 2005), suggesting that this period was favourable to a study about zooplankton. High zooplankton biomasses are classically recorded during spring in most regions of the western Mediterranean (Gaudy et al., 2003), where zooplankton peak twice: (i) in early spring, in response to the late winter phytoplankton bloom which follows the vertical

mixing of seawater allowing to cold, dense and nutrient-rich waters reach the surface, (ii) in late spring, as in the present study, following phytoplankton growth which results from intermittent fertilization pulses associated with seasonal hydrographic perturbations (Fernandez de Puelles et al., 2007).

Zooplankton biomass and abundance in Toulon Bay (mean = 130 mg DW m<sup>-3</sup> and 21 742 ind m<sup>-3</sup>, respectively) remained very high in comparison with those reported, at this period, from other mediterranean study sites (Fernandez de Puelles et al., 2007; Gaudy et al., 2003; Riandey et al., 2005; Yilmaz and Besiktepe, 2010). However, they are less than those observed in specific ecosystems, such as the Izmit Bay (NE Marmara Sea) where biomass reached up to 1400 mg DW m<sup>-3</sup> in March 2002 (Isinibilir et al., 2008). The closed and shallow Izmit Bay has been affected by anthropogenic influences during the last decade, which increased its nutrient and pollutant inputs. These disturbances have modified the zooplankton diversity in this ecosystem, as indicated by the low Shannon Index H' there recorded (Isinibilir et al., 2008). We reported similar results, with a drop in H' in the sampling stations that were closer to the port (minimal H' in S<sub>13</sub>).

On average, the total zooplankton abundance was twice as high in LiB than in LaB. These results are similar to those that we have previously reported (Jamet et al., 2001, 2005). The anthropogenic inputs into LiB, related to its semi-closed configuration, induce a greater eutrophication and a higher primary productivity, previously measured in this ecosystem (Jamet et al., 2001, 2005). By comparing zooplankton structures in LiB and LaB, different proportions of Cyclopoids and Calanoids have been found among Copepods

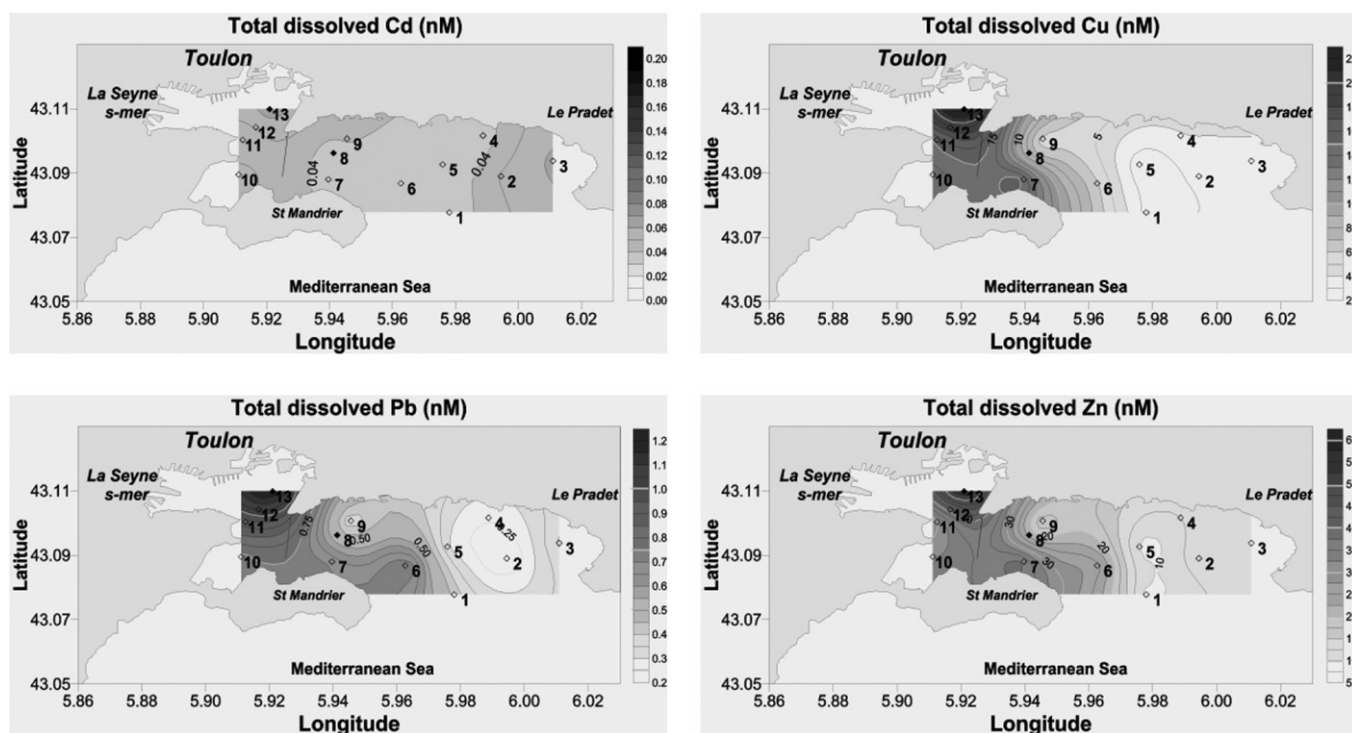


Fig. 5. Total dissolved trace metal concentrations (Cd, Cu, Pb and Zn) for sampling stations  $S_1$ – $S_{13}$  in Toulon Bay. (For logistic reasons, sampling for total dissolved trace metal analysis was not carried out in  $S_{12}$ .)

which always constituted the majority of zooplankton. Many other mediterranean studies also report Copepods to be the most abundant zooplankton taxon (Fernandez de Puelles et al., 2007; Gaudy et al., 2003; Riandey et al., 2005; Yilmaz and Besiktepe, 2010). In LiB, Cyclopoids made up most of a small-sized Copepod population predominated by *O. nana* (in  $S_{11}$  and  $S_{12}$ ), with females of this species more abundant than males. This latter result supports previous studies in Toulon Bay where the sex ratio of *O. nana* was clearly in favour of females in LiB (Richard and Jamet, 2001).

In LaB, far away from the port and open to the sea, Calanoids were main contributors to Copepod biomass, although Cyclopoids were more numerous. Many studies have mentioned that the Calanoid assemblage *Paracalanus/Clausocalanus* is generally the more abundant population in the Mediterranean, followed by the Cyclopoids *Oithona*, *Oncaea*, or *Corycaeus* (Fernandez de Puelles et al., 2007; Gaudy et al., 2003; Riandey et al., 2005). This was not observed in Toulon Bay, but the predominant Calanoid biomass measured in LaB in comparison with LiB, could show evidence that zooplankton structure of LaB was closer to other Mediterranean areas than LiB.

#### 4.3. Trace metals in Toulon Bay

With the exception of Cd present at very low levels, the significant and strong correlations between the metals studied suggest its similar sources and behaviours. Values measured in the  $S_1$  station of LaB were comparable to levels already reported for the western Mediterranean Sea (Morley et al., 1997). In contrast, higher values recorded in the north of LiB reflect significant contamination, due to numerous anthropogenic activities and low seawater renewal. Understanding the metal toxicity to marine organisms requires knowledge of the bioavailability of metals, which is linked to speciation. Some chemical forms, such as the free hydrated ion metal, are more easily assimilated by biota (Hirose, 2007). Determination of metal speciation needs specific analytical tools and







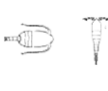

mathematical calculations using speciation codes (MINEQL, FITEQL, CHES) (Herbelin and Westall, 1999; Van der Lee and De Windt, 2000; Westall et al., 1976). It is well known that, in aquatic systems, the DOM controls the metal speciation (Bruland and Lohan, 2004). We recently characterized the interactions between  $\text{Cu}^{2+}$  and marine DOM from Toulon Bay (Louis et al., 2009b). From the calculated DOM binding parameters, the Cu speciation and the concentration of free  $\text{Cu}^{2+}$  in the natural environment have been estimated. These parameters have been recently used to evaluate the effect of ocean acidification on the DOM control of metal speciation in seawater (Millero et al., 2010). Taking into account these data, free  $\text{Cu}^{2+}$  concentrations were calculated at each sampling station of Toulon Bay using the MINEQL program. These ranged from 0.8 to  $6.5 \times 10^{-11}$  M in LaB, and 5.3 to  $12.5 \times 10^{-11}$  M in LiB. Except in some stations of LaB ( $S_1$ ,  $S_2$  and  $S_5$ ), all of the calculated values of free  $\text{Cu}^{2+}$  exceeded the defined toxicity limits for marine zooplankton (Sunda et al., 1987). Metal contamination could therefore be an important disturbance for these microorganisms in Toulon Bay.

#### 4.4. Relationships between zooplankton and trace metals

The development of *O. nana* in LiB, suggests that this species could benefit from the primary productivity there measured (Jamet et al., 2001, 2005), but also that it could tolerate the level of contamination in this ecosystem. The large number of positive correlations between *O. nana* and the concentrations of Cu, Pb, Zn could confirm this latter possibility, especially regarding females of this species, which were predominant in LiB. In contrast, Calanoids, Harpacticoids and Cladocerans, main contributors to Copepod biomass in LaB, showed many negative correlations between their representative species *Acartia* spp., *E. acutifrons*, *Microsetella rosea*, *M. norvegica*, *E. nordmanni* and concentrations of Cu, Pb and Zn. These observations suggest that *O. nana* and the species *E. acutifrons*, *M. rosea* and *M. norvegica* could oppositely respond towards



**Table 4**  
Positive and negative correlations ( $p < 0.05$ ) between biomasses of zooplankton species, genera, or groups from sampling stations  $S_1$  to  $S_{13}$  in Toulon Bay and total dissolved trace metal concentrations (Cd, Cu, Pb and Zn). Zooplankton group 1 is composed of Cladocerans, Calanoids, Harpacticoids and Cirripede nauplii. Zooplankton group 2 is comprised of Mollusca larvae and Cyclopoids.

Metal	(+) Correlations (with zooplankton group 2)
Cd	 Cyclopoida copepodites ( $r = 0.74$ ) <i>Oithona</i> genus ( $r = 0.66$ ) Cyclopoids ( $r = 0.64$ ) Copepods ( $r = 0.63$ )
Cu	 Gastropoda larvae ( $r = 0.62$ ) Mollusca larvae ( $r = 0.61$ ) <i>Oithona nana</i> females ( $r = 0.79$ ) <i>Oithona nana</i> males ( $r = 0.66$ ) <i>Oithona nana</i> ( $r = 0.62$ )
Pb	 Gastropoda larvae ( $r = 0.63$ ) Mollusca larvae ( $r = 0.62$ ) <i>Oithona nana</i> females ( $r = 0.88$ ) <i>Oithona nana</i> males ( $r = 0.72$ ) <i>Oithona nana</i> ( $r = 0.59$ )
Zn	 Gastropoda larvae ( $r = 0.65$ ) Mollusca larvae ( $r = 0.67$ ) <i>Oithona nana</i> females ( $r = 0.82$ ) <i>Oithona nana</i> ( $r = 0.73$ )
(–) Correlations (with zooplankton group 1)	
Cd	 <i>Oikopleura</i> spp. ( $r = -0.69$ )
Cu	 <i>Euterpina acutifrons</i> ( $r = -0.69$ ) <i>Microsetella norvegica</i> ( $r = -0.70$ ) <i>Microsetella</i> genus ( $r = -0.70$ ) Harpacticoids ( $r = -0.67$ )
Pb	 <i>Acartia</i> spp. ( $r = -0.65$ ) <i>Acartia</i> genus ( $r = -0.62$ ) <i>Euterpina acutifrons</i> ( $r = -0.58$ ) <i>Microsetella rosea</i> ( $r = -0.65$ ) <i>Microsetella norvegica</i> ( $r = -0.62$ ) <i>Microsetella</i> genus ( $r = -0.64$ )
Zn	 <i>Evadne nordmanni</i> ( $r = -0.59$ ) <i>Euterpina acutifrons</i> ( $r = -0.67$ ) <i>Microsetella norvegica</i> ( $r = -0.68$ ) <i>Microsetella</i> genus ( $r = -0.67$ ) Harpacticoids ( $r = -0.60$ )

metal stress. At the time of the sampling, these Copepods probably have been exposed to environmental stresses other than trace metals. These potential stresses have not been evaluated in the present study, as a result, we cannot predict their impact on zooplankton communities. However, a nycthemeral study carried out during 27 h on September 2007 in LiB and LaB, reported no marked changes in salinity, and only minor changes in seawater temperatures (0.5 °C at the most), whatever the seawater depth studied (Rossi, 2008). Our sampling has taken place in similar climatic conditions, consequently salinity and temperature stresses can be minimized, whereas the possibility of other stresses, especially contaminant, cannot be ruled out.

4.5. Relationships between zooplankton proteins and trace metals

Of the zooplankton proteins isolated by 2-DE, several were sequenced by LC-MS/MS, mainly indicating the presence of

**Table 5**  
Positive correlations ( $p < 0.05$ ) between changes in the intensities of zooplankton protein bands detected on the 1D-gels, and biomasses of zooplankton species, genera, or groups from sampling stations  $S_1$  to  $S_{13}$  in Toulon Bay. In grey, protein bands positively correlating with some members of zooplankton group 1 (Cladocerans, Calanoids, Harpacticoids and Cirripede nauplii). In black, protein bands positively correlating with some members of zooplankton group 2 (Mollusca larvae and Cyclopoids).



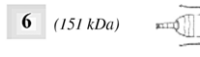
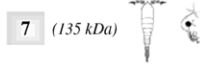

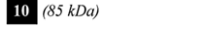
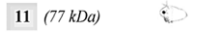

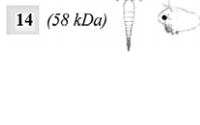

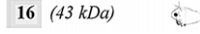

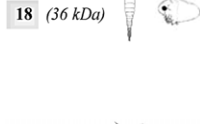

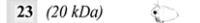

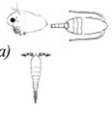
Protein band n°	Correlations with 4zooplankton
<b>1</b> (237 kDa) 	<i>Evadne nordmanni</i> ( $r = 0.64$ ) <i>Harpacticoida</i> spp. ( $r = 0.56$ ) Harpacticoids ( $r = 0.62$ )
<b>4</b> (176 kDa) 	<i>Euterpina acutifrons</i> ( $r = 0.62$ ) <i>Microsetella norvegica</i> ( $r = 0.59$ ) <i>Microsetella</i> genus ( $r = 0.59$ ) Harpacticoids ( $r = 0.64$ )
<b>6</b> (151 kDa) 	<i>Acartia</i> spp. ( $r = 0.61$ )
<b>7</b> (135 kDa) 	<i>Evadne nordmanni</i> ( $r = 0.65$ ) <i>Harpacticoida</i> spp. ( $r = 0.79$ )
<b>8</b> (118 kDa) 	Bivalvia larvae ( $r = 0.62$ ) Cyclopoida copepodites ( $r = 0.69$ ) <i>Oithona nana</i> copepodites ( $r = 0.61$ ) <i>Oithona nana</i> males ( $r = 0.55$ ) <i>Oithona nana</i> ( $r = 0.61$ ) <i>Oithona</i> genus ( $r = 0.58$ ) Cyclopoids ( $r = 0.57$ ) Copepods ( $r = 0.60$ )
<b>10</b> (85 kDa) 	Copepods ( $r = 0.64$ )
<b>11</b> (77 kDa) 	<i>Evadne nordmanni</i> ( $r = 0.65$ )
<b>12</b> (72 kDa) 	<i>Evadne nordmanni</i> ( $r = 0.63$ ) <i>Euterpina acutifrons</i> ( $r = 0.66$ ) <i>Microsetella norvegica</i> ( $r = 0.69$ ) <i>Microsetella</i> genus ( $r = 0.68$ ) Harpacticoids ( $r = 0.61$ )
<b>14</b> (58 kDa) 	<i>Evadne nordmanni</i> ( $r = 0.64$ ) <i>Harpacticoida</i> spp. ( $r = 0.76$ ) <i>Microsetella norvegica</i> ( $r = 0.66$ ) <i>Microsetella</i> genus ( $r = 0.65$ ) Harpacticoids ( $r = 0.82$ )
<b>15</b> (51 kDa) 	<i>Evadne nordmanni</i> ( $r = 0.62$ ) <i>Harpacticoida</i> spp. ( $r = 0.60$ )
<b>16</b> (43 kDa) 	<i>Evadne nordmanni</i> ( $r = 0.63$ )
<b>17</b> (40 kDa) 	<i>Evadne nordmanni</i> ( $r = 0.64$ ) <i>Harpacticoida</i> spp. ( $r = 0.72$ )
<b>18</b> (36 kDa) 	<i>Evadne nordmanni</i> ( $r = 0.63$ ) <i>Harpacticoida</i> spp. ( $r = 0.77$ ) <i>Microsetella norvegica</i> ( $r = 0.56$ ) <i>Microsetella</i> genus ( $r = 0.56$ ) Harpacticoids ( $r = 0.58$ )
<b>19</b> (34 kDa) 	<i>Evadne nordmanni</i> ( $r = 0.58$ ) Cirripedia nauplii ( $r = 0.61$ )
<b>23</b> (20 kDa) 	<i>Evadne nordmanni</i> ( $r = 0.63$ )

Table 5 (continued)

Protein band n°	Correlations with 4zooplankton
<b>24</b> (15 kDa) 	Gasteropoda larvae ( $r = 0.60$ ) Mollusca larvae ( $r = 0.63$ ) <i>Oithona nana</i> females ( $r = 0.57$ )
<b>25</b> (12 kDa) 	<i>Penilia avirostris</i> ( $r = 0.56$ ) <i>Acartia</i> spp. ( $r = 0.59$ ) <i>Microsetella norvegica</i> ( $r = 0.69$ ) <i>Microsetella</i> genus ( $r = 0.70$ ) Harpacticoids ( $r = 0.65$ )












cytoskeleton proteins, above all actin, and also tubulin. Cytoskeleton proteins were also predominant in the proteome from the marine bivalve *Ruditapes decussatus* exposed to Cd contamination (Chora et al., 2009). Actin, one of the most abundant proteins in cell, is a fundamental component of cytoskeleton in muscular and non muscular cells (Manduzio et al., 2005). It has roles in movement, phagocytosis, endocytosis, exocytosis, vesicular transport and cellular plasticity (Thompson et al., 2011). Regarding tubulin, it is also involved in the structure and the function of the cytoskeleton (Chora et al., 2009). It has been shown evidence that cytoskeletal proteins had a role as target of metal-related oxidative stress (Rodriguez-Ortega et al., 2003). Exposure of *Mytilus galloprovincialis* to  $\text{Cu}^{2+}$  ( $5 \mu\text{g L}^{-1}$ ) caused severe perturbations on haemocyte cytoskeletal architecture, due to disorganization of the cortical actin (Fagotti et al., 1996). Actin filaments attain different shapes and structure when exposed to  $\text{CuSO}_4$  2 mM (Kaur et al., 2011). At high concentrations (0.8–1 mM),  $\text{CdCl}_2$  caused a reversible actin denaturation (Dalle Donne et al., 1997), and in a same way, the actin down-regulation has been reported in *Mytilus edulis* from contaminated sites (Manduzio et al., 2005). Decrease in tubulin expression has been observed after oxidative stress (Miura et al., 2005), metal contamination inducing such stress.

In the present study, spots 1 and 4, respectively assigned to actin and tubulin, were less expressed in the most contaminated stations (spots 1 and 4 negatively correlated with Cu and Zn). This suggests that metal stress could alter cytoskeleton protein expression from zooplankton. Different results were obtained for spots A, C and D, also assigned to actin, and however showing stronger expression in the most contaminated stations (A, C and D positively correlated with Cu, Pb and Zn). Opposite actin expressions recently have been mentioned for *R. decussatus*, in gill from which, actin was 8-fold up-regulated after Cd exposure, whereas actin from the digestive gland was oppositely down-regulated, and even suppressed (Chora et al., 2009). Such changes in actin expression also have been reported for the clam *Chamaelea gallina*, in which two actin isoforms were respectively increased and decreased after exposure to a same contaminant treatment (Rodriguez-Ortega et al., 2003). Other studies mentioned the up-regulation of the cytoskeletal proteins actin (7.7-fold), tropomyosin (4.0-fold), myosin (2.8-fold) in the haemolymph from the oyster *Saccostrea glomerata* exposed to Pb and/or Zn (Thompson et al., 2011). Some hypothesis could explain such cytoskeleton protein up-regulation: (i) the damages caused by metal stress to cytoskeleton proteins could then induce their increased expression (Rodriguez-Ortega et al., 2003); (ii) metals could act as effective actin polymerizing agents, as shown for  $\text{CdCl}_2$  at concentrations from 0.25 to 0.6 mM (Dalle Donne et al., 1997), since metal ions are able to affect the nucleation step of actin polymerization (Maruyama, 1981).

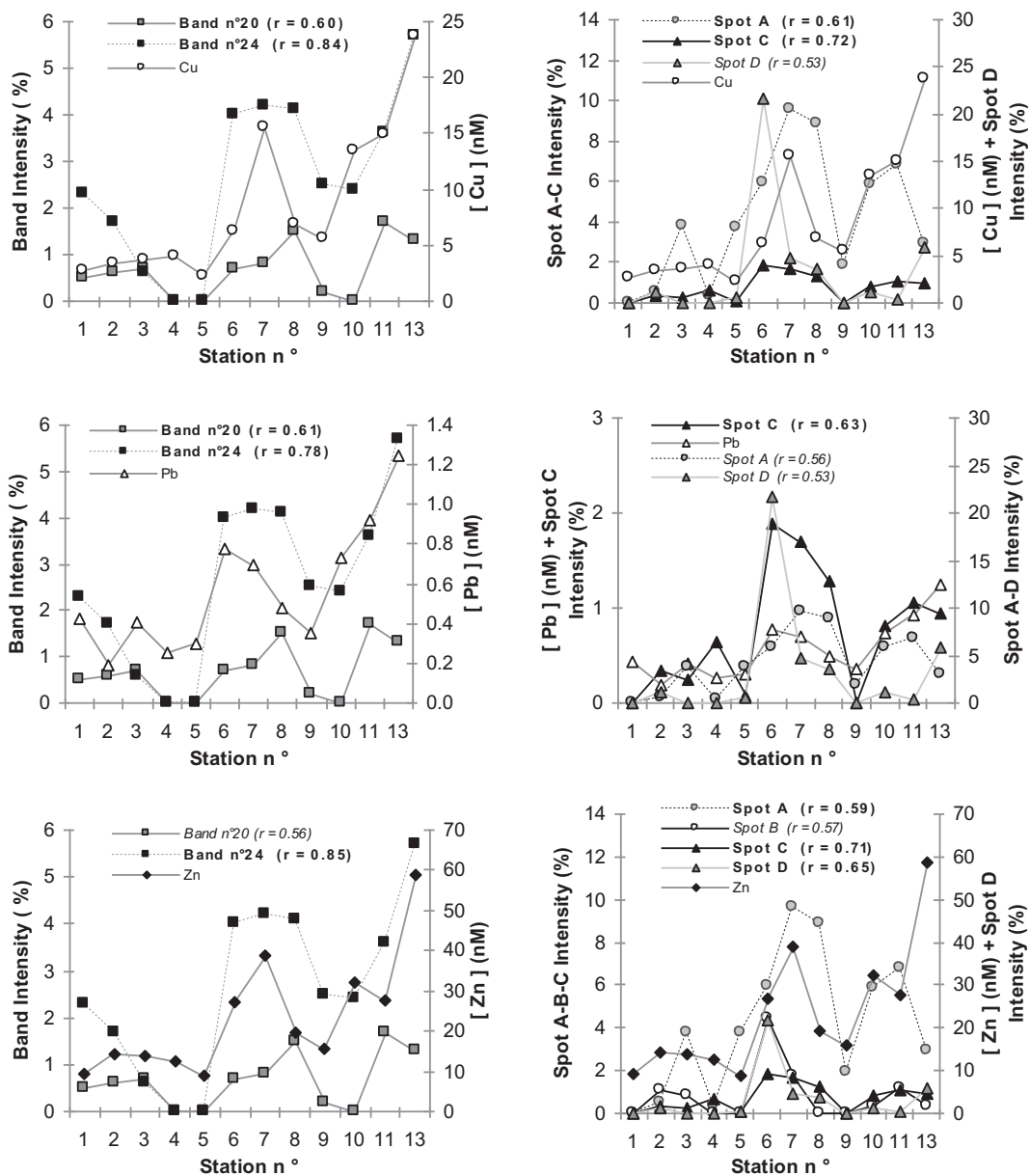
Spots A, C and D, assigned to actin, showed positive correlations with Cu, Pb and Zn, and with *O. nana* (for C and D). Another protein, calreticulin identified from spot B, also maintained such positive

Table 6

Positive correlations (in bold) ( $p < 0.05$ ) and relations (in italics) ( $0.05 < p < 0.10$ ) between changes in the intensities of zooplankton protein spots detected on the 2D-gels, and biomasses of zooplankton protein spots, genera, or groups from sampling stations  $S_1$  to  $S_{13}$  in Toulon Bay. In grey, spots positively correlating with some members of zooplankton group 1 (Cladocerans and Harpacticoids). In black, spots positively correlating with some members of zooplankton group 2 (Mollusca larvae and Cyclopoids).

Protein spot n°	Correlations or relations with zooplankton
<b>a</b> (31.2 kDa) 	<i>Oithona nana</i> females ( $r = 0.52$ )
<b>b</b> (20.1 kDa) 	<i>Penilia avirostris</i> ( $r = 0.63$ ) Cladocerans ( $r = 0.74$ )
<b>c</b> (22.6 kDa) 	Cyclopoida copepodites ( $r = 0.54$ ) <i>Oithona</i> genus ( $r = 0.68$ ) Cyclopoids ( $r = 0.61$ ) Copepods ( $r = 0.51$ )
<b>e</b> (14.5 kDa) 	Cyclopoida copepodites ( $r = 0.56$ ) <i>Oithona</i> genus ( $r = 0.54$ ) Cyclopoids ( $r = 0.70$ )
<b>B</b> (48.8 kDa) 	Bivalvia larvae ( $r = 0.65$ ) <i>Oithona nana</i> ( $r = 0.56$ ) <i>Oithona nana</i> females ( $r = 0.60$ )
<b>C</b> (38.2 kDa) 	<i>Oithona nana</i> females ( $r = 0.62$ )
<b>D</b> (38.3 kDa) 	<i>Oithona nana</i> females ( $r = 0.50$ )
<b>1</b> (14.2 kDa) 	<i>Penilia avirostris</i> ( $r = 0.65$ ) Cladocerans ( $r = 0.54$ ) <i>Euterpina acutifrons</i> ( $r = 0.51$ ) <i>Microsetella</i> genus ( $r = 0.53$ )
<b>2</b> (13.9 kDa) 	<i>Euterpina acutifrons</i> ( $r = 0.72$ ) <i>Microsetella norvegica</i> ( $r = 0.84$ ) <i>Microsetella</i> genus ( $r = 0.84$ ) Harpacticoids ( $r = 0.84$ )
<b>3</b> (13.7 kDa) 	<i>Euterpina acutifrons</i> ( $r = 0.66$ ) <i>Microsetella norvegica</i> ( $r = 0.85$ ) <i>Microsetella</i> genus ( $r = 0.84$ ) Harpacticoids ( $r = 0.84$ )
<b>4</b> (13.8 kDa) 	Cladocerans ( $r = 0.51$ )

relations with *O. nana* and trace metals, Zn more precisely. As a result, actin and calreticulin could be regarded as zooplankton markers of metal stress. Calreticulin, known as an endoplasmic reticulum (ER) molecular chaperone, is an ubiquitous multifunctional calcium-binding protein (Ryu et al., 2012). Calreticulin is



**Fig. 6.** On the left, positive correlations (in bold) ( $p < 0.05$ ) and relations (in *italics*) ( $0.05 < p < 0.10$ ) between total dissolved trace metal concentrations (Cu, Pb and Zn) in sampling stations  $S_1$ – $S_{13}$  in Toulon Bay\* and intensities of the zooplankton protein bands n°20 (30 kDa) and n°24 (15 kDa) present on the 1D-gels. On the right, positive correlations (in bold) ( $p < 0.05$ ) and relations (in *italics*) ( $0.05 < p < 0.10$ ) between total dissolved trace metal concentrations (Cu, Pb and Zn) in sampling stations  $S_1$ – $S_{13}$  in Toulon Bay\* and intensities of the zooplankton protein spots A, B, C and D present on the 2D-gels. \*For logistic reasons, sampling for total dissolved trace metal analysis was not carried out in  $S_{12}$ .

needed in  $Ca^{2+}$  buffering and in quality control process during protein synthesis and folding, in particular during refolding of misfolded proteins and ER stress responses (Leung et al., 2011). In addition to bind  $Ca^{2+}$ , calreticulin also would be able to bind  $Zn^{2+}$  (Michalak et al., 1992), and calreticulin requires Zn for its interaction with the ERp57 thiol oxidoreductase (Leach et al., 2002). Studies showed evidence that Cd stress induced the calreticulin up-regulation, in hepatopancreas of *Perna viridis* (Leung et al., 2011), and in leaf of *Phytolacca americana* (Zhao et al., 2011). As a result, it has been suggested that elevated levels of calreticulin could serve to protect the cells against Cd cytotoxicity (Zhao et al., 2011). The metal stress inducing ER stress, the resulting misfolded proteins would need the up-regulation of ER chaperone, like calreticulin, to be refolded (Leung et al., 2011). Although this has not directly been shown in the present study, the possible up-regulation of structural

and chaperone proteins such as actin and calreticulin in zooplankton cells could provide strength and protection to the predominant *O. nana* during the oxidative stress induced by metal contamination in LiB. In these conditions, the development of *O. nana*, and the subsequent decrease in zooplankton diversity that we observed in LiB, could be partly due to differences in protein expression.

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## Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.marenvres.2012.06.004.

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