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Proteomics-Based Method for the Assessment of Marine Pollution Using Liquid Chromatography Coupled with Two-Dimensional Electrophoresis

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Using a proteomic approach, we have developed a new method for the assessment of marine pollution that generates highly reproducible protein expression patterns and it is simple and scalable. The protocol is based on applying liquid chromatography (LC) coupled with two-dimensional electrophoresis (2-DE) to analyze changes in the protein expression pattern after exposure to marine pollution. The digestive gland of the sentinel "blue mussel" (*Mytilus edulis*) was batch-processed through a simple cell fractionation followed by ion-exchange chromatography and 2-DE. The selection of ligands, elution method, and small volume design was carefully considered to define a protocol that could be mainly robotized. A pilot study with samples collected from different Gothenburg harbor areas indicated that the clean area could be distinguished from the polluted ones based on a protein expression pattern (PES) composed of 13 proteins. Principal component analysis (PCA) and hierarchical clustering confirmed that the PES was sufficient to discriminate polluted and unpolluted areas and to provide a spatial gradient from the polluted source. Several proteins from the PES were identified by electrospray ionization tandem mass spectrometry (ESI−MS/MS), and they are involved in β-oxidation, amino acid metabolism, detoxification, protein degradation, organelle biogenesis, and protein folding. In the near future, this methodology could show potential advantages to assess marine pollution and could become a stable platform to elucidate ecotoxicological questions.

Keywords: proteomics • biomarker profiling • liquid chromatography • two-dimensional electrophoresis • marine pollution assessment

Introduction

Pollution in the aquatic environment is a problem of huge proportion, and therefore, improving the marine pollution assessment methodology is a major environmental concern. The environmental monitoring field has been transformed from a traditional panel of single-parameter biomarkers into new methods that can assemble multivariate information. Applying proteomics to monitor marine pollution is a new approach that can provide links between contaminants and ecological responses. However, thus far, several attempts have failed to develop a highly robust method with applicability to field experiments and large biomonitoring programs. Thus, the development of protein profiles or molecular signatures requires establishing well-defined protocols to allow adequate comparison of results.

Mussels are used worldwide as sentinel organisms in pollution monitoring.[1] Biological monitoring involves the evaluation of the physiological status of sentinel organisms living in the monitored environment, by determining the values of selected biological parameters that are known to vary in response to the toxic effects of pollutants.[2] Among the traditional biomarkers often reported, metallothionein induction, acetylcholinesterase inhibition, cytochrome P450 system induction, glutathione transferase, imposex, lysosomal membrane destabilization, and peroxisome proliferation compose a battery of techniques applied in biomonitoring programs.[3] The traditional biomarkers present some general disadvantages. (i) The difficulties in discriminating between pollution-related changes from "natural" occurring variations limit the robustness of single-parameter biomarkers. Marine bivalve mollusks suffer seasonal metabolic and enzyme activity variations related both to physical changes in the environment, such as changes in temperature, food availability, and oxygen levels, and to physiological factors such as gonadal development.[4] Hormonal changes governing the reproductive cycle have been reported to be linked to seasonal variations of several enzymes of *Mytilus galloprovincialis*.[5] (ii) Inter-individual variability is another issue

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to be considered when single biomarkers are applied. Differences between individuals in the response to pollutants would lead to misinterpretation of the data originated from a pool of individuals.[6,7] The significance of specific changes and the performance of a new method must be fully understood to avoid misinterpretations of data that lead to an inappropriate interpretation of the pollutants' mechanisms of action in the sentinel organism.

Proteomics-based methods that allow global analysis of cellular constituents provide molecular signatures that could overcome the disadvantages of single-parameter biomarkers. Initially, the attention was focused on the detection of proteins from sentinels commonly expressed after exposure to toxic contaminants. A two-dimensional electrophoresis (2-DE)-based approach was explored in mussels exposed to copper, Aroclor, and salinity stress;[8,9] in the marine alga *Nannochloropis oculata* exposed to cadmium;[10] in rainbow trout treated with diazinon, nonylphenol, propetamphos, and exposed to sewage treatment plant effluents,[11] as well as in clams exposed to model pollutants.[12] The analysis of embryonic zebrafish exposed to endocrine disrupting chemicals[13] and the differential expression of gill proteins in mussels exposed to crude oil in a laboratory experiment was reported.[14] Surface-enhanced laser desorption/ionization time-of-flight was applied to establish a protein expression pattern (PES) in mussels from areas contaminated with heavy metals and polyaromatic hydrocarbons as well as in laboratory experiments.[15,16] Higher level of carbonylated proteins have been detected by 2-DE and Western blotting in mussels from polluted sites.[17,18] In our previous work, peroxisomal proteomics was applied to obtain PES of exposure to individual pollutants, and field experiments were carried out to validate this approach. We provided the largest number of identified proteins from molecular signatures that could serve to identify novel and poorly understood pathways affected by xenobiotics.[19,20]

Improvements in the classical 2-DE by reducing the proteome complexity provide a powerful strategy to define robust PESs for environmental proteomics. Developments of such methodologies should aim for the inclusion of a powerful separation method that should be fast, allow removing the highly abundant proteins, and be scalable to high-throughput. The goal of this study was to develop a novel proteomics-based method for the assessment of marine pollution. We aimed, first, to obtain an organelle-enriched fraction by a simple fractionation procedure that would be easily robotized. Second, we included protein separation by liquid chromatography (LC) coupled with 2-DE and the partial identification of the PES by electrospray ionization tandem mass spectrometry (ESI−MS/MS). Finally, we assessed the performance of the method in a field experiment where samples were collected in several areas around the estuary of Göta älv river, Gothenburg, Sweden. This area hosts Gothenburg's harbor, the largest harbor in Scandinavia and the biggest oil terminal in Sweden. The contamination in this area is well-documented, including anthropogenic compounds such as organic chlorines, polychlorobiphenyls, petroleum hydrocarbons, polyaromatic hydrocarbons, furans, dioxins, and heavy metals.[21] A method for the assessment of marine pollution should be highly reproducible, easy to use, robust, affordable, and scalable up to automation. The method that we describe here satisfies these criteria, and the pilot study proves that this subproteomic strategy could provide a robust protein profile of exposure to a pollutants' mixture.

Experimental Section

Sampling. The sampling sites were Fjällbacka (58° 36′N, 11° 16′E), Hjuvik (57° 41′65″N, 11° 42′37″E), Nordre älv (57° 46′93″N, 11° 44′43″E), and Fiskebäck (57° 38′54″N, 11° 51′05″E) (Figure 1). Fjällbacka (Figure 1, no. 1) was selected as the reference site because it is regarded as uncontaminated and neither is close to heavy polluted areas nor influenced by major river outlets.[22] Hjuvik (no. 2) and Fiskebäck (no. 4) are located around the estuary of Göta älv river and in the vicinity of Gothenburg, Sweden (Figure 1). The harbor activities as well as the anthropogenic pollution are the main pollution sources in the areas of Hjuvik and Fiskebäck. Nordre älv is a river located in the north of Gothenburg, and although it has been considered as a control area in reported studies,[23] it is closer to the Gotheburg harbor activities than Fjällbacka. It has been recently reported that this coastal zone in the Gothenburg region acts as a trap for contaminants that are discharged from the urbanized regions and toxic compounds have been detected in the sediments.[24] Busy harbors should be considered under risk of accidental spills, as happened at the end of June 2003 when approximately 10−100 tons of bunker oil were spilled in the inner harbor. The sampling time was November 2003. Mussels (*Mytilus edulis*) of around 5 cm were collected, and the digestive glands were isolated *in situ* and frozen in liquid nitrogen. For the pilot study, 15 digestive glands were collected, 3 from Fiskebäck and 4 from each of the other sampling sites. Tissues from each individual sample were treated independently; therefore, the data obtained correspond to biological replicates.

Prefractionation and Ion-Exchange Chromatography. All the steps were carried out at 4 °C. The homogenization of individual digestive glands (100 mg) was performed with a grinding kit (Amersham Biosciences, San Francisco, CA) in homogenization buffer containing 250 mM sucrose, 5 mM MOPS, 1 mM EDTA, Na_2, 0.1% ethanol (v/v), and protease inhibitors, pH 7.2. The homogenate was centrifuged at 100*g* for 10 min; the pellet was dissolved in homogenization buffer and centrifuged again at 100*g* for 10 min. The supernatants were collected together and followed by a centrifugation at 1950*g* for 10 min, and the final supernatant corresponded to an organelle-enriched fraction. Several ion-exchange matrices were utilized for the method development including: DEAE-, CM-, S-, and Q-Sepharose Flast Flow (Amersham Biosciences, Uppsala, Sweden). The Q-Sepharose Fast Flow matrix (Amersham Biosciences, Uppsala, Sweden) was equilibrated three times with 40 mM Tris-HCl buffer, pH 8. One volume of the organelle-enriched fraction was combined with another volume of dilution buffer, 40 mM Tris-HCl, pH 9.0, to obtain a final pH of 8. This fraction was loaded onto a tube containing equilibrated Q-Sepharose beads and incubated on ice for 15 min. The flow-through fractions were discarded, and Q-Sepharose beads were washed three times with 40 mM Tris-HCl, pH 8, and finally, it was eluted twice with 300 μL of elution buffer containing 40 mM Tris-HCl and 1 M KCl, pH 8 (Figure 2). Equivalent steps of equilibration, wash, and elution were performed for the ion-exchange matrix DEAE-Sepharose Flast Flow. For cationic-exchange chromatography, the equilibration buffer was 30 mM sodium acetate, pH 4.5, and the elution buffer was 30 mM sodium acetate and 1 M KCl, pH 4.5.

Sample Preparation and 2-DE. The eluted fractions were precipitated by 20% TCA in 100% cold acetone with 0.07% β-mercaptoethanol and washed with 1 mL of acetone and 0.07% (v/v) β-mercaptoethanol. Proteins extracted by this

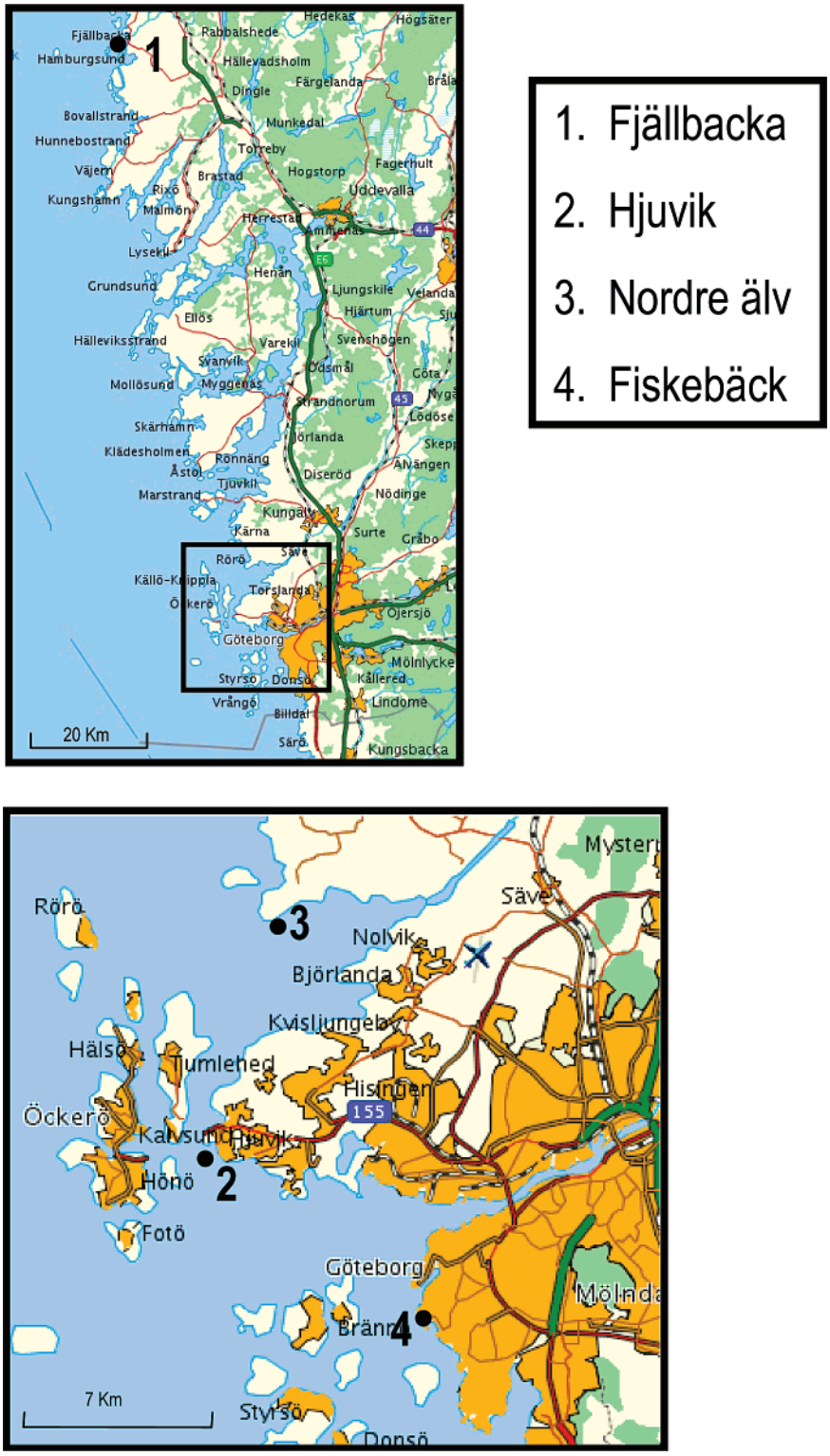

Figure 1. Map showing sampling sites in the Gothenburg harbor area: 1, Fjällbacka; is considered the control site for this study; 2, Hjuvik; 3, Nordre älv; and 4, Fiskebäck.

method were solubilized in a solubilization buffer (7 M urea, 2 M thiourea, 2% CHAPS (w/v), 0.5% Triton X-100, 1% β-mercaptoethanol, 1% (v/v) Pharmalyte (3−10), and 1% DTT (w/v)), modified from Rabilloud.[25] Afterward, samples were alkylated with 30 mM IAA for 15 min in darkness and then mixed with a rehydration solution containing 8 M urea, 2% CHAPS (w/v), 15 mM DTT, 1% β-mercaptoethanol (v/v), and 0.2% Pharmalyte (v/v) (3−10). Solubilized samples were applied onto 11 cm IPG strips, pH 4−7 (Bio-Rad, Hercules, CA). Protein concentrations were measured according to Bradford[26] using bovine serum albumin as a standard. The total protein applied per gel was 300 μg. Isoelectric focusing was performed on a Protean IEF Cell (Bio-Rad) at 20 °C using the following program: passive rehydration for 12 h; rapid voltage slope at all the steps; step 1, 250 V for 15 min; step 2, 8000 V for 2.5 h, and step 3. at 8000 V until it reached 35 000 Vh. After this, the

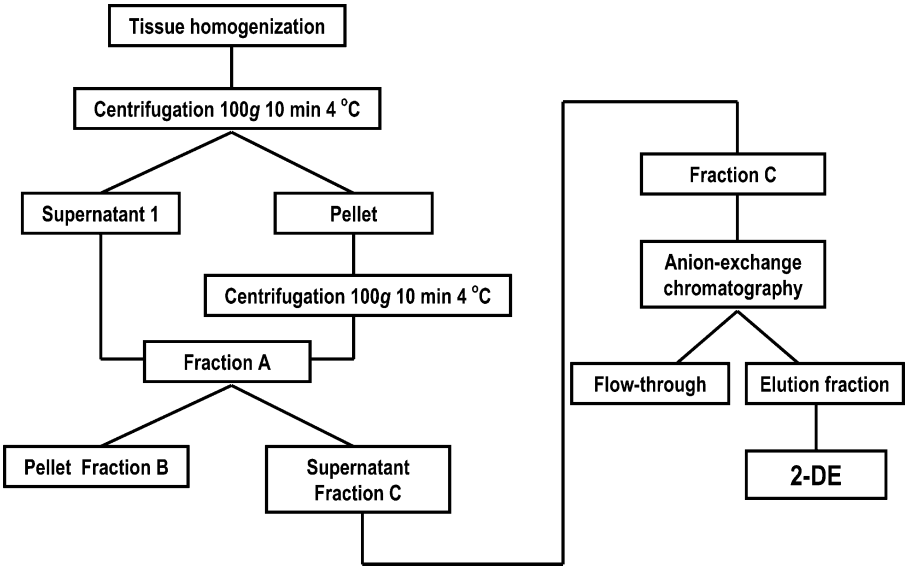

Figure 2. Schematic representation of the protein fractionation method depicting the individual steps of the sequential centrifugation and ion-exchange chromatography.

IPG strips were reduced (1% DTT (w/v)) and then were alkylated (4% IAA (w/v)) in equilibration buffer (6 M urea, 50 mM Tris, pH 8.8, 30% glycerol (v/v), 2% SDS (w/v), and 0.002% CBB (w/v)). The second dimension was carried out on homogeneous 12.5% T Criterion precast gels (Bio-Rad, Hercules, CA), at 120 V for 2 h using a Criterion Dodeca Cell (Bio-Rad).

Image Acquisition and Analysis. The protein spots in the gels were visualized by staining with CBB G-250, and the gel images were obtained using Image Scanner (Amersham Biosciences). The data was analyzed using Image Master 2D Platinum 6.0 from Amersham Biosciences. Image analysis included spot detection, spot quantification and normalization, background subtraction, and spot matching and was followed by statistical analysis. The amount of protein per spot was defined as the sum of the intensities of all the pixels that make up the spot. To correct the variability due to CBB staining and to reflect the quantitative variations between spots, spot volumes were normalized as a percentage of the total volume of all spots in the gel. Thereby percent values for all protein-spots were generated that were subsequently evaluated for significant differences between groups. Therefore, 2-DE maps from polluted sites were matched to the reference 2-DE map (uncontaminated station). To focus on the spots with drastically altered protein expression, the statistical two-sample *t* test with a significance level of 95% or higher was utilized.

Cluster Analysis Methods. By the use of the Image master Platinum 6.0 and MINITAB 14 statistical software, the data was processed using two different kinds of multivariate analysis. Initially, principal component analysis (PCA) was performed, including proteins present in at least 80% of the 2-DE maps and after gap-ratio filtering (gap > 1). Secondary, a hierarchical clustering was performed using the same spot selection criteria.

Protein Identification by ESI−MS/MS and Database Searching. The protein identification was accomplished by ESI−MS/MS analysis of peptides produced by proteolytic digestion of the excised spots from the 2-DE maps. Sequence information of several peptides was obtained, and homology searches were performed in available databases and in our data from predicted peroxisomal proteomes.[27] In detail, the differentially expressed spots were excised from gel, destained twice with 25 mM NH_4HCO_3 in 50% acetonitrile (ACN), dried once with 100% ACN, and digested overnight at 37 °C with sequencing grade modified trypsin (Promega, Madison, WI) in 50 mM NH_4HCO_3. The peptides were extracted twice with 0.1% TFA in 50% ACN and dried in a Speed Vac. HPLC−ESI−MS/MS analysis of extracted peptides was performed at the Lund University Swegene Proteome Centre (http://www. swegene.se). Sequence tags from the ESI−MS/MS spectra were interpreted, and identifications were performed using Masslynx software, UCSF facility's MS-homology search (htpp://prospector.ucsf.edu), and mass-spectrometry-driven BLAST (MS BLAST) and FASTA to search through the protein and genome databases (PIR, Swiss-Prot, and NCBI). Analysis of hypothetical proteins was conducted using software at servers accessible on the Internet: BLAST (http://www.ncbi.nlm.nih.gov/Education/BLASTinfo/information3.html), Pfam (http://pfam.wustl.edu/), PROSITE (http://www.expasy.org/prosite/), PSORT (http://wolfpsort.seq.cbrc.jp/), and ProteinProspector (http://prospector.ucsf.edu/ucsfbin4.0/mshomology.cgi).

Results

Preliminary Consideration: Sample Fractionation and Ion-Exchange Chromatography. Our aim to improve a 2-DE-based proteomic method for marine pollution assessments was supported on our previous experience applying peroxisomal proteomics in biomonitoring. Subproteomic approaches would offer greater ability to discover patterns than analyzing cellular proteomes because they enhance the possibility to quantify changes in low-abundance proteins, belonging to biochemical pathways directly affected by the exposure to chemicals. With this focus, we developed a method to obtain an organelle-enriched fraction after a short number of bench centrifugations in a buffer that maintained the membrane stability. The experimental flow chart of this approach is shown in Figure 2.

The organelle-enriched fraction was obtained after a simple centrifugation protocol. Considering the complexity of this fraction, a batch-wise LC step was included. This step revealed a larger number of low-abundance proteins and removed proteins that would interfere with the resolution. The LC matrix selection was based on the quality of the 2-DE maps: resolu-

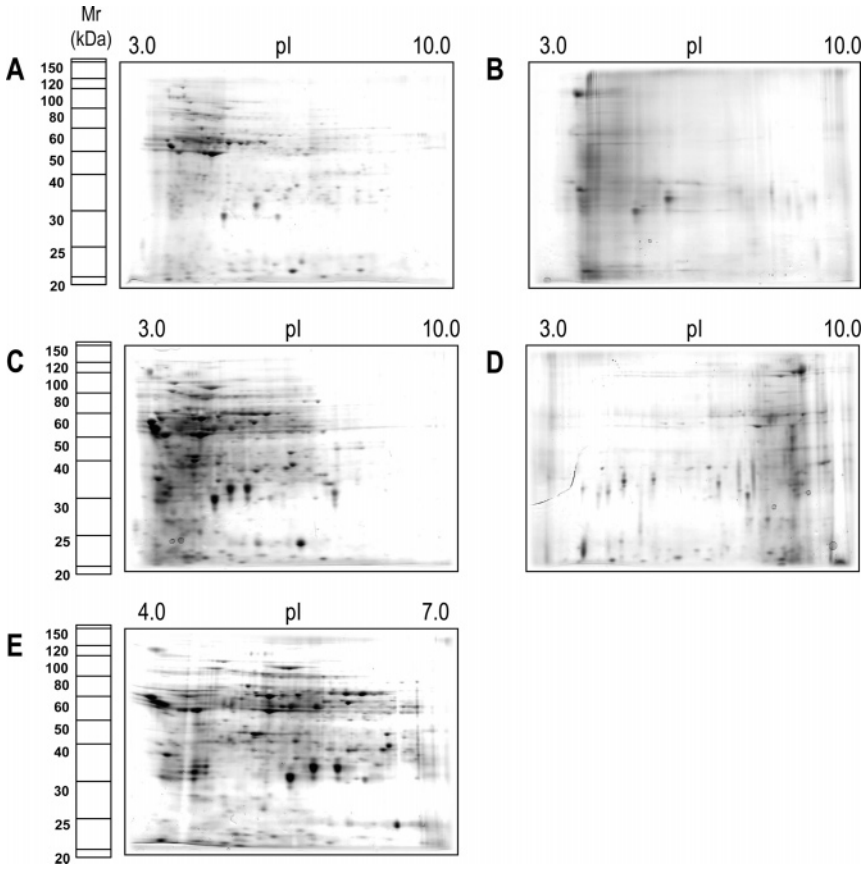

Figure 3. Representative 2-DE maps from the eluted fractions of each of tested ion-exchange matrix. The horizontal axis of the gels is the isoelectric focusing dimension, which stretches in the range of pH 3 to 10 in the maps marked as A−D and pH 4 to 7 in the map marked as E. The vertical axis is the polyacrylamide gel electrophoresis dimension, which covers from 20 kDa (bottom) to about 150 kDa (top). (A) Corresponded to DEAE-Sepharose Fast Flow; (B) SP-Sepharose Fast Flow; (C) Q-Sepharose Fast Flow (pH 3−10); (D) CM-Sepharose Fast Flow; and (E) Q-Sepharose Fast Flow (pH 4 to 7). (Sepharose Fast Flow products were supplied by Amersham Biosciences.)

tion, number, and distribution of the spots. The average bead diameter of 90 μm from Sepharose Fast Flow provided good flow for future automation with gravidity flow microplates. After testing several matrices, fractionation based on the protein charge was selected (data not shown). Four different ion-exchange matrices were tested: two anionic and two cationic exchange matrices. The base matrix for Q-Sepharose Fast Flow is a hydrophilic resin, which is completely stable in the pH range 2−12 which is ideal for almost any separation. In Figure 3, representative 2-DE maps from each of the eluted fractions analyzed are shown. In the maps from DEAE-Sepharose elution fractions, an average number of 186 spots were detected; for Q-Sepharose, 274 spots; for SP-Sephorose, 85 spots; and for CM-Sepharose, 188 spots. On the basis of our previous experience with 2-DE separation in the 11 cm format, we aimed to obtain no more than 300 spots in the maps. Therefore, we selected the anionic exchange matrix, Q-Sepharose. Initially, the 2-DE maps were separated over a range pH 3−10; however, it was observed that more than 90% of the proteins were concentrated between 20 and 120 kDa in the acidic-to-neutral pH range. The separation in a pH range of 4−7 increased the resolution of the 2-DE map. and therefore, this p*I* range was the final choice.

Protein Expression Signature of Exposure to Marine Pollution from Anthropogenic Sources. As a proof of the robustness of this new method for marine pollution assessment, a pilot experiment was performed with samples from the harbor of Gothenburg, Sweden. Samples were collected in four sampling sites, including a control site from a well-documented clean area (Figure 1).[22] On average, about 300 spots were analyzed in each gel, and they were evenly distributed along the pH range (4−7) and molecular weight (20−150 kDa). Statistical analyses were utilized to compare the average ratio of expression from the protein-spots on the 2-DE maps. We found that the expression of 13 protein-spots was drastically altered in all three sampling sites: 5 spots were down-regulated from 2- to 12-fold and 8 spots up-regulated up to 15-fold (Figure 4). The down-regulated spots were concentrated in the upper-half section of the 2-DE map with pH from 5.3 to 6.2 and molecular weight from 62 to 80 kDa. The up-regulated spots were distributed in the lower-half part of the 2-DE map with pH from 4.6 to 6.6 and molecular weight from 21 to 47 kDa. The highest changes in protein expression both, by up-regulation (15-fold) or by down-regulation (11-fold), corresponded to spots from the 2-DE maps from Fiskebäck (sampling site no. 4), which is the site closer to the harbor. In general, Hjuvik (sampling site no. 2) showed less dramatic increases and decreases up to 5-fold in both directions. Nordre älv (sampling site no. 3) was the site with the spot that showed the most dramatic down-regulated effect (12-fold) (Figure 5).

PCA and Hierarchical Clustering. To validate whether the PES obtained in this pilot experiment could constitute a robust set of biomarkers, PCA and clustering were performed. PCA is a useful tool for data categorization, since it separates the

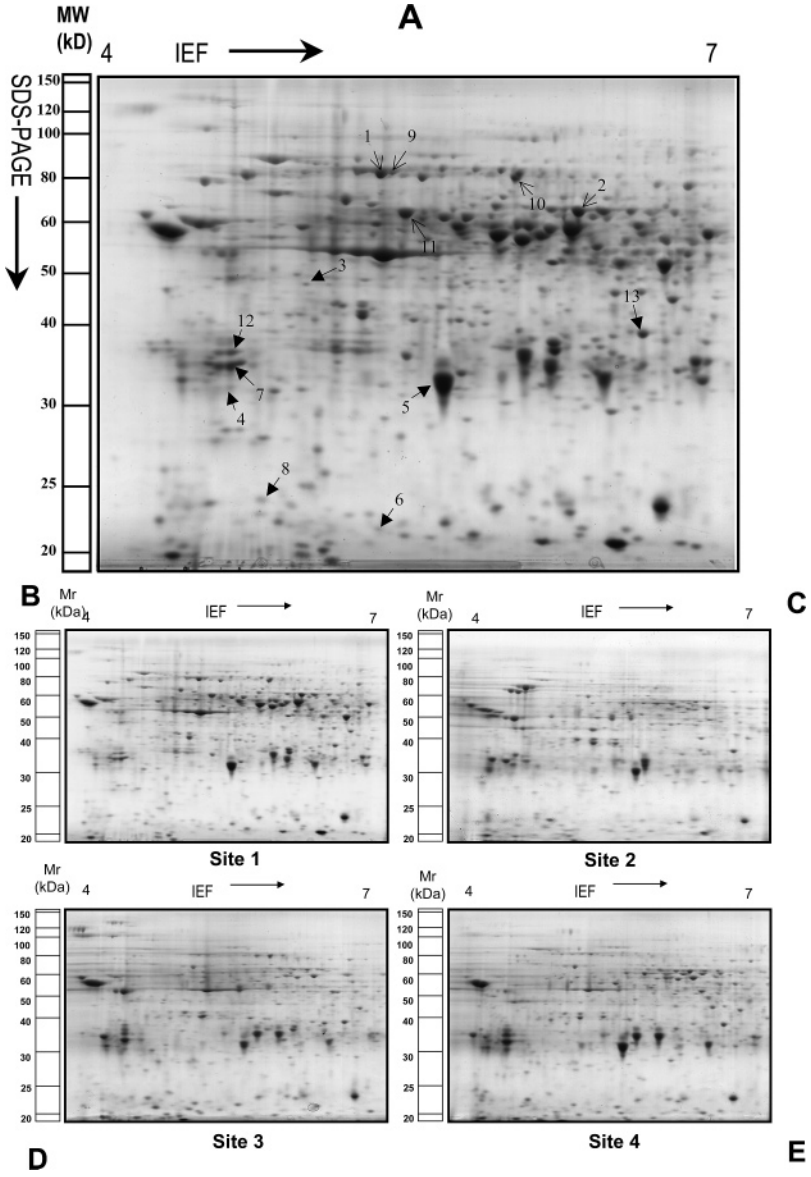

Figure 4. Representative 2-DE gels from different sampling sites stained with CBB G-250 C. The horizontal axis of the gels is the isoelectric focusing dimension, which stretches in the range of pH 4−7, and the vertical axis is the polyacrylamide gel dimension, which covers from 20 kDa (bottom) to about 150 kDa (top). (A) Representative 2-DE gels stained with CBB G-250 C showing the labeled spots. Numbers refer to proteins in other figures and Table 1. The proteins marked with opened arrows correspond to the down-regulated spot-proteins and the closed arrows to up-regulated ones. The gels were calibrated for molecular mass (in kDa) and p*I* (in pH units) by external pH and mass standards. (B−E) Representative 2-DE gels from sampling sites 1−4, respectively.

dominating features in the data set. PCA clearly distinguishes all the gels into four populations (Figure 6A). Therefore, the four sampling sites were clearly separated by the first component. The control group occupies the positive side of the *x*-axis, and the rest of the groups are mainly in the negative side. The gels from Fiskebäck (site 4) and Hjuvik (site 2), sites with the highest level of pollution, were separated by the second component. They are close to each other and occupy the positive side of the *y*-axis, whereas the gels from the Nordre Älv (site 3), a much cleaner site, occupy the negative side of the *y*-axis together with the Fjällbacka (site 1, control site). In addition, hierarchical clustering was used to blindly classify similar gels into classes. Noteworthily, all the gels from the control site were clustered together in a separate cluster with similarity level up to 62.5%. The gels from site 4 were clustered together in a separate cluster with similarity level 57% and simultaneously, constitute a cluster with 3 gels from site 2, similarity level 43.4%. In agreement with PCA, all the gels from site 3 were clustered together with similarity level up to 45.6%. One gel from site 2 was clustered separately and has only 33% of similarity.

Identification of Proteins Composing the PES. Table 1 summarizes the data from the identification of spots composing the PES. In total, 10 proteins were identified corresponding to the 5 down-regulated proteins and 5 up-regulated proteins. The identification was accomplished by ESI−MS/MS analysis and homology search. The protein position in the map is indicated with M_r in kilodaltons (kDa) and p*I*. In the group of up-regulated proteins, spot 3 (47/5.0) was identified as a glutathione *S*-transferase (GST) by homology with a protein of *Mesocricetus aureus*. The identification of spot 6 (21/5.4) as cathepsin B like cysteine proteinase was obtained from *Cean-*

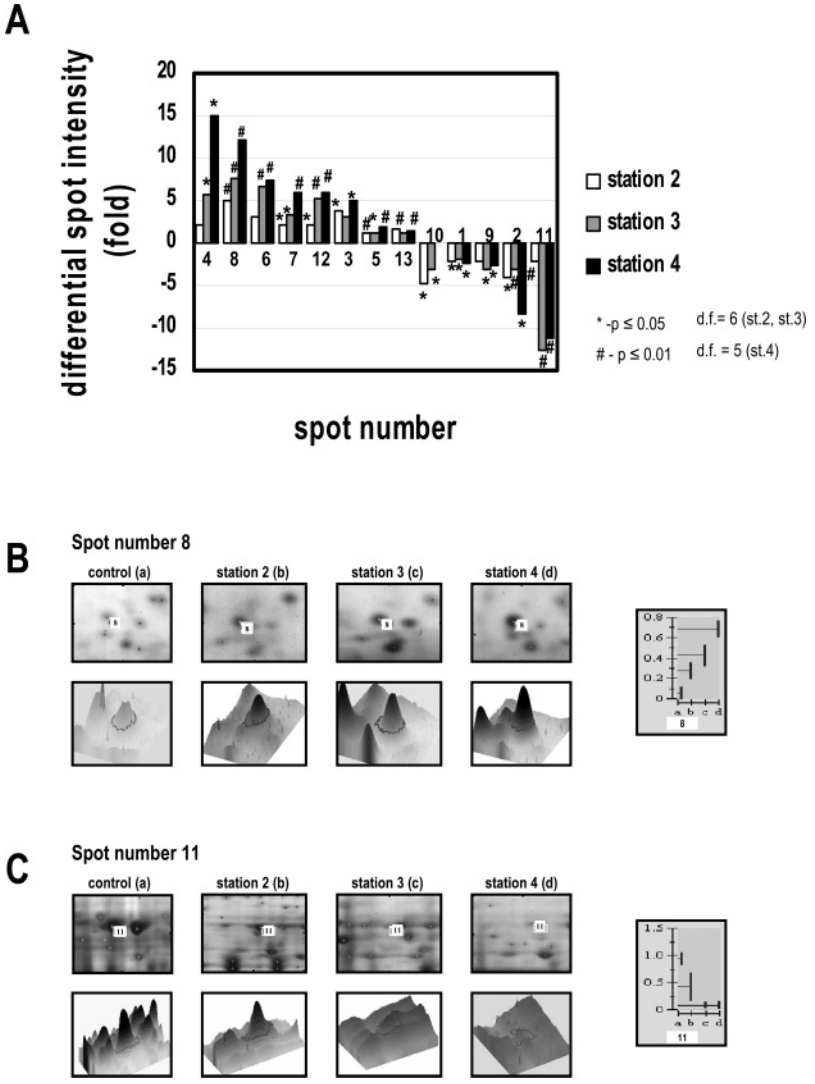

Figure 5. Proteins differentially expressed in sites 2−4 in comparison with the control site 1. (A) The vertical axis corresponds to differential spot intensity, above the 0 value for the up-regulated spots and below the 0 value for the down-regulated ones. In the horizontal axis, the up-regulated spots are organized with the highest values on the left side and the down-regulated ones show the highest values on the right side. (B and C) Two- and three-dimensional views from a representative up-regulated protein corresponding to spot number 8 (B) and a representative down-regulated protein corresponding to spot number 11 (C). The graph in the right shows in a different grayscale, the mean, and the standard deviation values of spot volume percentages (vol %) for each of the sites a−d, corresponding to the sites 1−4, respectively.

orhabditis elegans; spot 7 (35/4.7) was similar to a putative AMP binding protein of *Oryza sativa*; spot 12 (37/4.7) was homologous to acyl-CoA dehydrogenase of *Sus scrofa*, and spot 13 (38/6.6) was identified as phosphoglycerate kinase by homology with the protein of *Trypanosoma brucei*.

All the down-regulated proteins were identified; spot 1 (80/5.3) and spot 9 (80/5.49) were identified as heat shock proteins (HSP) by homology to *M. edulis* protein and *M. galloprovincialis*, respectively; spot 2 (62/6.29) corresponded to fascin-like protein of *Drosophila melanogaster*; spot 10 (78/5.9) corresponded to peroxisome biogenesis factor 1 of *Homo sapiens*, and spot 11 (62/5.4) was identified as aldehyde dehydrogenase 1A2 of *Danio rerio*.

Discussion

Method Development. Protein expression profiling can identify alterations in protein expression associated with changes in the environment such as increase in pollution. An ideal method should allow the simultaneous analysis of hundreds of molecular parameters within a single experiment, in a medium to high-throughput level at an affordable price. The method that we present here fulfills these requisites and demonstrates the utility of proteomics-based techniques for the assessment of marine pollution.

The first prerequisite for the comprehensive analysis of complex proteomes is an extensive fractionation; therefore, several approaches have been investigated that deal with this problem.[28,29] In the method presented here, an organelle-enriched fraction was obtained by a simple fractionation procedure. Fractionation was based on a differential centrifugation that could be performed with a standard bench centrifuge and it could easily scale up to the automation required in large and periodically biomonitoring campaigns. Other alternative methods have been recently reported, but they do not provide a solution to easily separate a fraction that could contain novel biomarkers.[20] Membrane organelles solubiliza-

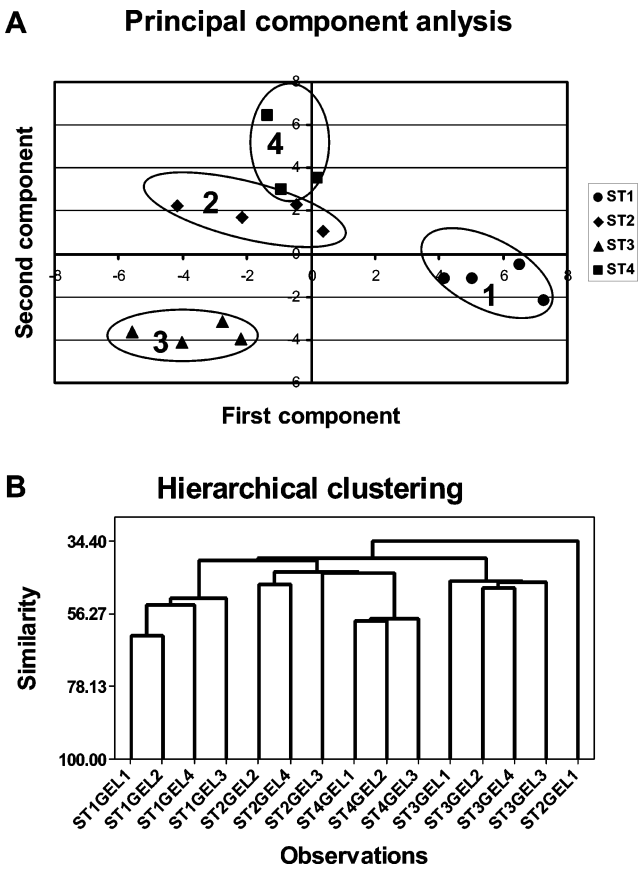

Figure 6. Organization of data by multivariate analyses. (A) Principal component analysis performed on correlation matrix. The plot shows the clustering of the 2-DE maps from the four sampling sites analyzed. In the plot, circles represent the gels from site 1; rhombuses correspond to gels from site 2; triangles for site 3; and squares for site 4. (B) Hierarchical clustering plot with single linkage and Pearson distance.

tion by extraction with buffers containing surfactants is a fractionation strategy recently reported.[30] The separated nuclei from the organelle fraction in a single centrifugation step has also been applied.[31] In our previous work, we isolated peroxisomal-enriched fractions and obtained a PES composed of proteins affected by pollution exposure. Although our method was applied to find specific PES of exposure to xenobiotics or in field experiments with a few sampling sites, the automation required in large biomonitoring programs would not be compatible with that complex cellular fractionation.[19,20,32]

The second requisite to consider in 2-DE-based proteomic method is the resolution limit. The best 2-DE gels can routinely resolve no more than a few thousand proteins and in the small format 2-DE maps no more than a few hundreds.[19] Therefore, only the most abundant proteins can be visualized from a complex fraction, but low-abundance proteins can only be visualized after the inclusion of an additional fractionation step. In the method presented here, the ion-exchange chromatography was selected because, first, it rendered a set of proteins whose distribution and level of intensity were adequate for an accurate quantification. Second, the elution was highly reproducible, and finally, we could obtain a few proteins that vary in protein abundance after exposure to pollutants. Several chromatography principles have been applied to visualize low-copy-number gene products. The enrichment by hydroxyapatite of *Escherichia coli* proteins showed to be useful to search for novel drug targets.[33] The enrichment of human fetal brain proteins by heparin chromatography has been applied to study neurological disorders.[34] To summarize, the inclusion of a simple fractionation method by bench centrifugation and a batch-wise ion exchange LC aided in the resolution of low-abundance proteins from an organelle-enriched fraction by 2-DE.

Pilot Experiment. The PESs obtained by proteomics are a new concept in the field of environmental sciences and sometimes are still controversial.[11] The field experiment data presented here indicated that the polluted sampling sites could be distinguished from the control site based only on a pattern (increase or decrease in protein abundance) of 13 discriminatory proteins. Moreover, this method could predict a gradient from higher to lower level of pollution from the inner to the outer harbor and detect sampling sites with similar pollution levels. This pollution gradient was based on the quantitative data. The up- or down-regulation levels were correlated with the pollution degree of the sites. Protein expression changes over 10-fold were solely obtained in site 4, which is the closest to the effluent from the estuary and the areas of harbor activities. In addition, PCA and hierarchical clustering confirmed that the pollution gradient could be predicted, and moreover, those techniques depicted the similarity between the sampling sites situated at a medium distance to the main pollution source. Finally, the use of biological replicates relates data from a specimen that could respond differently than the rest of the item. That was the case of one gel from site 2 that did not show similarity with any of the gels of the study.

Identification of PES. The protein identification was not required to assess marine pollution with this method; however this is useful information for environmental toxicology. Several proteins were identified among the up-regulated proteins. Glutathione *S*-transferases (GSTs) are involved in the detoxification of many chemical compounds including hydrocarbons, organochlorine insecticides, and polychlorinated biphenyls. The existence of a peroxisomal GST has been discussed in recent years, and evidence has been accumulated in that direction.[35−37] In mussels, a previous homologous sequence to GST has been identified, and it contained a predictable peroxisomal targeting signal type 2 (PTS2).[20] The PTS2 is composed of a nonapeptide with the sequence (R/K)(L/V/I)-X_5(H/Q)(L/A), which is quite conserved among peroxisomal proteins but fairly rare.[38] In mammals, the peroxisomal GST is associated with peroxisomal membranes. In this case, an obvious targeting motif for peroxisomal membrane proteins, like the PTS1 and PTS2 for matrix proteins, does not exist in the peroxisomal membrane proteins.[39] The GST activity has been widely used as a biomarker of exposure to these substances in mollusks.[40] The GST up-regulation has been reported both in laboratory and field experiments.[41,42] The response to oil exposure of the GST in freshwater prawns has been reported. The enhanced levels of this and other biotransformation enzymes in oil-exposed prawns demonstrate a well-established detoxifying mechanism and utility as biomarker for the early detection of oil pollution.[43] On the contrary, in laboratory experiments where mussels were exposed to phthalates, an inhibitory effect in the GST activity has been observed, and it has been suggested that phthalates may cause alteration in detoxification capacities of mussels.[20] In mammals, the peroxisomal GST probably catalyzes the glutathione peroxidase activity toward cumene hydroperoxide,[44] and an interaction with fatty aldehyde dehydrogenase was suggested.[45] This

Table 1. Putative Identification of Spots from the 2-DE Maps by ESI−MS/MS[a]

Spot no	Obs. pI	Obs. Mr	St.2	St.3	St.4	Putative identification	Homology to protein (Genbank)	Pfam domain	Peptide fragments	Functional pathway	Subcellular localization	↑↓
4	4.6	32000	2	5.8	15	-						
8	4.8	24000	4.9	7.6	12.2	-						
6	5.4	21000	3.2	6.6	7.3	cathepsin B like cysteine proteinase	gi:25146613 *Caenorhabditis elegans*	peptidase C1	DECGIESGVVGGIPK	protein degradation	lysosome	↑
7	4.7	35000	2.2	3.4	6.0	putative AMP binding protein	gi:34895038 *Oryza sativa*	AMP binding	GTNVCVRRVTAAA	AMP binding	peroxisome	↑
12	4.7	37000	2.2	5.3	6.0	acyl-CoA dehydrogenase	gi:1703065 *Sus scrofa*	acyl-CoA	LATDAVQVFG	fatty acid ß-oxidation	mitochondrion	↑
3	5	47000	3.8	3.2	4.9	glutathione S-transferase	gi:2117750 *Mesocricetus auratus*	GST	RGLTXPIRM	detoxication	cytosol and peroxisome	↑
5	5.6	32000	1.3	1.2	1.9	-						
13	6.6	38000	1.6	1.1	1.5	phosphoglycerate kinase	gi:129912 *Trypanosoma brucei*	PGK	TGGVPGFQQK	glycolytic pathway	cytosol and peroxisome	
10	5.9	78000	-4.8	-3.2	-	Pex1	gi:4505725 *Homo sapiens*	AAA	KGMMKELQTKQ	peroxisomal biogenesis	peroxisome	↓
1	5.3	80000	-2.2	-2.0	-2.3	HSC70	gi:18076565 *Ostrea edulis*	Hsp 70	PFTIINDGTKPK KNQVAMNPTNTIFDAKR KSTSGDTHLGGEDFDNRM KSINPDEAVAYGAAVQA	chaperone	cytosol	↓
9	5.4	80000	-2.1	-3	-2.6	HSC71	gi:76780612 *Mytilus galloprovincialis*	Hsp 70	KTGPAIGIDLGTTYSCVGVFQHGKVEIIANDQGN KNQVAMNPVNTVFDAKR KETAEAYLGKLVNNSVITVPAYFNDSQRQ KDAGTISGMNVLRIINEPTAAAIAYGLDKK RMVNHFIQEFK KAAVHEIVLVGGSTRI KSINPDEAVAYGAAVQAAILSGDK KNSLESYSFNMKQ KELEGVCNPIITKL	chaperone	cytosol	↓
2	6.2	62000	-4.0	-3.2	-8.3	fascin-like protein	gi:24640473 *Drosophila melanogaster*	fascin	YMTAETFGFK	actin binding protein	cytosol	↓
11	5.4	62000	-2.2	-12.5	-11	aldehyde dehydrogenase 1A2	gi:16565463 *Danio rerio*	aldedh	RYYAGWADK KVAFTGSTEVGKL	amino acid metabolism	mitochondrion	↓

[a] Obs., observed; St., site. Differential spot intensity expressed in fold.

interaction could prevent lipid peroxidation and to scavenge harmful metabolites. In yeast, the role of the peroxisomal GST was connected to the redox regulation of the cystathionine beta-lyase protein.[36]

Another up-regulated protein was cathepsin B, a papain-family cysteine protease that is normally located in lysosomes. It is involved in the turnover of proteins and plays various roles in maintaining normal cellular metabolism. In disease conditions, increases in the expression of cathepsin B occur at both gene and protein levels. At the gene level, the altered expression results from gene amplification, elevated transcription, use of alternative promoters, and alternative splicing. These molecular changes lead to increased cathepsin B level and in turn redistribution, secretion, and increased activity.[46] In fish, cathepsin D, an aspartic protease, participated in intracellular breakdown of tissue proteins.[47] This enzyme has also been localized in granular haemocytes of the marine mussel *M. edulis* by immunolocalization.[48] Although this protein has not been reported before as a possible biomarker, in general, enlargement of digestive cell lysosome has been reported on exposure to metals and different chemicals both in laboratory and field experiment and it has been used as an early warning signal of exposure to pollution.[49,50]

The acyl-CoA dehydrogenase catalyzes the first step in each cycle of fatty acid β-oxidation in mitochondria.[51] The increase in the acyl-CoA dehydrogenase could be associated with an adaptive response to exposure to pollution. Sponges, which are sessile filter feeders, have developed an efficient defense system against environmental stressors. Those organisms contain genes involved in an adaptive response against xenobiotics.[52] Among them, a protein homologous to the mammalian acyl-CoA dehydrogenase has been found.

Among the down-regulated proteins, we found two putative HSPs. Members of the HSP70 family are strongly up-regulated by heat stress and chemical toxins, particularly heavy metals such as arsenic. Those proteins are synthesized in response to changes that cause proteins denaturation in vivo.[53] In terms of ecotoxicological research, these proteins have been broadly used as biomarkers for a wide range of stressors including metal pollution, and their induction is accordingly a sign for adverse effects of the substances tested.[54] The use of mussels HSP70 as a biomarker for marine metals contamination has been reported.[55] However, no effect was detected on sponge HSP expression transplanted to an environment with a moderated concentration of metals.[56] On the earthworm *Lumbricus terrestris,* in the proximal and midbody wall muscle tissues of animals exposed to heavy metals, a decrease in expression of

HSP70 was observed.[57] In the evaluation of toxic effects of insecticides in cultured human cells, HSP72/73 was found to be down-regulated by all insecticides tested.[58] In fish, the HSP70 content in an animal body is not clearly correlated with exposure to harmful chemicals, and therefore, it has been argued that it cannot be considered as an universal biomarker for ecotoxicology.[59] As intertidal organisms, mussels live in rapidly fluctuating habitats where protection from environmental stressors including changes in temperature, salinity, and anthropogenic contaminants is essential for survival. High HSP70 expression was detected in mussels under natural environmental conditions where it was considered a form of endogenous protection.[60]

Finally, the toxicological roles of fascin-like protein and aldehyde dehydrogenase down-regulation have not been reported in mussels earlier. The aldehyde dehydrogenase is mainly localized in mitochondria; however, the peroxisomal subcellular location is still an open possibility after the detection of peroxisomal-associated aldehyde dehydrogenases in rat liver[61] and both organelles comprising the subproteomic fraction studied here. From studies of multiple cell types in invertebrates and vertebrates, fascin has emerged as an actin-binding protein of general importance for a diverse set of cell protrusions with functions in cell adhesion, cell interactions, and cell migration.[62] Specific inhibition of fascin by phosphorylation increased the formation of cell protrusions and the rate of cell migration.[63] In mollusks exposed to pollution, down-regulation of proteins from the cytoskeleton have been reported.[12,20] In general, the structure and function of the cytoskeleton has been proposed to be one of the first targets of oxidative stress related to exposure to pollution.

Conclusions

We have implemented a 2-DE-based proteomic method by coupling a simple cellular fractionation and a step of LC in batch. Our studies revealed that a combination of several factors is crucial for a successful proteomic method for marine pollution assessment. First, in relation to the sample, it should be considered minimizing the amount of required sample and increasing the homogeneity and quality of the sample. Second, considering the method design, it should aim for increasing the robustness against biotic factors including inter-individual variations and abiotic factors such as salinity, pH, and seasonality and achieve reproducibility for inter-laboratory validation. Finally, it is recommended to simplify the procedure to be scalable to automation and affordable enough to cover large monitoring campaigns. We have demonstrated with a field experiment that the method presented here fulfils all those considerations. Moreover, identification of the putative proteins comprising the PES from a not fully sequenced model organism could provide useful information to further studies. This methodology shows a potential not only to assess marine pollution but also to become a stable platform to elucidate ecotoxicological questions.

Abbreviations: LC, liquid chromatography; PES, protein expression signature; 2-DE, two-dimensional electrophoresis; GST, glutathione *S*-transferase; ROS, reactive oxygen species; HSP heat shock proteins; ESI−MS/MS, electrospray ionization tandem mass spectrometry; PCA, principal component analysis; PTS2, peroxisomal targeting signal type 2.

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