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Peroxisomal proteomic approach for protein profiling in blue mussels (*Mytilus edulis*) exposed to crude oil

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Abstract

Peroxisomal proteomic protein profiles of exposure to marine pollution have been recently introduced in biomonitoring experiments. However, laboratory experiments to study the independent effect of common pollutants are needed to define a minimal protein expression signature (PES) of exposure to a specific pollutant. The aim of this study was to obtain PESs in blue mussels (*Mytilus edulis*) exposed to two different crude oil mixtures for future application in biomonitoring areas affected by oil spills. In the study, peroxisome-enriched fractions from digestive gland of *M. edulis* (L., 1758) were analysed by two-dimensional fluorescence difference electrophoresis (DIGE) and mass spectrometry (MS) after 3 weeks of exposure to crude oil mixtures: crude oil or crude oil spiked with alkylated phenols (AP) and extra polycyclic aromatic hydrocarbons (PAH) in a laboratory flow-through system. A minimal PES composed by 13 protein spots and unique PESs of exposure to the two different mixtures were identified. A total of 22 spots from the two-dimensional maps that had shown a significant increase or decrease in abundance in each of the exposed groups exposed were analysed. The hierarchical clustering analysis succeeded in discriminating the exposed groups from the control groups based on the unique PES. The PESs obtained were consistent with protein patterns obtained in previous field experiments. The results suggest that the protein profiles obtained by peroxisomal proteomics could be used to assess oil exposure in marine pollution assessments.

Keywords: *Biomarker, peroxisomal proteomics, two-dimensional fluorescence difference electrophoresis (DIGE), marine pollution, polycyclic aromatic hydrocarbons (PAH), crude oil*

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Introduction

There is a general need of proteomic methods development for protein profiling in the field of marine pollution assessments. Contamination of the coastal environmental by chemical contaminants such as hydrocarbons is a major environmental concern. In particular, petroleum-based products are the major source of energy for industry and daily life. Petroleum is also the raw material for many chemical products such as plastics, paints and cosmetics. In addition, the transport of petroleum across the world is frequent and, consequently, the potential for oil spills and its devastating effects on the marine ecosystem is significant. The recent black tides from the *Erika* and *Prestige*

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vessels in the European Atlantic provide evidence for the high risk of accidents and serious ecological impact in the area (Le Hir & Hily 2002, Laffon et al. 2005). The use of biomarkers has been proposed as a sensitive 'early warning' tool for biological effect measurement in environmental quality assessments (Cajaraville et al. 2000). Biological monitoring involves evaluation of the physiological status of 'sentinel' organisms (bioindicators) living in the monitored environment by determining the values of selected biological parameters (biomarkers) that are known to vary in response to the toxic effects of pollutants (Viarengo & Canesi 1991, Shugart et al. 1992). Sessile molluscs, such as marine mussels (*Mytilus* spp.), are commonly used as bioindicators of pollution in marine and estuarine environments (Goldberg 1986) due to their wide geographical distribution in both hemispheres. They live throughout the middle and low inter-tidal zones and are common on rocky shores. The blue mussels have been chosen as bioindicators for their distribution, abundance, sessility and filter-feeding activity response, rapid response to xenobiotics (Fahimi & Cajaraville 1995) and great bioaccumulators to environmental pollutants (Widdows & Donkin 1991).

Variations in specific protein expression are commonly used as indicators of exposure to pollution. However single-parametric biomarkers are more sensitive to variation dependent on biotic or abiotic factors and show a lack of robustness against mixed pollutants, making marine pollution assessments much more uncertain (Cancio & Cajaraville 1999, Cancio et al. 1999). The implementation of biomonitoring techniques is among the most urgent task to improve the marine risk-assessment analysis (Beliaeff & Bocquene 2004, Narbonne et al. 2005). The introduction of high-throughput techniques such as proteomics could provide a more complex expression pattern of exposure to a unknown mixture of pollutants or certain classes of chemicals (Mi et al. 2005, Apraiz et al. 2006). In laboratory experiments, PES of mussels exposed to copper, Aroclor and salinity stress has been made (Shepard & Bradley 2000, Shepard et al. 2000), in rainbow trout treated with diazinon, nonylphenol, propetamphos, and to sewage treatment plant effluents (Bradley et al. 2002), and in clams exposed to model pollutants (Rodriguez-Ortega et al. 2003) and in mussels exposed to oil (Manduzio et al. 2005). The use of surface-enhanced laser desorption/ionization-time of flight has been also applied to establish PES in mussels from areas contaminated with heavy metals, polyaromatic hydrocarbons and exposure to oil (Knigge et al. 2004, Bjornstad et al. 2006). We have recently introduced peroxisomal proteomics to establish the PES of exposition to an unknown mix of pollutants, which offer a complex and reproducible expression pattern. Peroxisome proliferators comprise a heterogeneous group of compounds known for their ability to cause massive proliferation of peroxisomes and liver carcinogenesis in rodents. Aquatic organisms living in coastal and estuarine areas are exposed to a variety of pollutants of industrial, agricultural and urban origin, which are potential peroxisome proliferators. The identified proteins from the protein profile belong to several peroxisomal biochemical pathways that do not response to biotic factors in a unique manner (Mi et al. 2005). In laboratory experiment, peroxisomal proteomics has been applied to study the effects of several marine pollutants (diallyl phthalate, PBDE-47, bisphenol-A) on the peroxisomal proteome of the digestive glands from mussels, obtaining a minimal PES that could be used to distinguish exposure to different pollutants (Apraiz et al. 2006).

The aim of this work was to assess the utility of comparative proteomics to provide PES directly associated with the exposure to model pollutants under controlled laboratory conditions. In those experiments, *Mytilus edulis* was exposed at sublethal concentrations to crude oil or a mixture of crude oil spiked with a mixture of AP and extra PAHs. Peroxisomal matrix proteins from digestive glands were analysed by DIGE. The results suggested that PESs could be used as a multi-parametric type of biomarker to distinguish between different model pollutants. The work highlighted the suitability of comparative proteomics for assessing the initial or temporal impacts of marine pollution accidents, such as fuel oil spills and in the evaluation of the environmental recovery.

Materials and methods

Animals

Mussels, *Mytilus edulis*, 60–70 mm in length, were collected in December 2002 in a pristine site (Forlandfjorden, Norway). Experiments were performed on three different groups of animals, which were kept under controlled laboratory exposures for 3 weeks, under sublethal concentrations of different pollutants. Mussels used as control, group 1, were maintained under filtered seawater at 10–12°C with 34‰ of salinity. Mussels in group 2 were exposed to 0.5 ppm of crude oil; and the third group to 0.5 ppm of crude oil spiked with a mixture of 0.1 ppm alkylated phenols and 0.1 ppm polycyclic aromatic hydrocarbons. This experiment belonged to campaign number 6 of the European Project BEEP (Biological Effects of Environmental Pollution in Marine Coastal Ecosystems) under contract nEVK3-CT2000-00025 and sampling was carried out in RF-Rogaland Research Institute (Stavanger, Norway).

Cell fractionation and isolation of peroxisomes. The digestive glands from 10 mussels per experiment were dissected and used for cell fractionation and the isolation of peroxisomes. The homogenization of minced tissue and subcellular fractionation by differential and density gradient centrifugation in iodixanol were performed according to an established method (Ghosh & Hajra 1986) with a few modifications outlined below. The main subcellular fractions were termed according to the nomenclature established (Volkl & Fahimi 1985). Thus, the total homogenate was termed fraction A, the heavy mitochondrial fraction B, the light mitochondrial or enriched peroxisomal fraction D, the cytosolic fraction E and the microsomal fraction F. A total of 2 ml of the resuspended D fraction were layered carefully on the top of 15 ml of 28% iodixanol (v/v), 5 mM MOPS (3–[N-Morpholino]-propanesulfonic acid), 0.1% ethanol, 1 mM tetrasodium ethylenediamine tetra-acetic acid (EDTA) solution (pH 7.3, density 1.16 g ml^{-1}) and 2 ml of 50% iodixanol (v/v) cushion (density 1.27 g ml^{-1}) and centrifuged at 40 000 rpm (131 000g_{avg}) for 2 h in a Beckman L7-55 centrifuge using a TFT50.2 Ti rotor. The peroxisome-enriched fractions were obtained from the interface between 28 and 50% of iodixanol. The activities of following marker enzymes were measured in the different main fractions across the fractionation procedure: catalase for peroxisomes, succinate dehydrogenase for mitochondria, and acidic phosphatase for lysosomes (Graham 1993). Protein was determined according to a colorimetric method (Bradford 1976). To analyse the quality of the peroxisome-enriched fractions, we conducted protein gel blot analysis with different commercial polyclonal antisera, according to standard procedures,

using chemoluminescence for detection and enzymatic marker analysis (Mi et al. 2005).

Protein extraction, Cy-dye labelling and two-dimensional electrophoresis (2-DE). Proteins were precipitated in a phenol extraction-ammonium precipitation procedure with some modifications (Mangolin et al. 1999). Briefly, the sample was mixed with 1 vol. of Tris-buffered phenol (pH 8.0) and centrifuged for 5 min at 11 300*g*. Proteins extracted in the phenolic phase were collected and re-extracted three times by the addition of 1 vol. of a back-extraction buffer (10 mM Tris-HCl, 20 mM KCl, 10 mM EDTA, 0.4% mercaptoethanol, pH 8.4) and centrifuged for 5 min at 11 300*g*. Phenol extracted proteins were precipitated with 5 vols of ammonium acetate in methanol, at -20°C for 30 min and washed with 100% cold acetone.

The peroxisomal proteins were solubilized in a solubilization buffer (7 M urea, 2 M thiourea, 2% 3-[(3-chloroamidopropyl)dimethylmmonio]-1-propane-sulfonate (CHAPS) (w/v), 0.5% Triton X-100, 1% β-mercaptoethanol, 1% dithiothreitol, 1% Pharmalyte pH 3–10, and 0.001% bromophenol blue) (Rabilloud 1998), to a final protein concentration of 2.5 mg ml^{-1}. The mixed internal standard methodology used was described by Alban et al. (2003). Samples containing 25 μg of peroxisomal proteins were labelled with 200 pmoles of Cy-Dye reconstituted in 99.8% anhydrous dimethylformamide, following the protocol described by the manufacturer (Amersham Biosciences, Uppsala, Sweden) and explained elsewhere (Unlu et al. 1997). Cy2 Dye was applied to the internal standard sample and Cy3 and Cy5 were used to label experimental samples. Three to four replicates of peroxisome-enriched fractions were performed per experimental condition. The number of replicates to fulfil the statistical requirements can be reduced to three or four replicates using DIGE because inverse labelling of replicates and randomization is applied (Alban et al. 2003). In order to fulfil the requirements for the IEF running, a second buffer was added to the labelled samples to get a final concentration of 7 M urea, 2 M thiourea, 2% CHAPS (w/v), 0.5% Triton X-100, 1% (v/v) Pharmalyte (3–10), 1% dithiolthreitol (DTT) and 1% β-mercaptoethanol. Afterwards, samples were alkalised with 30 mM iodoacetamide (IAA) (Herbert et al. 1998) and then mixed with a rehydration solution containing 8 M urea, 2% CHAPS (w/v), 15 mM DTT, 1% β-mercaptoethanol, and 0.2% (v/v) Pharmalyte pH 3–10. Solubilized samples were applied onto 11 cm IPG strips (pH 3–10 non-linear (nl)). Iso-electric focusing was performed on a Protean IEF Cell (Bio-Rad) at 20°C using the following programme: passive rehydration for 12 h, rapid voltage slope in all steps, step 1: 250 V for 15 min, step 2: 8000 V for 2.5 h and step 3: at 8000 V until reached 35 000 Vh. After this, immobilized pH gradient strips were reduced (1% dithiothreitol) and then alkalised (4% iodoacetamide) in equilibration buffer (50 mM Tris, 6 M urea, 30% glycerol, 2% sodium dodecylsulphate (SDS), pH 8.8). The second dimension run was carried out on homogeneous 12% T Criterion™ XT BIS-TRIS gels, at 120 V for 2 h using a Criterion Cell (Bio-Rad). DIGE gels were fixed in a 7.5% acetic acid and 30% ethanol solution overnight. Before the image analysis, gels were rinsed with distilled water. As alternative method to obtain the representatives gels showed in Figure 1, the protein spots in the analytical gels (not for quantitative applications) were visualized by staining with silver ammonia (Hochstrasser & Merril 1988).

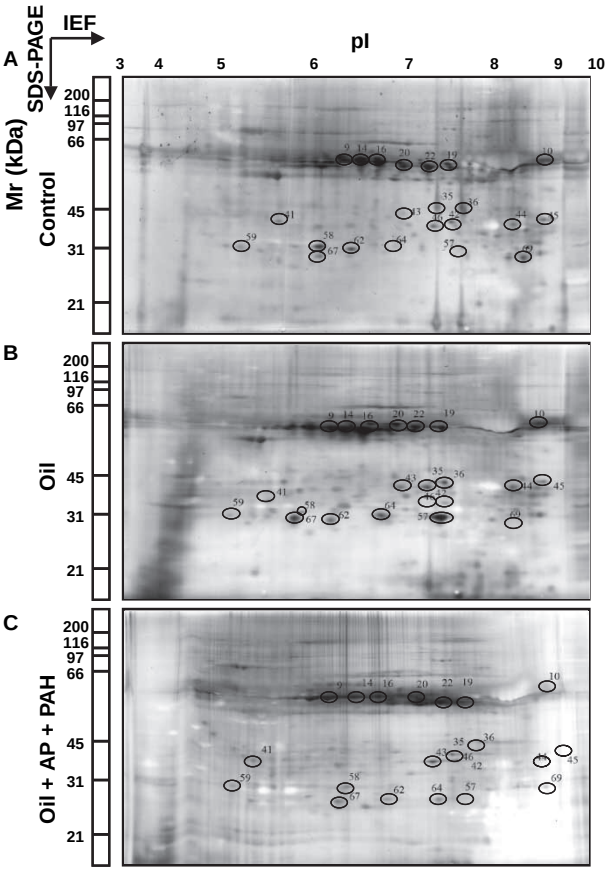

Figure 1. Representative two-dimensional electrophoresis (2-DE) maps showing labelled spots. The horizontal axis of the gels is the iso-electric focusing dimension, which stretches in the pH 3–10 range in a non-linear fashion, and the vertical axis is the polyacrylamide gel dimension, which stretches from 10 kDa (bottom) to about 250 kDa (top). Numbers refer to proteins in other figures and tables. The proteins marked with numbers increase or decrease in the expression level in the two experiments showed. (A) Representative 2-DE map from control sample; (B) representative 2-DE map from exposure to crude oil; and (C) representative 2-DE map from exposure to crude oil spiked with AP and PAH. Gels were calibrated for molecular mass (in kDa) and pI (in pH units) by external pH and mass standards.

Image acquisition and DIGE analysis. Labelled proteins were visualized using a Typhoon imager and images were analysed with the help of the DeCyder^{TM} software platform version 4.0 and 6.5 (Amersham Biosciences). Gel image pairs were processed by the DIA (Differential In-gel Analysis) software module to co-detect and differentially quantify the protein spots in the images, taking the internal standard sample as a reference to normalize the data, so the rest of the normalized spot maps could be compared among them. At a second stage, the BVA (Biological Variation Analysis) software module was applied. BVA performs a gel-to-gel matching of the internal standard spot maps from each gel. The average ratios of expression were analysed by one-way analysis of variance (ANOVA) ($p \leq 0.05$). Proteins showing differences in expression were further analysed in couples, comparing the different experimental exposure groups with the control group, using the Student's *t*-test ($p \leq 0.05$).

Clustering analysis

Hierarchical clustering was performed using 'cluster software' (Eisen et al. 1998). All the spots and experiments were set as the same weight, and the similarity metrics was selected based on the Pearson correlation coefficient.

Results

Protein expression pattern of exposition to different marine pollutants

In this study, sentinel animals *Mytilus edulis* were exposed for 3 weeks to crude oil and a mixture of crude oil spiked with a mixture of AP and PAHs. The first group corresponded to a control group, which was fed by filtered seawater. The second group was exposed to 0.5 ppm of crude oil and the third group to 0.5 ppm of crude oil spiked with a mixture of 0.1 ppm alkylated phenols and 0.1 ppm polycyclic aromatic hydrocarbons. On average, 100–150 protein spots were analysed in each of the gels. Representative 2-DE maps from the control and exposed groups are shown in Figure 1, A–C. The PESs of exposure to crude oil and crude oil mixture were composed by 22 protein spots. The expression of these spots increased up to 2.6-fold or decreased up to 5.3-fold in comparison with the control group's average ratio of expression. The representative 2-DE maps showed protein spots with molecular weights from 30 to 60 kDa and pIs from 4.0 to 8.5. Among the proteins composing these PESs, a group of 13 spots was differentially expressed in both experimental groups. Six up-regulated spots and seven down-regulated spots characterized the minimal protein expression pattern of exposure to crude oil or crude oil mixed with other pollutants (Figure 2, C). Interestingly, those spots modulated its expression in response to both types of crude oil exposures. However, only three of the spots changed the expression by up- or down-regulation in both experimental groups. The five up-regulated spots after exposure to crude oil corresponded to the same spots that were down-regulated after the exposure to the crude oil mixture with AP and extra PAHs. The other five down-regulated spots by the exposure to crude oil turned into up-regulated spots in the PES of exposure to the crude oil mixture. It is relevant the spot-protein number 67 with the highest up-regulated effect in the crude oil experiment and the highest down-regulated effect in the crude oil mixture group (Table I, A, B).

The changes in the expression profiles after exposure to crude oil are shown in Table I, A, and Figures 1, B, and 2, A, C. In this experimental group, 16 protein spots were detected with a significant increase or decrease in protein expression. The exposure to oil did not show a specific up- or down-regulation tendency. Here, seven stops were up-regulated up to 2.5-fold and nine spots were down-regulated up to 3.5-fold. It is remarkable the spatial distribution of the relevant spots in the 2-DE maps. Most of the down-regulated spots were aligned with pIs from 5.5 to 8.5 and in the range of 60 kDa. Those spots were distributed in the upper half section of the gel. The up-regulated spots were situated in the bottom half part of the gel, corresponding to molecular weight around 30–40 kDa and pIs from 5.2 to 8.5. Three spots composed a unique protein signature from this group; one up-regulated spot and two spots were down-regulated.

The oil mixture group showed stronger variation in the protein expression, both in the amount of protein spots that were affected by the exposure and in the levels of protein expression variation (Table I, B, and Figures 1, C, and 2, B, C). In this

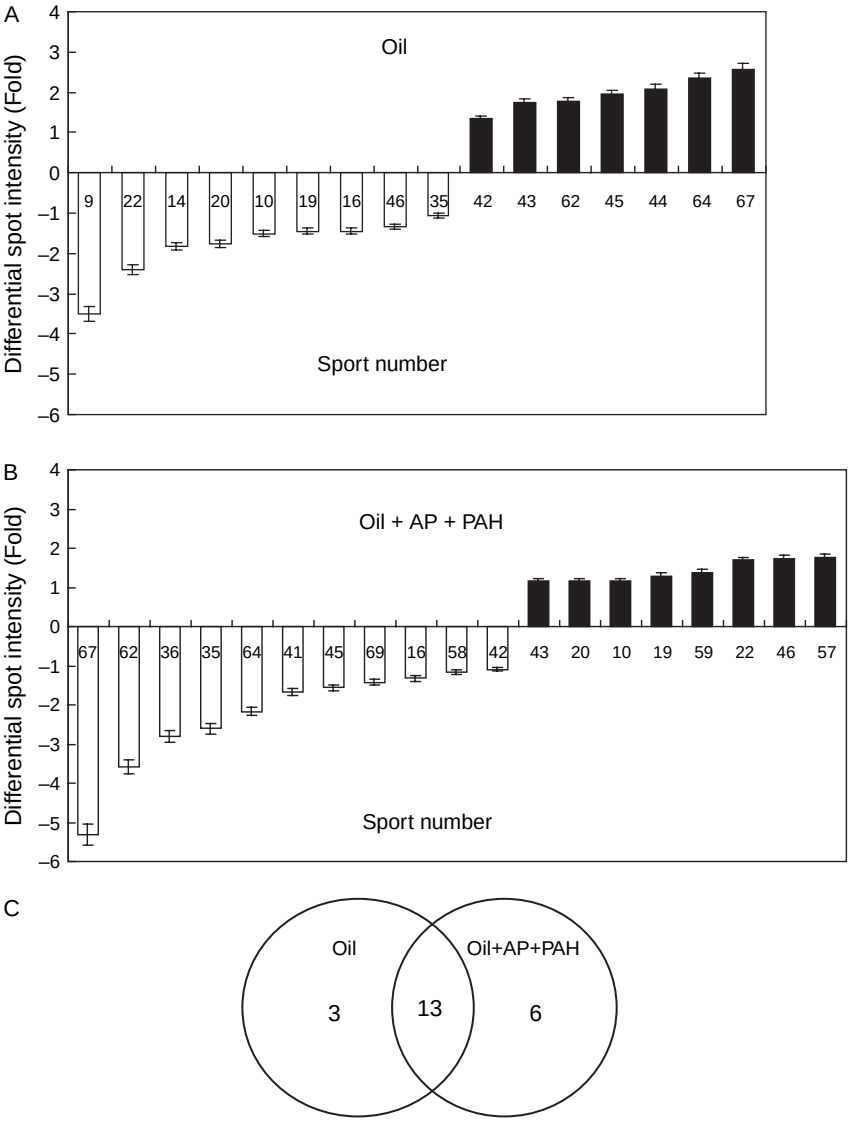

Figure 2. Proteins differentially expressed in the two experimental groups in comparison with the control group. The vertical axis corresponds to the average ratio of expression, above the zero value for the up-regulated proteins and below the zero value for the down-regulated ones. In the horizontal axis, the up-regulated proteins are organized with the highest values on the left-hand side and the down-regulated ones show the highest values on the right-hand side. (A) Control group against the group exposed to crude oil; (B) control group against the group exposed to crude oil spiked with an AP and PAH mix; and (C) a Venn diagram representing differentially expressed spots common between the different experimental groups.

experimental group, 19 spots showed changes in proteins expression, eight spots were up-regulated and 11 spots were down-regulated up to 5.3-fold. The differential expressed spots from the oil mixture group followed the opposite spatial distribution in the 2-DE maps than in the previous experimental group. Here, the down-regulated spots were concentrated in the bottom half section of the maps and the up-regulated

Table I. Differential protein expression of the spots from the exposure groups against the control group classified by fold and indicating the spot number, its position in the gel and fold regulation. (A) Crude oil exposure and (B) crude oil spiked with alkylated phenols (AP) and extra polycyclic aromatic hydrocarbons (PAH) exposure.

A Spot Number	Mw ob.(kDa)	pI ob.	Fold
9	60	6.4	−3.51
22	58	7.2	−2.41
14	60	6.5	−1.82
20	58	7	−1.76
10	60	9	−1.51
19	58	7.6	−1.45
16	60	6.7	−1.44
46	41	7.4	−1.34
35	48	7.5	−1.06
42	42	7.6	1.34
43	42	7	1.75
62	32	6.4	1.78
45	42	9	1.96
44	42	8.5	2.08
64	32	7	2.35
67	30	6	2.58
B Spot Number	**Mw Ob.(kDa)**	**pI Ob.**	**Fold**
67	30	6	−5.30
62	32	6.4	−3.59
36	48	7.8	−2.81
35	48	7.5	−2.61
64	32	7	−2.17
41	43	5.5	−1.68
45	42	9	−1.56
69	30	8.6	−1.42
16	60	6.7	−1.32
58	32	6.1	−1.16
42	42	7.6	−1.09
43	42	7	1.15
20	58	7	1.15
10	60	9	1.17
19	58	7.6	1.30
59	32	5.2	1.39
22	58	7.2	1.69
46	41	7.4	1.73
57	33	7.3	1.76

ones were distributed along the entire gel. Two up-regulated spots and four down-regulated spots composed the unique PES of exposure to oil mixed with AP and PAHs. All of them were localized in the bottom half-section of the 2-DE maps with molecular weight from 33 to 48 kDa and pIs from 3.9 to 7.4.

Hierarchical clustering

Cluster analysis is an exploratory data analysis tool used to solve classification problems and each cluster thus describes, in terms of the data collected, the class to which its members belong. We have used it here to validate the specific PES of both

experimental groups and the effect of the individual changes triggered by spiking the crude oil with AP and PAHs. Data were analysed by cluster software (Eisen et al. 1998), as shown in Figure 3. Data analysis showed a clustering in a hierarchical manner (dendrogram) where the down-regulated spots from the crude oil group at the left branch of the dendrogram were clearly separated from the down-regulated spots from the experimental group at the right branch of the dendrogram.

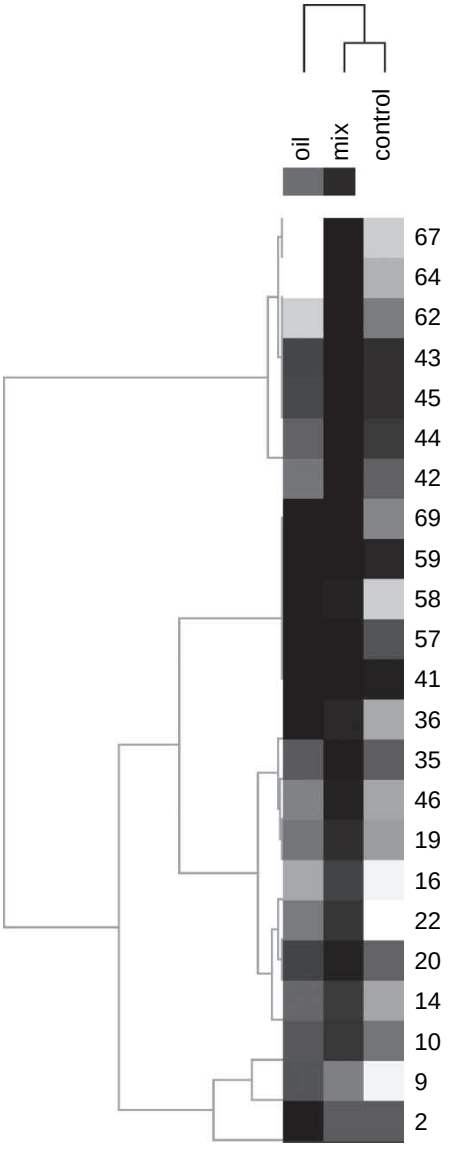

Figure 3. Organization of data by cluster analysis. The proteins that participated in the cluster analysis were present in at least 80% of the spot maps and passed the filter of the analysis of variance (ANOVA) ($p < 0.01$) test. The dendrogram shows the separation of the different experimental groups after hierarchical cluster analysis. Every coloured square represents a protein that is up-regulated or down-regulated, or a protein that does not show any differences in expression. Very short branches join items if they are very similar to each other, and by increasingly longer branches as their similarity decreases.

Discussion

Accident spills of crude oil during transportation during daily harbour activities or from the oil industry are often the cause of tremendous ecological damage at different levels, as well as causing a considerable economical impact on fisheries resources. In this context, improving the biomonitoring programme with novel multivariable biomarkers is one of the present goals in this field. The present study has applied a relatively new proteomic approach, DIGE (Unlu et al. 1997), to analyse changes in protein expression associated with exposure to crude oil and a oil spiked with AP and PAHs. The aim of this work was to apply peroxisomal proteomics for obtaining PESs of exposure to a mixture of chemicals. Recently, several groups have been focused on searching for new biomarkers. Among them, the 2-DE based methods have been applied in *Chamelea gallina* or *Mytilus edulis* (Rodriguez-Ortega et al. 2003, Manduzio et al. 2005). The use of surface-enhanced laser desorption/ionization-time of flight has been used to obtain PES in mussels from field and laboratory experiments. In this case, the corresponding peptides or protein fragments have not been identified (Knigge et al. 2004, Bjornstad et al. 2006). The data presented herein were in agreement with our previous application of peroxisomal proteomics in field experiments, which provides addition support to suggest the utility of this proteomics-based method in environmental risk assessments (Mi et al. 2005).

The present experimental approach called peroxisomal proteomics is based on the established knowledge that aquatic organisms living in coastal and estuarine areas are specially threatened by peroxisome proliferation (Cajaraville et al. 2003). Peroxisome proliferation involves morphological and functional changes in the peroxisomes. Both laboratory and field studies have shown that phthalate ester plasticizers, PAHs and oil derivatives, PCBs, certain pesticides, bleached kraft pulp and paper mill effluents, alkylphenols, and oestrogens provoke peroxisome proliferation in different fish or bivalve mollusc species (Cajaraville et al. 2003). Peroxisome proliferation has already been assessed in aquatic sentinel species such as mussels (Cajaraville et al. 1997) and it was introduced several years ago as a biomarker of environmental pollution (Cajaraville et al. 2000). Measuring peroxisome proliferation in mussels' digestive glands was achieved by the application of various biochemical methods (Small et al. 1985, Cajaraville et al. 1997, Orbea et al. 1999). However, the detection of peroxisome proliferation by proteomics is another proposal that provides a PES instead of a single-parameter biomarker. The robustness of this subproteomic approach is based, on the one hand, on the fact that low abundant cellular proteins can be detected and on the other hand, that changes in the peroxisomal proteome are mainly dependent on changes in peroxisomal pathways (Mi et al. 2005, Apraiz et al. 2006).

In this laboratory experiment, the aims have focused on the effects of exposure to crude oil mixtures. Mussels bioconcentrate petroleum hydrocarbons from the aqueous phase by two to five orders of magnitude in proportion to petroleum hydrocarbons' concentration in the ambient water (Widdows & Donkin 1992). The partition behaviour of PAH in mussels is known and allows one to estimate equivalent water PAH concentrations compared with mussels' tissue PAH concentration (Neff & Burns 1996). Mussels, unlike many other invertebrates and all vertebrates, have a limited ability to metabolize PAH (Stegeman 1981). When ambient concentrations of bioavailable PAH decrease, mussels passively release accumulated hydrocarbons back into the water. Therefore, at any given time, the steady-state concentration of

PAH in mussels at a given location reflects the concentrations of all bioavailable PAH derived from all sources at the site (Baumard et al. 1999). In the present study, the pollutant mixture composition differs between both experimental groups because the crude oil is spiked with AP and extra PAHs. Although the addition is relatively small compared with the concentration of crude oil, two differential responses in the peroxisomal proteome were obtained. The response is stronger in the crude oil mixture group, with a higher number of proteins and a stronger variation in protein expression, especially at the down-regulated level. In agreement with the present results, this experimental group has also reported a double number of mass peaks than the crude oil in plasma protein expression profile obtained by surface-enhanced laser desorption/ionization-time-of-flight (SELDI-TOF) (Bjornstad et al. 2006).

It is remarkable that most of the proteins composing the common PES that were up-regulated in exposure to only crude oil showed a down-regulated effect in the mixture group. First, it has been discussed recently that the mechanism of mixture toxicity cannot be easily estimated as addition of independent actions and novel approaches have been developed for the prediction hazardous assessment of realistic mixtures of pollutants (Junghans et al. 2006). Second, this response could indicate that the oestrogenic effect of AP had a prevalent response over other biochemical pathways affected by exposure to crude oil. Alkylphenolic compounds were first found to be oestrogenic in the 1930s (Dodds 1938) and more recently it has been reported in mussels' expose to AP that hormonal disturbance could be a direct action of the pollutant (Aarab et al. 2004). The potential mixture toxicity is still an unresolved issue.

From the comparison between the 2-DE maps from peroxisomal proteomics from field and laboratory experiments, strong coincidences in the protein distribution were observed. Taking in consideration that two different species collected for these two studies were different, *Mytilus galloprovincialis* in the field experiment and *Mytilus edulis* in this laboratory experiment, the PES obtained was very consistent in both experiments (Mi et al. 2005). The positional difference in some proteins should be ascribed to differences between species (Lopez et al. 2002). Acyl-CoA oxidase was identified in that experiment in an equivalent position as spot 67 in this study. This protein response was up-regulation in the oil experimental group and down-regulation in the oil mixture group. It has been recently published that the increase of acyl-CoA oxidase, which is a crucial peroxisomal proteins, is lower in the oil mixture group than in the oil group, suggesting a possible antagonist effect of AP over the PAHs (Orbea et al. 2002, Ortiz-Zarragoitia & Cajaraville 2005). The position and response of the peroxisomal sarcosine oxidase identified in our field experiments strongly resemble spots 44 and 45 from this laboratory experiment (Mi et al. 2005). This enzyme, which is involved in amino acid metabolism, shows a strong over-expression in our previous work and could be an interesting candidate for deeper studies. This protein combines characteristic required for the design of a possible protein chip. It is highly specific in biochemical pathway, subcellular location and low abundance.

Using the spots differentially expressed in these experiments: control, exposure to crude oil and exposure to crude oil, PA and extra PAHs, the three groups were clearly separated from each other based on the association between different spots. Cluster analysis is an ideal tool to evaluate the associations and structure and to classify gels blindly into classes. The data organized in four classification levels after clustering corroborate the important of PESs characterization to identify the possible source of

contamination in field experiments and in biomonitoring programmes. It is also remarkable that the unique PES from the crude oil mixture group succeeded in clustering together at the fourth level of the cluster. Taken together, the unique spots could represent a valuable multi-parameter biomarker to distinguish both experimental groups.

In summary, the data presented in this work show that peroxisomal proteomics provide PESs that were sensitive multivariable indicators of exposure to crude oil or crude oil mixtures and were consistent with protein expression patterns obtained in field experiments. The identification of the unique proteins from those protein profiles could provide valuable information for ecotoxicological studies, although they are beyond the aim of this work.

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