Bivalves and especially mussels are very good indicators of marine and estuarine pollution, and so they have been widely used in biomonitoring programs all around the world. However, traditional single parameter biomarkers face the problem of high sensitivity to biotic and abiotic factors. In our study, digestive gland peroxisome-enriched fractions of *Mytilus edulis* (L., 1758) were analyzed by DIGE and MS. We identified several proteomic signatures associated with the exposure to several marine pollutants (diallyl phthalate, PBDE-47, and bisphenol-A). Animals collected from North Atlantic Sea were exposed to the contaminants independently under controlled laboratory conditions. One hundred and eleven spots showed a significant increase or decrease in protein abundance in the two-dimensional electrophoresis maps from the groups exposed to pollutants. We obtained a unique protein expression signature of exposure to each of those chemical compounds. Moreover a set of proteins composed a proteomic signature in common to the three independent exposures. It is remarkable that the principal component analysis of these spots showed a discernible separation between groups, and so did the hierarchical clustering into four classes. The 14 proteins identified by MS participate in α- and β-oxidation pathways, xenobiotic and amino acid metabolism, cell signaling, oxiradical metabolism, peroxisomal assembly, respiration, and the cytoskeleton. Our results suggest that proteomic signatures could become a valuable tool to monitor the presence of pollutants in field experiments where a mixture of pollutants is often present. Further studies on the identified proteins could provide crucial information to understand possible mechanisms of toxicity of single xenobiotics or mixtures of them in marine ecosystems. *Molecular & Cellular Proteomics* 5:1274–1285, 2006.

Contamination of the coastal environment by chemical contaminants such as hydrocarbons (oil spills and wastes), pesticides (crop treatment), heavy metals, and various organic pollutants in dredged sediments and wastewaters is a major environmental concern. During the last years, the detection of these pollutants and their effects in living organisms has become very important. In this context, many biomonitoring programs have been focused on the water quality control all around the world; among them, many are based on "Mussel Watch"-like programs (1). Bivalves and especially mussels have been widely used as indicators of marine and estuarine pollution due to their capacity to bioaccumulate and concentrate organic and metallic pollutants, thus providing temporally and spatially integrated levels of contamination (2). To show an integrated measure on effects of pollutant exposure in biological systems, the use of different biomarkers has been introduced in monitoring programs. Biomarkers are measures at a cellular, biochemical, or molecular level and show whether a key organism has been exposed to toxic chemicals and the magnitude of the organism’s response to the contaminant (3). Each biomarker shows stronger specificity for certain types of xenobiotics, and thus the use of a battery of biomarkers has been strongly recommended to be included in monitoring programs (4).

Among the chemical compounds affecting the marine environment, it has been verified that some can induce morphological and functional changes in peroxisomes and also peroxisome proliferation. This proliferation can be the cause of long term liver carcinogenesis processes in some rodent species (5). Peroxisome proliferation has been assessed in aquatic sentinel species like mussels and applied as a biomarker of environmental pollution in biomonitoring programs (4). Measuring peroxisome proliferation in the digestive glands of mussels has been achieved by various biochemical methods (6, 7). Recently the detection of peroxisome proliferation by proteomics has been proposed; this approach provides a more complex protein expression signature (PES)1 and can be applied to field experiments (8).

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1 The abbreviations used are: PES, protein expression signature; BPA, bisphenol A; DAP, diallyl phthalate; HAO1, hydroxy-acid oxidase 1; SOD, superoxide dismutase; PBDE-47, 2,2',4,4'-tetrabromodiphenyl ether; PCA, principal component analysis; PCB, polychlorinated biphenyl; ROS, reactive oxygen species; 2-DE, two-dimensional electrophoresis; BEEP, Biological Effects of Environmental Pollution in Marine Coastal Ecosystems; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; ANOVA, analysis of variance; EDA, extended data analysis; BVA, biological variation analysis; PTS2, peroxisomal targeting signal type 2; PEX, peroxin.
The traditional biomarkers present some important drawbacks. The difficulties in discriminating between pollution-related changes from “naturally” occurring variations or the presence of interindividual variability are still unsolved issues (4). Recently proteomics-based techniques have successfully solved similar problems faced in medical studies; thus, the application of proteomics to environmental monitoring has begun to be explored. The first proteomic studies reported in this field were focused on the identification of fish species (9, 10). However, the attention is now moving to the identification of the critical set of mussel proteins commonly expressed after exposure to toxic contaminants (11). In several laboratory experiments, the utility of PES has been reported. This methodology has been explored in mussels exposed to copper, Aroclor, and salinity stress (12, 13); in rainbow trout treated with diazinon, nonylphenol, propetamphos, and sewage treatment plant effluents (14); and in clams exposed to model pollutants (15). More recently, the use of SELDI-TOF has been introduced in the first field experiment to establish PES in mussels from areas contaminated with heavy metals and polyaromatic hydrocarbons (16). One of the advantages of using comparative proteomics instead of conventional biomarkers is that proteomics does not require previous knowledge of pollutant mechanisms, and because many xenobiotics alter protein expression, it may serve to identify novel and unbiased biomarkers and to establish their mechanisms of toxicity (17). Although proteomics has been well developed in model organisms creating extensive 2-DE databases of proteins (18, 19) and characterizing the response to peroxisome proliferators (20), its feasibility and utility for environmental pollution assessment based on non-model organisms like mussels has yet to be demonstrated.

In this project, *Mytilus edulis* (L., 1758) were exposed at sublethal concentrations to specific pollutants such as diallyl phthalate (DAP), 2,2′,4,4′-tetrabromodiphenyl ether (PBDE-47), and bisphenol A (BPA). Phthalates, which are widely used as plasticizers in products molded with polyvinyl chloride, are suspected of disrupting the endocrine system, especially by mimicking estrogens (21, 22). Polybrominated diphenyl oxide (ether) is commercially used as a flame-retardant on a number of products including textiles and electronic devices, and leakage of the compound from dump sites into the environment has been demonstrated (23, 24). The toxicity of PBDEs is not as well understood as that of polychlorinated biphenyls (PCBs). PBDEs are endocrine disruptors and neurotoxins. They are believed to cause liver tumors and neurodevelopmental and thyroid dysfunctions (25). This group of pollutants is highly resistant toward acids, bases, heat, and reducing and oxidizing compounds; as a result, they are extremely persistent when released in the environment. In mussels, field studies have reported that the bioaccumulation potential of PBDE-47 is higher than that of PCBs, but both type of components are bioavailable substances that are rapidly taken up and bioaccumulated by blue mussels, indicating that these substances may reach high concentrations in aquatic organisms (26). Bisphenols are monomers (building blocks) of polycarbonate plastics, and they are utilized for packing, for coating of food cans, and in epoxy resins (27). Bisphenols have become well known targets for environmental research because of their potential activity as endocrine disruptors in mammals (28) and in aquatic organisms (29). The proteome from digestive gland peroxisome-enriched fractions were analyzed by DIGE. Our results showed that the proteins constituting a unique PES can be used as novel biomarkers to distinguish between these three model pollutants and moreover that a minimal PES common to all of them could be applied to field experiments. Our approach offers a fast and sensitive method with applicability in marine pollution monitoring. The identification of the proteins that change in expression level after exposure to pollutants opens up the possibility to improve our understanding of toxicological pathways.

**MATERIALS AND METHODS**

**Animals—**Mussels (*M. edulis*) 60–70 mm in length were collected in March 2003 in a pristine site (Forlandfjorden, Norway). Experiments were performed on four different groups of animals, which were kept under controlled laboratory exposures for 3 weeks, under sublethal concentrations of different pollutants (at a concentration of 1% of the LC50 value). Group 1, the mussels used as control, was maintained under filtered seawater at 10–12 °C with 34% salinity. Group 2 was exposed to 38.3 μg/liter DAP, group 3 was exposed to 0.23 μg/liter PBDE-47, and group 4 was exposed to 59.4 μg/liter BPA. This experiment belongs to campaign number 6 of the European Project Biological Effects of Environmental Pollution in Marine Coastal Ecosystems (BEEP), and the sampling was carried out in RF-Rogaland Research Institute (Stavanger, Norway).

**Cell Fractionation and Isolation of Peroxisome-enriched Fractions—**The digestive glands from 50 mussels per experiment were dissected and used for cell fractionation and isolation of peroxisome-enriched fractions. The homogenization of minced tissue and subcellular fractionation by differential and density gradient centrifugation in iodixanol was performed according to an established method (30) with a few modifications outlined below. The main subcellular fractions were termed according to the nomenclature used by Völkl and Fahimi (31). Thus, the total homogenate was termed A, the heavy mitochondrial fraction was termed B, the light mitochondrial or enriched peroxisomal fraction was termed D, the cytosolic fraction was termed E, and the microsomal fraction was termed F. Two milliliters of the resuspended D fraction were layered carefully on the top of 15 ml of 28% iodixanol (v/v), 5 mM MOPS, 0.1% ethanol, 1 mM tetradsodium EDTA solution (pH 7.3; density, 1.16 g/ml) and 2 ml of 50% iodixanol (v/v) cushion (density, 1.27 g/ml) and centrifuged at 40,000 rpm (131,000 × gav) for 2 h in a Beckman L7-55 centrifuge using a TFFT50.2 Ti rotor. The purified peroxisomes were obtained from the interface between 28 and 50% iodixanol. The activities of the 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 (32). Protein concentration was determined according to Bradford (33). To analyze the purity of the peroxisomal preparations, we conducted protein gel blot analysis with different commercial polyclonal antisera according to standard procedures using chemoluminescence for detection.

**Protein Extraction, Cy Dye Labeling, and 2-DE—**Proteins were extracted using a modified version of the protocol described by Völkl and Fahimi (31). The homogenate from 200 mussels was sonicated in lysis buffer (50 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 2.5 μg/ml leupeptin, 1 μg/ml pepstatin A, and 0.5 mM pepstatin A) for 15 min on ice. The sample was then centrifuged at 12,000 rpm for 20 min at 4 °C, and the supernatant was saved for 2-DE. The pellets were then resuspended in lysis buffer and sonicated for another 15 min on ice. After centrifugation, the supernatant was saved for 2-DE. The protein concentration was determined using the Pierce BCA assay (Thermo Scientific). The proteins were labeled with Cy2 and Cy3 using the Multiplex Protein Labeling Kit (GE Healthcare) following the manufacturer's instructions.

**Protein Electrophoresis—**Protein samples were subjected to 2-DE using the Enhanced PIgel PRE HD kit (GE Healthcare) and the Pro-Tab MAP kit (GE Healthcare) according to the manufacturer's instructions. The gels were stained with Coomassie Brilliant Blue R-250 and destained with methanol and acetic acid. The gels were scanned using a Typhoon 9400 variable laser array (GE Healthcare) and analyzed using the ImageQuant TL software (GE Healthcare). The proteins were identified using mass spectrometry.
precipitated in a phenol extraction-ammonium precipitation procedure with some modifications (34). Briefly the sample was mixed with 1 volume of Tris-buffered phenol (pH 8.0) and centrifuged for 5 min at 11,300 × g\text{avg}. Proteins extracted in the phenolic phase were collected and re-extracted three times by addition of 1 volume of a back-extraction buffer (10 mm Tris-HCl, 0.02 mm EDTA, 0.4% β-mercaptoethanol, pH 8.4) and centrifuged for 5 min at 11,300 × g\text{avg}. Phenol-extracted proteins were precipitated with 5 volumes of ammonium acetate in methanol at −20 °C for 30 min and washed with 100% cold acetone.

Proteins were solubilized in a solubilization buffer containing 7 M urea, 2 M thiourea, 2% CHAPS (w/v), 0.5% Triton X-100, 1% β-mercaptoethanol, 1% dithiothreitol, 1% Pharmalyte pH 3–10, and 0.001% bromophenol blue modified from Rabilloud (35) to a final protein concentration of 2.5 mg/ml. Samples containing 25 μg of solubilized proteins were labeled with 200 pmol of Cy dye reconstituted in 99.8% anhydrous dimethylformamide following the protocol described by the manufacturer (Amersham Biosciences) and explained elsewhere (36). Cy2 dye was applied to the internal standard sample, and Cy3 and Cy5 were used to label experimental samples. The mixed internal standard methodology used has been described by Alban et al. (37). Three to four replicates were performed per experimental condition. To fulfill the requirements for IEF, a second buffer was added to the labeled samples to get a final concentration of 7 M urea, 2 M thiourea, 2% CHAPS (w/v), 0.5% Triton X-100, 1% Pharmalyte pH 3–10, 1% DTT, and 1% β-mercaptoethanol. Afterward samples were alkali dized with 30 mm iodoacetamide (38) 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). 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 in all steps; step 1, 250 V for 15 min; step 2, 8000 V for 2.5 h; step 3, 8000 V until it reached 35,000 V-h. After this, immobilized pH gradient strips were reduced (1% DTT) and then alkali dized (4% iodoacetamide) in equilibration buffer (50 mm Tris, 6 M urea, 30% glycerol, and 2% 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 35,000 V-h. After this, immobilized pH gradient strips were reduced (1% DTT) and then alkali dized (4% iodoacetamide) in equilibration buffer (50 mm Tris, 6 M urea, 30% glycerol, and 2% 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.

Image Acquisition and DIGE Analysis—Labeled proteins were visualized using a Typhoon imager, and images were analyzed with the help of the DeCyder™ software platform versions 4.0 and 6.5 (Amersham Biosciences). Gel image pairs were processed by the DeCyder-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 DeCyder-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 analyzed by one-way ANOVA (p < 0.05). Proteins showing differences in expression were further analyzed in couples, comparing the different experimental exposure groups (DAP, PBDE-47, and BPA) with the control group using the Student’s t test (p ≤ 0.05).

Protein Identification by MALDI-TOF or ESI-MS/MS and Database Searching—Proteins were excised from gels and cleaved with trypsin by in-gel digestion (39). The peptide extract from each tryptic digest was crystallized in 0.5 μl of matrix solution (α-cyano-4-hydroxycinnamic acid in methanol) on the MALDI-TOF target plate. Molecular mass information of the peptides was obtained by using a MALDI-TOF mass spectrometer that was equipped with a nitrogen laser and operated in reflector/delay extraction mode (Voyager-DE-STR; Perseptive Biosystems Inc.). All MALDI-TOF spectra were externally and internally calibrated for the correction of masses. Alternatively peptides were analyzed by ESI-MS/MS according to Wilm et al. (40) on a Q-TOF mass spectrometer (Waters Ltd.). Sequence tags were interpreted from the ESI-MS/MS spectra, and identifications were performed using Masslynx software (40), the University of California San Francisco MS-Homology search (prospector.ucsf.edu), and mass spectrometry-driven BLAST (MS BLAST) (41) and FASTA to search through the protein and genome databases (Protein Information Resource (PIR), Swiss-Prot, and National Center for Biotechnology Information (NCBI)). Analysis of hypothetical proteins was conducted using software at servers accessible on the Internet: BLAST (www.ncbi.nlm.nih.gov/Education/BLASTinfo/information3.html), Pfam (pfam.wustl.edu/), PROSITE (www.expasy.org/prosite/), ProDom (protein.toulouse.inra/prodom/current/html/ hole.php), and PSORT (psort.nibb.sc.jp).

**RESULTS**

Protein Expression Pattern of Exposure to Different Marine Pollutants—This investigation was carried out following a 3-week exposure to several xenobiotics of four groups of mussels collected in a pristine site. The first group corresponded to a control group, which had been fed by filtered seawater. The second group was exposed to 38.3 μg/liter DAP, the third group was exposed to 0.23 μg/liter PBDE-47, and the fourth group was exposed to 59.4 μg/liter BPA. The selected concentrations correspond to sublethal concentrations of different pollutants (at a concentration of 1% of the LC50 value). We utilized DIGE techniques to analyze possible variations upon acclimatization to these specific pollutants. DIGE maps from digestive gland peroxisome-enriched fractions of M. edulis and the normalized average ratio of expression for the spots present in the maps were obtained.

On average, about 130 spots were analyzed in each gel, and they were distributed along the whole range of pI applied in this experiment (pI 3–10) with a higher concentration of proteins with a pI higher than 7. Statistical analyses were applied to compare the average ratios of expression from the spots in the control 2-DE maps and in the exposed groups (DAP, PBDE-47, and BPA). One hundred and eleven spots were chosen after showing a significance difference at the level of 0.05 as well as a 2-fold or higher change in expression compared with the control group. A master image showing the statistically relevant spots was therefore obtained (Fig. 1). The supplemental material summarizes the response of the 111 spots in the different groups. Comparing the spots high-
lighted in each exposure, we found 16 spots that were differentially expressed in the same direction in all three experimental groups; nine spots were up-regulated, and seven spots were down-regulated (Fig. 2). It was also remarkable that the highest increases, up to 11-fold, corresponded to the PBDE-47 group. However, the same set of up-regulated spots from the other two groups (DAP and BPA) reached values not higher than 4-fold. The contrary was observed among the common down-regulated spots: the level of down-regulation was higher in the BPA and DAP groups than in the PBDE-47 group where only one of the spots (spot number 23) decreased to a higher degree (Fig. 2B). This group of 16 spots

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**Fig. 1.** Representative 2-DE Cy dye-stained protein gel showing labeled spots. The horizontal axis of the gels is the isoelectric focusing dimension, which stretches in the range of pH 3–10 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 a circle changed in the expression level in the three experiments. Gels were calibrated for molecular mass (in kDa) and pI (in pH units) by external pH and mass standards.

**Fig. 2.** A, Venn diagram representing differentially expressed spots in common for the different experimental groups. B, positional identification of the 16 differentially expressed spots that are common to the three groups and their average ratios of expression. Obs., observed.
composed a minimal PES of exposure to a mixture of common marine pollutants.

Changes in the protein expression profile caused by exposure to DAP are shown in Figs. 3A and 4A and B. As a general tendency, this group showed a reduction in the protein expression level after the exposure. In total, 29 of 43 spots were down-regulated. This treatment caused a decrease in expression between 2- and 4-fold to 19 spots, over 4-fold to nine spots, and over 8-fold to one spot. The up-regulated set was formed by 14 spots; 13 of them increased in protein expression between 2- and 4-fold, and only one spot increased over 4-fold. Finally a set of eight spots composed a unique PES from the DAP group; one spot (spot number 43) was up-regulated 3-fold, and seven spots were down-regulated between 2- and 7-fold.

The PBDE-47 group showed the strongest variation in the peroxisome-enriched fraction proteome with a higher tendency toward up-regulation. The nominal concentration of PBDE-47 in the exposure water was supposed to reach 5 ng/liter (ppb); however, after monitoring the water quality a lower concentration (0.23 ± 0.189 ng/liter) of the component was revealed. Here 27 spots showed an increase in protein expression over 4-fold. In total 59 spots showed changes in expression after exposure to PBDE-47. Thirty-eight of them were up-regulated: 11 spots were up-regulated between 2- and 4-fold, 22 spots were up-regulated between 4- and 8-fold, and five spots were up-regulated over 8-fold. Twenty-one spots were down-regulated: 15 spots were down-regulated between 2- and 4-fold, five spots were down-regulated between 4- and 8-fold, and one spot was down-regulated over 8-fold (Figs. 3B and 4B and C). A set of 25 spots is unique to this group; 18 of them were up-regulated up to 9-fold.

Finally the BPA group showed the highest number of statistically significant differentially expressed spots with at least nine spots down-regulated over 4-fold (Figs. 3C and 4C and D). The reduction of the expression of 41 of 71 spots was the general pattern and was distributed as follows: 32 spots were down-regulated between 2- and 4-fold, six spots were down-regulated between 4- and 8-fold, and three spots were dras-

\[ \text{O-K Andersen, personal communication} \]
A set of 30 spots was up-regulated in a more moderate manner: 18 spots were up-regulated between 2- and 4-fold, and only two spots were up-regulated between 4- and 8-fold. The BPA-specific PES was composed of 32 spots: 11 spots were up-regulated, and 21 spots were down-regulated, one of them up to 11-fold. Studying in parallel the common expression pattern between two groups, the BPA and DAP groups shared 28 spots, mainly down-regulated (17 spots). Groups BPA and PBDE-47 presented also 27 spots in common. Those were equally distributed between up- and down-regulation.

**Identification of Proteomic Signatures**—We assigned putative protein identifications to a set of differentially expressed proteins. The strong up- or down-regulation indicated that those proteins could become biomarkers of exposure to a single pollutant or to a combined mixture. Table I summarizes the data from 16 identified spots corresponding to 13 different proteins. The identification was accomplished by either ESI-MS/MS or MALDI-TOF analysis of peptides produced by proteolytic digestion of spots excised 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 (42). Proteins identified in this work can be divided into two classes. The first class comprised proteins differentially expressed in all the groups, and the second class was formed by proteins common to two groups. In the first class, six of a total of 16 spots forming the minimal PES were identified: spots 49 (425.95), 103 (44.5/8.4), 95 (53/7.55), 97 (54/7.9), 98 (54/8.5), and 99 (54/8.8) (numbers in parentheses correspond to observed Mr and pI). The first two spots corresponded to two proteins from the common PES that were down-regulated after the exposure, and the last four spots corresponded to a unique protein with an up-regulated effect. Spot 49 was identified as β-tubulin by homology to a Crassostrea gigas protein. β-Tubulin forms the microtubules from the cytoskeleton after polymerization. Spot 103 was identified as being related to the enzyme alcohol dehydrogenase of Saccharomyces cerevisiae that participates in the functional pathway of the oxidation of ethanol to acetaldehyde and has a cytosolic subcel-
lular localization. Spots 95, 97, 98, and 99 corresponded to catalase of *M. edulis*; catalase is a peroxisomal enzyme that converts hydrogen peroxide (H$_2$O$_2$) to water.

In the second class, five different proteins that are common to the groups DAP and BPA were identified. Spots 20 (69/4.6), 32 (44.5/5.32), 36 (44/8.2), and 67 (29.5/7.17) were down-regulated, and spot 104 (43.5/9.3) was up-regulated. Spot 20 corresponded to hydroxy-acid oxidase 1 (HAO1) by homology with the protein of *Strongylocentrotus purpuratus*. This peroxisomal enzyme oxidizes a broad range of /H$_2$O$_2$/-hydroxy acids to /H$_2$O$_2$/-keto acids and concomitantly reduces molecular oxygen to H$_2$O$_2$. Spot 32 was identified as GST by homology with the protein of *Oryza sativa*. GST is involved in the detoxification and metabolism of xenobiotics and is possibly localized in the cytosol or peroxisome. The identification of spot 36 as aldehyde dehydrogenase was obtained from homology to the enzyme of *Schistosoma japonicum*. This is a mitochondrial matrix NAD-dependent enzyme that catalyzes the second step of the proline degradation pathway, converting pyrroline 5-carboxylate to glutamate. Spot 67 was assigned to carbonic anhydrase by homology search with the protein of *Homo sapiens*. Carbonic anhydrases are cytosolic zinc-containing enzymes that catalyze the reversible reaction between carbon dioxide hydration and bicarbonate dehydration. Finally, spot 104 corresponded to Mn-superoxide dismutase (Mn-SOD), similar to the protein of *Candida albicans*. Mn-SOD is a mitochondrial and peroxisomal antioxidant metalloenzyme essential in the oxyradical metabolism. Five proteins with altered expression profiles in both the PBDE-47 and BPA groups were identified. Two of them were down-regulated, and three were up-regulated. Spot 30 corresponded to ATP synthase subunit of *M. edulis* that is a very abundant mitochondrial protein that catalyzes the synthesis of ATP from ADP and phosphate. Spot 38 was similar to *Gallus gallus* peroxin 10, which is involved in peroxisomal assembly. Spot 34 was homologous to cytochrome P450 2A3 (CYP2A3) of *H. sapiens*. This enzyme catalyzes coumarin 7-hydroxylation and is involved in the metabolism of xenobiotics. Spot 63 was identified as enoyl-CoA hydratase by its similarity with a homologous protein of *Mus musculus*. It is a crucial enzyme in the /H$_2$O$_2$/-oxidation of fatty acids localized in the matrix of the peroxisome. Spot 110 was identified as similar to a phospholipase A2 of *Apis mellifera*. Phospholipase A$_2$ is a crucial enzyme in the cell signaling pathways that has been found in the cytosol and is associated to organelles such as peroxisomes. Only one common protein from the DAP and PBDE-47 groups was identified. It was spot 21, and it corresponded to cytochrome c oxidase subunit II, which is an essential part of
the mitochondrial respiratory chain.

**Multivariate Analyses**—Data were analyzed by two different multivariate analysis methods with the aid of the DeCyder-EDA module. The results obtained by these two methods showed that the control group formed a separate cluster from the other three experimental groups. The PCA showed a very clear separation in the first component (PC1) of the loading plot of the spot map (Fig. 5A) where the control group occupied the positive side of the x axis and the rest of the groups were in the negative side. The second component (PC2) separated the rest of the experimental groups, which were also clustered individually. In addition, the pattern analysis showed a clustering in a hierarchical manner where the control group formed a cluster by itself separated from the exposed groups (Fig. 5B).

**DISCUSSION**

Recent proteomic studies in the environmental monitoring field have aimed to decipher changes in protein expression patterns and to identify proteins governing the mechanism of toxicity of marine environmental xenobiotics. However, alterations in the protein expression profile affect all cell compartments through many diverse biochemical pathways. Several approaches have been followed that deal with the problem of the complexity of the cellular proteome and the difficulty to isolate homogeneous samples for these type of studies (43). In our approach, we tried to circumvent this problem by utilizing fractions highly enriched in peroxisomes. It is recognized that a broad variety of pollutants of industrial, agricultural, and urban origin are potential peroxisome proliferators. Significant variations in peroxisomal proteins of mussels exposed to chemical pollutants and in the peroxisomal proteome of animals collected in a polluted environment have been reported previously (8, 44, 45). In our previous work, we have applied this method in a field experiment where the purification quality and reproducibility of the peroxisome-enriched fraction proteome was assessed (8). To date, few reports have described PES in mussels exposed to individual pollutants (16). In the present study, we applied a relatively new proteomic approach, DIGE (36), for the identification of proteins that are up- or down-regulated in response to the exposure to several model pollutants. The aim of this study was to explore peroxisomal proteomics to identify PESs that could characterize the exposure to a specific pollutant. This could be a starting point for the definition of new biomarkers in biomonitoring programs.

The three European marine mussel species, *M. edulis*, *Mytilus galloprovincialis*, and *Mytilus trossulus*, which are differentially distributed along the European coasts, are widely used as sentinel species in biomonitoring programs. The 2-DE maps obtained from digestive gland peroxisome-enriched fractions of *M. edulis* and the ones of *M. galloprovincialis* obtained in our previous work were comparable (8). The similarity between the 2-DE maps from different mussel species would suggest the potential of this technique because it could be applied to geographical areas where only one of these mussel species grows natively. In addition, it would also help improve protein identification due to the poor representation of mussel proteins in databases (10). On the other hand, the PES was concentrated in the upper half of the 2-DE map. The proteins in the PES did not exhibit low molecular weight or...
extreme pl, indicating that the 2-DE-based proteomic techniques are an appropriate framework for the development of novel biomarkers.

By comparing all 2-DE maps, we obtained a minimal set of proteins with an altered expression profile. This group of proteins defined a minimal PES that was common to the three exposed groups. Nevertheless important differences in the general expression level were observed. In the PBDE-47 group, a marked overexpression was seen, whereas expression was only at a moderate level in the BPA and DAP groups. Therefore, the detection of the presence of any of those pollutants in a field experiment might be assessed based on the minimal PES expression level. Some evidences in favor of this hypothesis arose from a field experiment where a similar strategy was applied (8). First, the PES obtained in both cases showed strong resemblance, although different mussels species were used (M. galloprovincialis and M. edulis). Second, the same biochemical pathways were altered, and lastly, several of the identified proteins such as catalase and SOD were common to both studies. No more coincidences were observed probably because few proteins were identified in both studies. In addition, blind tests are being carried out to confirm this hypothesis.

**Minimal Protein Expression Signature Identification**—Among the identified proteins from the minimal PES, β-tubulin is related to structure and function of the cytoskeleton, which has been proposed to be one of the first targets of oxidative stress (46). α- and β-tubulins form tubulin heterodimers, which get assembled into linear protofilaments and in turn assemble into microtubules. An important function of microtubules is to move around cellular structures such as chromosomes, mitotic spindles, and other organelles inside the cells (47). The effects of phthalates and bisphenols in microtubules have been reported to affect both polymerization and inhibition of microtubule assembly in culture cells (48, 49). The mechanisms of growth inhibition and interference of microtubules by BPA are not well known, but this effect has never been reported in PBDE exposures (50). It has been proposed that a direct actin-small heat shock protein interaction occurs to inhibit actin polymerization and to participate in the in vivo regulation of actin filament dynamics. In such a way, the cytoskeleton is protected against the disruption induced by various stressful conditions (51). In agreement with that, phosphorylation of small heat shock proteins has been described to be causally related to the regulation of microfilament dynamics following oxidative stress, and α-tubulin promotes phosphorylation to prevent the formation of microtubules under oxidative stress (46, 52). Alcohol dehydrogenase was also a part of this minimal PES. This isozyme preferentially catalyzes the conversion of acetaldehyde to acetoacetate, but it also acts on a variety of primary unbranched aliphatic alcohols. The interactions of phthalates with the pharmacological response and metabolic aspects of ethanol in mice have been investigated. Intraperitoneal doses of phthalates have been shown to significantly increase the sleeping time and decrease the activity of alcohol dehydrogenase (53). In yeast, the decrease in alcohol dehydrogenase activity was not a consequence of an increasing degradation rate. This protein is transcriptionally regulated; thus, the decline in protein synthesis was correlated with a 6–10-fold decrease in the amount of functional alcohol dehydrogenase I (54). Finally catalase, the most representative enzyme from the peroxisome, was identified both by ESI-MS/MS and by MALDI-TOF analysis. Catalase activity increases in response to pollutants have been well reported. Three- to 6-fold increases in the enzymatic activity of acyl-CoA oxidase, other peroxisomal oxidases, and catalase have been observed in liver from low molecular weight phthalate ester-fed rats (55). In mussels exposed to phthalates and other pollutants, catalase and glutathione peroxidase increases have been reported. Moreover peroxisome proliferation, measured as an increase of the peroxisomal enzyme acyl-CoA oxidase, has been proposed as a specific biomarker of organic pollutants in mussels (56).

**Protein Expression Signature Common to Groups DAP and BPA**—One of the proteins in the PES common to the groups DAP and BPA turned out to be GST, an enzyme that plays an important role in the detoxification and metabolism of many xenobiotics and endobiotic compounds. The glutathione adducts produced in the detoxification process have an increased solubility in water and are subsequently enzymatically degraded to mercapturates and excreted. Recent studies have shown that several phthalates caused a significant decrease in the GST activity in rats and hamsters (57–59). This inhibitory effect of phthalates in the GST activity permits the modulation of the biological response and toxicity of chemicals that are detoxified or metabolized via GST. It has been suggested that the direct effects of phthalates on xenobiotic-metabolizing enzymes may be independent of the peroxisome proliferation effect and the oxidative stress and be dependent on the structure of the particular chemical compound interacting with the enzyme (57). Our results indicate that exposure to phthalates may also contribute to the detoxification capacities in the metabolism of mussels. The subcellular localization of the putative GST was not possible to determine because neither N-terminal nor C-terminal amino acid sequence was obtained from the ESI-MS/MS analysis. Therefore, the possible peroxisomal localization of this protein in mussels is still an open discussion. It should be emphasized that the analysis of the identified homologous sequence contained a predictable peroxisomal targeting signal type 2 (PTS2). The PTS2 is an N-terminal nonapeptide with a consensus motive (R/K)(L/A)(H/Q)(L/A) quite conserved among peroxisomal proteins but fairly rare (60). In this PES, the observed up-regulation of Mn-SOD and down-regulation of HA01 were two strategies to activate an adaptive response to oxidative stress. Recently it has become evident that contaminant-stimulated reactive oxygen species (ROS) production and resulting oxidative damage may be a mechanism of
toxicity in aquatic organisms exposed to pollution (61). ROS produced in biological systems are detoxified by antioxidant defenses, among them Mn-SOD, which is the primary antioxidant enzyme in the mitochondria of all eukaryotic cells. This enzyme has also been reported in mammalian peroxisomal membranes (62). The increased ROS formation and oxidative damage may also occur not because of direct pro-oxidant properties of the contaminant but rather because of some tissue injury caused by the pollutant that in turn can lead to the release of ROS (63). The more than 2-fold increase of Mn-SOD coincides with previous results where an activation of the antioxidant defense was observed as an effective adaptive response to oxidative stress (64, 65). The peroxisomal HAO1 catalyzes a broad range of 2-hydroxy acid oxidations. In mammals, it is a liver-specific enzyme, and in plants, the enzyme is well studied as a component of the glyoxylate cycle in glyoxysomes (66). The down-regulation of this enzyme in liver has been associated with oxidative injury markers, and there are evidences supporting the role of oxidative stress and ROS in the transcriptional control of this enzyme. Therefore, during oxidative stress when the high catalase content was not sufficient to prevent the release of H2O2 in the cytoplasm, down-regulation of HAO1 expression may represent a mechanism to prevent excessive H2O2 formation. Our results add new evidences that indicate that down-regulation of ROS-producing enzymes may represent a poorly recognized but potentially relevant response to oxidative stress. Finally the toxicological roles of down-regulation of carbonic anhydrase and aldehyde dehydrogenase have not been reported in mussels. However, carbonic anhydrases have been found in all kingdoms of life and play essential roles in facilitating the transport of carbon dioxide and protons in the intracellular space, across biological membranes, and in the layers of the extracellular space. In relation to the aldehyde dehydrogenase subcellular localization, the peroxisome is still an open possibility, especially after the detection of peroxisome-associated aldehyde dehydrogenases in rat liver (67).

Protein Expression Signature Common to Groups PBDE-47 and BPA—The highest up-regulated tendency detected in this study corresponded to the PBDE-47 group. This is especially remarkable after observing the low concentration of PBDE-47 diluted in the water in this laboratory experiment; it only reached 4.6% of the nominal concentration. In general, the metabolism of mussels is not considered important for aromatic substances. In fish, it has been concluded that the uptake of these substances from the gastrointestinal tract may be facilitated by co-transport with lipids and proteins through a mediated active transport mechanism (25). What could be the active metabolism of PBDEs in mussels could begin to be clarified by the identification of the PES obtained in this study. Enoyl-CoA hydratase is a peroxisomal protein from the β-oxidation pathway. The effect of peroxisome proliferators, such as bisphenol, in the β-oxidation of fatty acids is well described as an up-regulated response in mice and rats (68) as well as mussels (45). Another up-regulated protein was phospholipase A2, a key enzyme involved in the release of arachidonic acid from the cell membrane. Its activation in response to peroxisome proliferation as well as that oxidative stress could be a factor for the release of arachidonic acid has been widely reported (69, 70). The subcellular localization in mussels has not been studied; however, there are previous evidences of a mammalian calcium-independent phospholipase A2 from the hepatic peroxisomal compartment. It has been suggested that phospholipase A2 can contribute to the lipid second messenger generation by hydrolysis of peroxisomal arachidonic acid-containing phospholipids (71). An increase in total cytochrome P450 content from the digestive glands in M. edulis has been detected after exposure to PCBs and other xenobiotics, hence supporting the theory of an inducible P450 system (72). Among the down-regulated proteins in this group, a homologous protein to peroxin 10 (PEX10) was also found. PEX10 encodes an integral membrane protein required for peroxisome biogenesis in mammals and yeast. This protein plays an important role in the protein interaction network by connecting the ubiquitin conjugation enzyme PEX4 to other members of the protein import machinery (73). Our results suggest that a decrease in the peroxisomal import might be associated to the mussels’ exposure to pollution. Whether the peroxisome biogenesis is retarded or halted or whether this affects the distribution of peroxisomal matrix proteins after exposure remains to be elucidated.

In summary, the protein reference maps of subfractions of non-sequenced organisms could become an important tool for understanding changes in the proteomes. The higher resolution of the 2-DE maps enhances the detection of novel biomarkers and shows great potential in the assessment of anthropogenic pollution in the marine environment. The protein expression signatures obtained and partially identified provide new information to elucidate possible mechanisms of toxicity of xenobiotics in mussels, which are used worldwide as “sentinels” in environmental monitoring. The identified proteins indicate that the main cause of the changes in the enriched peroxisomal proteome after exposure to those three pollutants was adaptive responses to oxidative stress.

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