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**Research Paper**

# Accumulation of Polychlorinated Biphenyls in Mussels: A Proteomic Study

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## ABSTRACT

Polychlorinated biphenyls (PCBs) are environmental pollutants of industrial origin that can contaminate food, mainly food of animal origin. Although production of PCBs has been banned in many countries since the 1980s, they are still present in the environment and are considered dangerous pollutants for human health. In fact, they can bioaccumulate in living organisms such as marine organisms because of their chemical and physical properties. New analytical approaches are useful to monitor the presence of such contaminants in seafood products and in the environment. In this work, we evaluate changes in protein expression of *Mytilus galloprovincialis* (Lam.) experimentally exposed to a PCB mixture and identify chemically specific protein expression signatures by using a proteomic approach. In particular, we identify 21 proteins whose levels of expression are sensibly modified after 3 weeks of exposure. The present work shows that a proteomic approach can be a useful tool to study alterations of protein expression in mussels exposed to PCBs and represents a first step toward the development of screening protocols to be used for biomonitoring surveys of fishery products.

Key words: Food control; *Mytilus galloprovincialis*; Polychlorinated biphenyls; Proteomics

Fish, shellfish, and crustaceans are important components of the human diet; however, consumers could be exposed to various contaminants through the consumption of seafood products if the latter have accumulated these toxins in polluted waters. Contamination of the marine environment from organochlorine compounds and other organic pollutants is of great concern because of their toxicological properties and the high frequency of detection of their residues in aquatic ecosystems *(49, 55, 56)*. The consumption of fish, shellfish, and fishmeal feeding contaminated with polychlorinated biphenyls (PCBs) represents an important source of accumulation in both humans and food-producing animals *(21, 34)*.

PCBs include a group of 209 different congeners that differ in the number and the position of chlorine atom substituents *(51),* and they are classified as (i) dioxin-like PCB (12 DL-PCB) and (ii) non–dioxin-like PCB (197 NDL-PCB) congeners. Because of their physicochemical properties, such as chemical stability, low heat conductivity, and high dielectric constants, PCBs were widely used in many industrial applications. Recently, the production and the use of PCBs have been banned in the majority of industrialized countries *(14, 15, 49)*. Nevertheless, they are still present in the environment because of their high stability. Furthermore, because of both their lipid solubility and the absence of adequate metabolic pathways in the organisms, PCBs tend to accumulate in fatty tissues, and they biomagnify along the trophic chain *(51, 56)*.

Aquatic invertebrates, such as bivalves, often become reservoirs for many environmental pollutants because of their ability to accumulate trace contaminants from the aquatic environment. Mussels of the genus *Mytilus* are among the most commonly used sentinel organisms for the monitoring of biological effects of various contaminants in the marine environment because of their sedentary lifestyle, expressed filter-feeding activity, capacity to accumulate and tolerate chemicals, and wide geographical distribution *(13, 17, 23, 35, 38, 43)*. Among the molluscs, the edible mussel *Mytilus galloprovincialis* (Lam.) is the most consumed in Italy, and these animals come almost exclusively from mariculture *(29),* which is a branch of aquaculture involving the cultivation of marine organisms in an enclosed section of the sea.

Because the monitoring of all PCB congeners is not possible, surveillance activities target a limited number of congeners, considered as markers of the overall contamination *(21)*. Data on occurrence of NDL-PCBs in different food and environmental samples, provided by the European Food Safety Authority *(22),* have been reported as the sum of six PCB congeners (PCBs 28, 52, 101, 138, 153, and 180 according to the International Union of Pure and Applied

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Chemistry) often referred to as indicator PCBs. These PCBs are the most common NDL-PCB congeners found in food (approximately 50%) and are easily quantified, if compared with the other NDL-PCBs *(21, 22)*. Although there are different sources for the production of dioxins/furans (PCDD/Fs) and PCBs, including DL-PCBs and NDL-PCBs, a correlation between (i) the occurrence of NDL-PCBs and DL-PCBs and (ii) the occurrence of NDL-PCBs and total PCDD/F+DL-PCBs has been verified *(21)*. Maximum levels for PCDD/Fs, DL-PCBs, and NDL-PCBs in foodstuffs have recently been laid down in Commission Regulation (EC) No 1259/2011 *(19)*.

According to Commission Regulation (EC) No 589/2014, the use of screening methods is provided for analysis of PCDD/Fs and DL-PCBs in food *(20)*. A valid screening method should allow the fast analysis of a large number of samples. Moreover, it has to be simple and cheap and sensitive enough to detect PCDD/Fs and DL-PCBs at the level of interest. In this way, suspect samples are identified and undergo further analysis with a confirmatory method. The latter allows the unequivocal identification and quantification of PCDD/Fs and DL-PCBs in a sample, but it is more sophisticated and expensive and requires well-trained operators *(3, 34, 50)*. It usually features gas chromatography–high-resolution mass spectrometry (GC-HRMS) or gas chromatography–tandem mass spectrometry (GC-MS/MS).

Various screening methods have been used for the determination of DL-PCBs in different environmental and food matrixes, such as enzyme-linked immunosorbent assays, chemical-activated luciferase gene expression bioassays, and several types of sensors *(11, 40)*.

In contrast, because screening methods for the determination of NDL-PCBs are not yet widespread, the presence of these contaminants is usually determined by gas chromatographic methods *(20)*.

In this framework, a novel and cheap screening tool for preliminary screening of NDL-PCBs in fishery products may be useful. To fulfill this goal, one of the more innovative and promising techniques is proteomics. In fact, the proteome of living organisms responds even to the most subtle environmental changes *(37)*; therefore, proteomics allows for the measurement and identification of thousands of proteins without any prior assumption on their mechanisms of action *(24)*. Furthermore, a proteomic analysis elucidates the connection between proteins and toxicant exposure that has not been described previously *(16, 28, 31)*. The study of proteome alterations also explains the early molecular events involved in toxic responses, if such study is carried out at toxic concentrations that do not induce significant physiological alterations *(1)*. If applied to environmental toxicology, proteomics may be used to identify chemically specific protein expression signatures (PES). These PES could be used as biomarkers and provide useful molecular descriptions of the state of the cell and of the tissue. Therefore, PES are currently replacing single molecule biomarkers because they may be more robust indicators of stress exposure because of their higher specificity and sensitivity *(39)*. The identification of these PES may overcome the lack of genome information, which is characteristic of many species that are relevant for the environment *(7, 33)*. They have been determined for several aquatic organisms exposed to metals *(46)*, polychlorinated biphenyls *(43, 47)*, polyaromatic hydrocarbons *(30)*, physicochemical agents *(26)*, and even to natural contaminated environments *(44)*.

In this work, we evaluate changes in protein expression on *M. galloprovincialis* exposed to a NDL-PCBs mixture. The goal of the study was to identify PES that could characterize PCB exposure through MS. Further studies could allow us to characterize new biomarkers of early exposure by the identification of key proteins altered by the presence of these contaminants in bivalves. Overall, identified proteins, after validation, may represent a starting point for the development of new tools for surveying PCB levels in fish and fishery products, aimed to protect the health of consumers.

## MATERIALS AND METHODS

**Reagents.** The following reagents for electrophoresis and proteomic analyses were from GE Healthcare (Little Chalfont, Buckinghamshire, UK) and Sigma (St. Louis, MO): agarose ammonium carbonate; ammonium persulfate; analytical standards of PCBs 138, 153, and 180; bromophenol blue, 3-[(3-cholamido-propyl)-dimethylammonio]-propane-sulfonate (CHAPS); Coomassie brilliant blue; dithiothreitol; formic acid; glycerol; Immobiline DryStrip gels; iodoacetamide; β-mercaptoethanol; pharmalyte, pH 3 to 10; reagent of Bradford; sodium dodecyl sulfate; thiourea; trichloroacetic acid; trypsin; 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris); and urea. The following reagents for chemical analyses were from Carlo Erba (Milan, Italy): diethyl ether, anhydrous $Na_2SO_4$, petroleum ether, $H_2SO_4$ (96%), and isooctane. Extrelut NT3 columns were purchased from Merck (Darmstadt, Germany), and Florisil cartridges (1 g) were purchased from Isolute (Uppsala, Sweden). All the reagents were of molecular biology or analytical grade, if not otherwise stated.

**Exposure condition of mussels.** Adult *M. galloprovincialis* mussels (6- to 7-cm valve size) were collected from a mariculture farm in Pozzuoli, Napoli, Italy. The mussels ($n$ = 200) were transported alive immediately to the Pozzuoli Fish Market laboratory and divided into two equal groups: control (C) and exposed (E). Each group was placed in a 100-L tank for acclimation for 7 days at 15°C. The tanks were filled with PCB-free seawater and equipped with a continuous water recirculation system. After acclimation, group E was exposed to the three PCB indicators, PCB138, PCB153, and PCB180, contaminating the aquatic environment at a nonlethal concentration of 30 $\mu g\ L^{-1}$ for each congener for 3 weeks. This was achieved by adding a mixture of these PCB indicators to PCB-free feed. Group C was kept in seawater and fed with PCB-free feed added with the vehicle. To assess the effects of the contaminated environment on protein expression, 20 mussels were selected from each group, before (samples E0 and C0) and after (samples E3 and C3) contamination.

**Sample preparation and extraction of proteins.** The length of the mussels (shell) was measured. Their edible part was collected, completely homogenized, lyophilized, and then frozen at –80°C. The weights of the whole mussels, shells, fresh edible part, and lyophilized edible part were recorded. Proteins were extracted by suspending 30 mg of lyophilized tissue in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, and 3% dithiothreitol) to avoid proteolysis. The mixture was centrifuged at 12,000 × $g$ for 15 min.

Supernatants were either used immediately for electrophoresis or were stored at –80°C. The protein concentration of each extract was determined according to the Bradford method *(8)*.

**Chemical analysis of PCBs.** Homogenate (5 g) was extracted using a shaking machine for 12 h with 20 mL of diethyl ether. The extract was filtered through anhydrous $Na_2SO_4$, dried under nitrogen flow, and dissolved in 2 mL of petroleum ether. A two-step cleanup was performed using a diatomaceous earth solid support (Extrelut NT3, Merck) followed by a solid-phase extraction Florisil cartridge (1 g; Isolute). First, the extract was loaded on an Extrelut NT3 (3 g) column that was previously treated with 3 mL of $H_2SO_4$ (96%). After 20 min at room temperature, the compounds were eluted with 20 mL of petroleum ether, and the extract was cleaned up through a Florisil cartridge. The column was rinsed with petroleum ether (6 mL), and the extract was eluted with petroleum ether (20 mL) and then dried in the bath Rotavapor (Büchi, Assago, Italy) set at 40°C and dissolved in 1 mL of isooctane. The sample was filtered through a membrane of 0.45-μm nylon (Millipore, Billerica, MA) and injected in a gas chromatograph (Autosystem XL, PerkinElmer, Waltham, MA) equipped with an electron capture detector and a 35% phenyl–65% dimethylpolysiloxane–fused silica capillary column (30 m by 0.25 mm by 0.25 μm) *(45)*.

The injection volume of the sample was 0.5 μL, the temperature of the injector was 250°C, and the temperature of the detector was 380°C. Oven temperature was increased from 100 to 250°C at a rate of 15°C $min^{-1}$, from 250 to 300°C at a rate of 5°C $min^{-1}$, and then was held for 1 min at 300°C. PCBs in the sample were identified if their retention time (tR) was tR of standard PCB ± 0.5%. PCB concentration in the test solution was calculated by external calibration with a linear calibration curve constructed with three standard solutions: 1.0, 10.0, and 20.0 ng $g^{-1}$ PCB mixture in isooctane *(45)*.

**High-resolution two-dimensional electrophoresis.** Eighteen-centimeter immobilized pH gradient strips (pH 3 to 10) (GE Healthcare) were passively rehydrated for at least 12 h with 400 μg of protein in 350 μL of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mM dithiothreitol, and 0.5% ampholine, pH 3 to 10). First-dimension isoelectric focusing was carried out at 20°C by using a MultiPhor II system (GE Healthcare). The experiment was started with an applied potential of 500 V for 8 h. The voltage was gradually increased to 10,000 V for 3 h and finally raised to 10,000 V until a value of 80,000 V-h had been achieved. The immobilized pH gradient strips were first soaked for 15 min in an equilibration solution (50 mM Tris-HCl buffer [pH 8.8], 6 M urea, 30% [v/v] glycerol, 2% sodium dodecyl sulfate, and bromophenol blue traces) containing 25 mg·$mL^{-1}$ dithiothreitol and subsequently soaked for 15 min in an equilibration solution containing 45 mg·$mL^{-1}$ iodoacetamide.

Second-dimension separation was carried out at 20°C in 12.5% polyacrylamide gels by using an Ettan Dalt twelve gel tank (GE Healthcare) at a maximum output of 25 W per gel.

**Image acquisition and analysis.** Gels were fixed in 40% ethanol, 10% acetic acid for 3 h, stained in 0.1% Coomassie brilliant blue R-250, and destained in 30% ethanol and 10% acetic acid. Gel images were acquired using the Image Scanner III LabScan 6.0 (GE Healthcare) and analyzed using Image Master 2D Platinum 6.0 software (GE Healthcare) to achieve spot detection, quantification, normalization, and matching.

For each gel, the number of valid protein spots and the number of proteins matched were determined. Moreover, qualitative and quantitative differences in the protein patterns between the E and C groups were assessed.

The volume of protein spots from the E3 and the C3 groups was measured using the Image Master 2D Platinum 6.0 software; the achieved intensities were compared using the same software to perform Student's *t* tests. Spots showing a statistically significant difference (95% confidence level, $P \leq 0.05$) and a fold change $\geq$1.8 underwent further analysis.

**Identification of proteins.** Interesting stained protein spots underwent gel digestion with trypsin; the resulting peptide mixtures were analyzed by high-performance liquid chromatography–nano-electrospray ionization–tandem mass spectrometry (HPLC-ESI-MS/MS) on a quadrupole-time of flight mass spectrometer (Waters, Milford, MA) coupled with pumps and autosampler under the following standard conditions: capillary temperature of 90°C and source voltage of 3.5 kV. Argon was used as collision gas. The digests were separated by reverse-phase LC by using a 3-Å ethylene-bridged hybrid column (0.3 by 100 mm) in a nanoACQUITY LC system. Mobile phase A was 0.1% formic acid in water; mobile phase B was 0.1% formic acid in acetonitrile-water (80:20, v/v). The digest (5 μL) was injected, and the organic content of the mobile phase was increased linearly from 5% B to 40% in 60 min. In the survey scan, MS spectra were acquired for 0.5 s in the mass-to-charge (*m*/*z*) range between 500 and 2,000. The most intense peptide ions were sequenced. The collision-induced dissociation energy was set according to the (*m*/*z*) ratio and charge state of the precursor ion. Raw data MS/MS spectra were converted in PKL format with the ProteoLynxs data analysis software (Waters). Subsequent protein identification was carried out against the National Center for Biotechnology Information nonredundant protein database through the MS search algorithm on the Mascot search engine. Search parameters were set as follow: MS tolerance, 50 ppm; MS/MS tolerance, 0.25 Da; fixed modifications enzyme specificity, trypsin; one missed cleavage permitted; fixed modification, carbamidomethylation of cysteine; variable modification, methionine oxidation; and significance threshold, $P < 0.05$ and score $>50$. The taxonomy was limited to other metazoan species. The MS/MS spectra were used to perform a homology search in the National Center for Biotechnology Information database with the Basic Local Alignment Tool (BLAST) code *(4)*.

## RESULTS

Mussels belonging to group E were exposed to a PCB mixture for 3 weeks. Then, protein expression profiles of mussels belonging to group E and group C were analyzed and compared. Moreover, a complete comparative physical analysis was performed, showing for both groups (i) normal distributions of shell lengths, (ii) similar weights for both shell and homogenized edible parts, (iii) regular liquid inside half shells, and (iv) closed valves. The rate of mortality for both groups during the experiment was less than 5%.

A quantitative chemical analysis of the extracts obtained from group E mussels was performed to assess the concentration of PCBs. For this purpose, a novel validated method was adopted *(45)*. The measured concentrations in exposed mussel tissues were 76.41 ng $g^{-1}$ for PCB 138, 80.42 ng $g^{-1}$ for PCB 153, and 50.86 ng $g^{-1}$ for PCB 180. These results confirmed uptake of the three PCBs. Furthermore, these concentrations are comparable to the maximum allowed concentration listed in Commission

Regulation (EC) No 1259/2011 *(19)*. We calculated the bioconcentration factor (BCF) of each PCB ($BCF_{PCB\ 138}$ = 2.5; $BCF_{PCB\ 153}$ = 2.7; $BCF_{PCB\ 180}$ = 1.7) through the following equation: BCF = organism concentration/environment concentration. These values indicated that time of exposure and contamination through feed were efficient.

A classical two-dimensional difference gel electrophoresis based on a proteomic approach was performed to obtain a proteomic map of each sample *(25)*. Protein concentration into the different extracts was measured and ranged from 18 to 24 μg $\mu L^{-1}$. The quality of the achieved gels was determined considering the following set of parameters: resolution, definition, homogeneous distribution, morphology and clarity of the spots, minimum background, streaks or veined bands, and clear separation of proteins. Spots were almost homogeneously distributed across the entire isoelectric point range. Moreover, they were clear and well defined and had morphologies ranging from circular to oval. A few clusters formed by high-molecular-weight proteins were observed. In this regard, poorly defined regions, such as those including (i) spots at the boundaries of gels, (ii) overlapping proteins, and (iii) areas containing aggregates, were discarded. The number of spots detected on gels varied between 963 and 1,195 because of our choice to count only clearly defined spots. Proteins with a molecular mass >200 kDa could not be observed because of the limited capacity of large proteins to be introduced into the gel of first dimension *(52)*.

A detailed comparison between protein maps acquired for different samples was carried out, taking into account the volume of the corresponding spots (Fig. 1).

First, no statistically significant differences were observed for the proteomic profiles of the C0 and E0 groups; therefore, a comparison between the proteomic profiles of E3 and C3 was performed. According to our threshold, differences ($P < 0.05$) in the volume of 30 spots were observed. In particular, upon exposure, 8 spots showed an increased volume and 22 spots featured a smaller volume.

These spots were excised from the stained gels and underwent trypsin digestion. The resulting peptide mixtures were analyzed through HRMS. Twenty-one proteins were identified from the investigated spots (5 upregulated and 16 down-regulated) (Table 1 and Fig. 2). Notwithstanding the good quality of the MS data, other proteins were not identified. Indeed, because *M. galloprovincialis* is a non-model organism, databases do not include most of the sequences of the proteins expressed by this organism.

The proteins with modified levels of expression, upon exposure of the mussels, are mainly involved in the regulation and the maintenance of cell morphology *(36, 43)* (β-actin, raminin receptor, rootletin-like protein, gelsolin, collagen α-1(XII) chain, tropomyosin, paramyosin, elongation factor 1-β, myosin regulatory light chain A, myosinase-I) or in energy metabolism *(18)* (electron transfer flavoprotein subunit α, voltage-dependent anion channel 2, enolase, EP protein, cathepsin L, malate dehydrogenase, guanine nucleotide–binding protein). Furthermore, the identification of many proteins associated with cell survival and stress response (glutathione *S*-transferase, proliferating cell nuclear antigen, 14-3-3 protein, Rho GDP dissociation

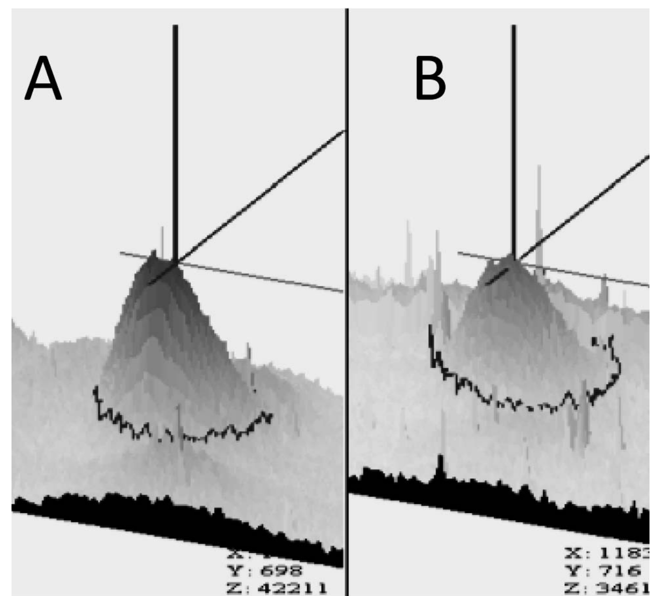


FIGURE 1. *Comparison between volume of spot 1117 in the two-dimensional gel of sample C3 (A) and sample E3 (B).*

inhibitor) was significantly affected by the exposure to PCBs. All the variations of volume value fall in the same range (Table 1 and Fig. 3) and variations higher than fivefold have not been recorded, regardless of the absolute abundance of the protein.

## DISCUSSION

PCBs are toxic compounds that can contaminate the environment (e.g., seawater and soil), and food, particularly products of animal origin. Because of their liposolubility and stability, PCBs tend to bioaccumulate along trophic chains where they are stored in fatty tissue. Therefore, their presence in food and environmental matrices has been monitored for many years in European countries. Among these matrices, fish products including mussels are subjected to analytical control for the presence of PCBs. Mussel is the common name used for members of several families of bivalve molluscs from saltwater and freshwater habitats. Some species of mussels are edible; in fact, humans have used mussels as food for thousands of years. One of the most commonly consumed species is *M. galloprovincialis*. It is widespread in European countries, especially in costal areas of France, Belgium, Holland, and Italy, particularly in the southern regions *(29)*.

Moreover, *M. galloprovincialis* was chosen as a bioindicator of marine pollution because it fulfills most of the criteria required for an acceptable bioindicator *(2, 32)*. In fact, it is sedentary, widespread, easy to collect, and able to accumulate large concentrations of pollutants. Furthermore, its life cycle is long enough to accumulate contaminants, and it possesses amounts of tissue that are sufficient for chemical analysis.

In the present work, we used *M. galloprovincialis* as an aquatic model by exposing it to a mixture of three NDL-PCBs to evaluate changes in protein expression. We chose three indicators considered as markers of the overall

TABLE 1. *Proteins identified by LC-MS in the gel spot whose intensity in sample E3 was significantly different from that observed in sample C3 (see Fig. 3)*

| Spot | National Center for Biotechnology Information accession | Identification | Organism | Level change ratio (E3/C3) |
|---|---|---|---|---|
| 677 | gi\|14161517 | Enolase | *Cryphalus abietis* | 2.3 |
| 700 | gi\|58306751 | Collagen α-1(XII) chain-like | *Anolis carolinensis* | 0.5 |
| 746 | gi\|72089178 | Gelsolin | *Strongylocentrotus purpuratus* | 0.4 |
| 790 | gi\|33469507 | β-Actin | *Euprymna scolopes* | 0.4 |
| 921 | gi\|126697324 | Raminin receptor | *Haliotis discus discus* | 1.8 |
| 950 | gi\|73656269 | Cytosolic malate dehydrogenase | *Mytilus trossulus* | 0.3 |
| 999 | gi\|34304719 | EP protein precursor | *Mytilus edulis* | 0.3 |
| 1004 | gi\|121014 | Guanine nucleotide-binding protein subunit β | *Euprymna scolopes* | 0.3 |
| 1030 | gi\|212815279 | Tropomyosin | *Mytilus galloprovincialis* | 0.4 |
| 1050 | gi\|145895072 | Proliferating cell nuclear antigen | *Litopenaeus vannamei* | 0.2 |
| 1051 | gi\|58307490 | Electron transfer flavoprotein subunit α | *Megachile rotundata* | 0.5 |
| 1081 | gi\|310706696 | 14-3-3 protein | *Chlamys farreri* | 0.4 |
| 1101 | gi\|145883962 | Cathepsin L | *Pinctada fucata* | 0.5 |
| 1113 | gi\|145896447 | Voltage-dependent anion channel 2 | *Haliotis diversicolor* | 0.5 |
| 1116 | gi\|58306972 | Myosinase-I | *Todarodes pacificus* | 0.4 |
| 1117 | gi\|42559342 | Paramyosin | *Mytilus galloprovincialis* | 0.4 |
| 1129 | gi\|291239961 | Rootletin-like | *Saccoglossus kowalevskii* | 4.0 |
| 1135 | gi\|223027747 | Elongation factor 1-β | *Danio rerio* | 1.8 |
| 1178 | gi\|22094809 | Glutathione *S*-transferase | *Mytilus galloprovincialis* | 3.5 |
| 1198 | gi\|149382257 | Rho GDP dissociation inhibitor | *Schistocerca gregaria* | 0.5 |
| 1359 | gi\|127163 | Myosin regulatory light chain A | *Placopecten magellanicus* | 0.4 |

contamination *(21),* and we used proteomic technology because of its ability to assess biochemical changes at the physiological conditions of the organisms.

Even if proteomic studies are still limited in the food control and ecotoxicological fields *(42),* some marine organisms, such as fish and molluscs, have already been used for proteomic investigations *(12, 35, 41).*

In the present study, we were able to identify 21 different proteins whose expression levels were significantly modified after prolonged exposure of *M. galloprovincialis* to a mixture of three NDL-PCBs. Our results indicated that exposure had a mainly down-regulating effect on the expression of protein, probably reflecting the potent inhibitory action of PCBs toward several biotransforming and detoxifying cellular systems *(5)*. Interestingly,

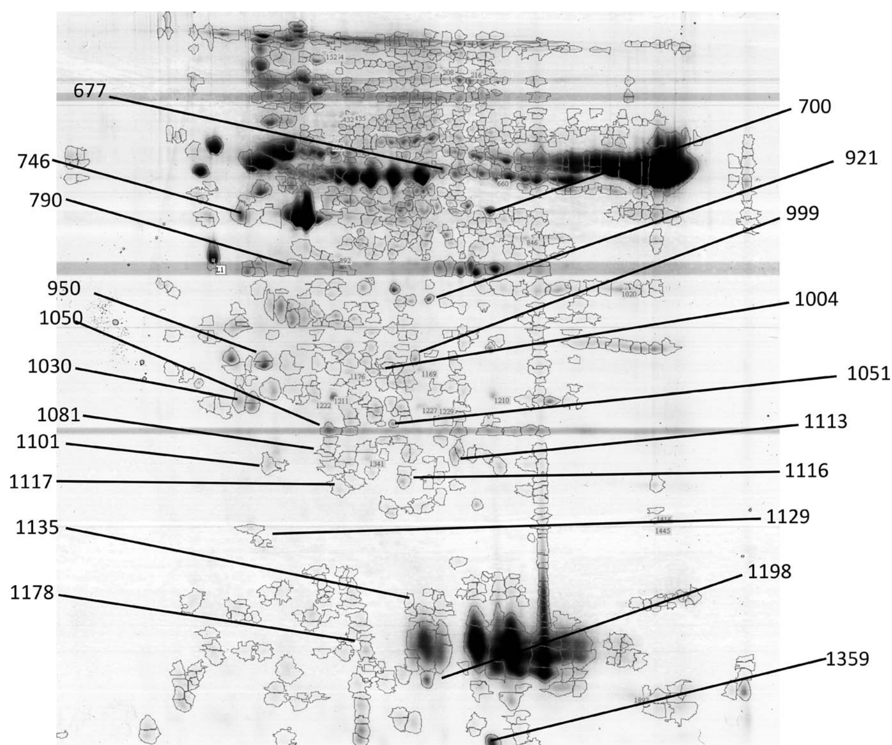


FIGURE 2. *Gel spots whose intensity in sample E3 were different from that observed in sample C3 that were successfully identified by LC-MS analysis (see Table 1).*

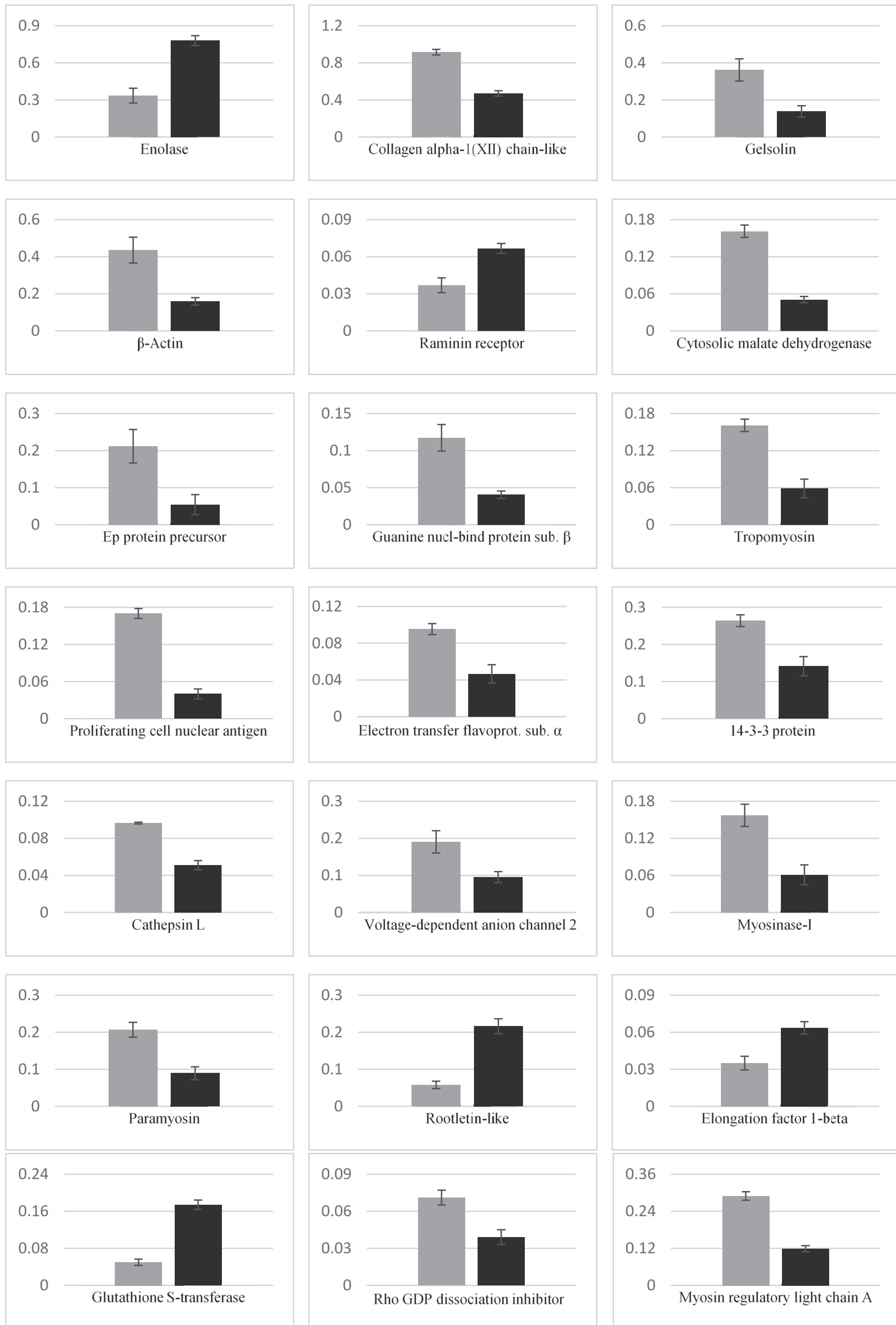


FIGURE 3. *Comparison of spot volume of proteins between C3 (grey bar) and E3 (black bar) samples.*

some of these proteins were found to be affected by exposure to specific pollutants also in different species. For example, it is well known that the expression levels and the activity of glutathione *S*-transferase significantly change because of environmental contamination by different toxic agents *(9, 48, 53, 54)*. Moreover, cathepsin L RNA levels have been demonstrated to be affected by PCB contamination in different marine species *(10)*. At variance, other proteins have never been suggested as possible exposure biomarkers, indicating that they could be selectively affected by the exposure to PCBs. The identification of both novel and well-known biomarker proteins indicates that the results presented in this work represent a promising starting point to define new PES for the exposure of marine organisms to PCBs.

Because different classes of contaminants are present in the environment, further experimental characterization is required to investigate the specificity of this approach that may also be used in conjunction with other analytical techniques. Nevertheless, protein patterns, composed by more than 10 different protein spots, have already enabled distinguishing organisms grown in unpolluted and polluted areas, thus proving the effectiveness of PES determination *(6)*. In fact, PES are currently replacing single molecule biomarkers because they may be a more robust indicator of stress exposure because of their higher specificity and sensitivity to mixed pollutants *(39)*. Because this approach involves the simultaneous measurements of changes in hundreds of proteins, it provides multiple endpoints. A multi-endpoint analysis is robust against external factors, such as age, season, or abiotic factors, other than the given stressor *(28)*. PES have been determined for aquatic organisms exposed to several pollutants *(30, 43, 46, 47)*, and the proteins that compose them were different for each case, showing the specificity of this approach.

The proteins with altered expression profiles identified in our study are related to the structure and function of cytoskeleton, which has been proposed as one of the first targets of oxidative stress *(36, 43)*. Cytoskeletal proteins are related to plasma membrane through which pollutants enter the cell. Therefore, our results suggest membrane labilization as a major cellular biomarker of environmental pollution *(27)*. However, PCB exposure also affected other biological processes, such as the general stress response and energy metabolism. Disruption of energy metabolism has been associated with exposure to xenobiotics *(18)*. To date, toxicological studies of the responses of metabolic enzyme activities to chlorinated compounds remain limited. Nevertheless, the exposure of some common aquatic species to different pollutants led to the down-regulation of genes encoding proteins that were mainly involved in energy metabolism and oxidative phosphorylation *(18)*. Overall, the identified proteins, after an adequate procedure of validation, could be used for the development of a screening method for the analysis of NDL-PCBs in fishery products.

In conclusion, the present results provide further evidence on the suitability of the proteomic approach in toxicology and food control. A challenge of proteomics is to correlate biological response with environmental quality conditions, and this work suggest that proteomic analysis can be used to identify species PES in response to pollutants. Therefore, this approach can be considered a valuable and promising tool for biomonitoring surveys of marine pollution and chemicals accumulation in mussels and other animals.

The identification of the obtained PES can represent a starting point in the search of new specific molecular biomarkers and may serve as the basis for future investigations aimed to further characterize and develop tools for the rapid screening of chemical contamination in *Mytilus* spp. as well as other fishery products during surveys and monitoring programs. Moreover, this method could enable elucidation of possible mechanisms of toxicity of xenobiotics in mussels, organisms that are used worldwide as sentinels in environmental monitoring.

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