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"A chemical-toxicological study of animal models exposed to organohalogen environmental contaminants"

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LIST OF ABBREVIATIONS

2-DE	Two-dimensional electrophoresis
AhR	Aryl hydrocarbon receptor
ANOVA	Aalysis of variance
ATSDR	Agency for toxic substances and disease registry
BLAST	Basic local alignment tool
BCF	Bioconcentration factor
BSA	Bovine serum albumin
CA	Carrier ampholyte
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CID	Collision-induced dissociation
DIGE	Difference gel electrophoresis
DL-PCB	Dioxin-like polychlorinated biphenyl
DTT	Dithiotreitol
EC	European Commission
EEC	European Economic Community
ESI	Electrospray
EFSA	European Food Safety Authority
FDR	False discovery rate
FT-ICR	Fourier transform ion cyclotron
GC-ECD	Gas chromatography-electron capture detector
ICAT	Isotope-coded affinity tag
IEF	Isoelectric focusing
IPG	Immobilised pH gradient
iTRAQ	Isobaric tags for relative and absolute quantitation

IZS	Istituto Zooprofilattico Sperimentale
MALDI	Matrix-assisted laser desorption ionization
MoA	Mode of action
MS/MS	Tandem mass spectrometry
NCBI	National center for biotechnology information
NDL-PCB	Non-dioxin-like polychlorinated biphenyl
PCA	Principal component analysis
PCB	Polychlorinated biphenyl
PCDD/F	Polychlorodibenzodioxin/furan
PES	Protein expression signatures
PFF	Peptide fragment fingerprinting
pI	Isoelectric point
PLSR	Principal least squares regression
PMF	Peptide mass fingerprinti
POP	Persistent organic pollutant
Q-TOF	Quadrupole time-of-flight tandem mass analyzer
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SILAC	Stable isotope labeling by amino acids in cell culture
SLE	Solid supported liquid/liquid extraction
TCA	Trichloroacetic acid
TMT	Tandem mass tags
WHO	World Health Organization

Abstract

Sensitive effect determination, the understanding of molecular toxicity mechanisms and the discovery of novel biochemical biomarkers are some of the major challenges in ecotoxicology in dealing with chemicals in the environment. Among several 'omics' tools, proteomic approaches are used to study the whole proteome of organisms and may provide novel insights into the functional molecular state of a biological system and for discovery of new sensitive biomarkers indicating exposure or effects at low toxicant concentrations.

In this study, a proteomic approach has been used in *Mytilus galloprovincialis* as a screening of changes in protein expression caused by a mixture of polychlorinated biphenyls (PCBs), in order to characterize the effects of these environmental contaminants on protein profile and to develop new molecular biomarkers through identification of more drastically altered proteins.

To achieve this objective, 100 mussels were exposed to PCB 138, 153 and 180 for 3 weeks under controlled conditions at the concentration of 30µg/l. An equal number of mussels was kept under the same conditions, but not treated, as control. The edible parts were homogenized and lyophilized. Extracted proteins were quantified and separated by two-dimensional electrophoresis (2-DE). It has been made a comparative study of two-dimensional electrophoresis gels obtained from proteomic analysis and the changes in protein expression were assessed by image analysis. Image analysis included spot detection, quantification, normalization and matching. On average more than 1000 spots were resolved and altered expression was

qualitatively detected. Stained protein spots of interest were excised from preparative gels and their tryptic digests were subjected to protein identification by mass spectrometry. It was used Matrix Science Mascot search engine, database NCBI and for a homology search the program BLAST.

Protein identification using mass spectrometry becomes a challenge when the proteins are from an organism whose genome is not yet sequenced, as is the case of the genus *Mytilus*. However, the identification of 36 proteins of 71 studied proteins was achieved directly with *Mytilus* spp. or with other invertebrate species.

Our results indicate that exposure of the mussel *M. Galloprovincialis* to PCBs had a mainly down regulating effect on protein expression level. Significantly, differentially expressed proteins, identified in the present work, turned out to be related mainly to structure/function of cytoskeleton, which has been proposed as one of the first targets of oxidative stress.

Hence, it was concluded that a toxicoproteomics approach in *Mytilus Galloprovincialis* enables the detection of substance specific effects and more general stress responses in the cellular protein pattern and allows the identification of candidate protein biomarkers.

With this work we demonstrated the high degree of sensitivity of proteomic approach and its utility in toxicological studies, as we have shown that protein expression varies significantly between examined group identifing specific PES in response to pollutants. This proteomics approach can be considered to be a valuable and promising tool for the development of environmental research.

Chapter I

1 Introduction

1.1 Ecotoxicology

Ecotoxicology is the study of the interactions between living organisms, their ecosystems and stressors (Lemos et al. 2010). This is a multidisciplinary field, which integrates toxicology and ecology. These two disciplines have largely evolved as separate sciences over the past century with unique journals, unique tools and a distinct jargon that has reinforced an allopatric evolution of ideas: while ecologists have focused on how biotic and abiotic factors affect species distribution and species interactions, toxicologists have traditionally focused on single-species toxicity tests (Relyea and Hoverman 2006). The rise of ecotoxicology, also termed “environmental toxicology", is generally associated with the 1960s and the first formal definition came from Truhaut who considered ecotoxicology to be the branch of toxicology concerned with the effects of pollutants on the constituents of an ecosystem in an integrated context (Truhaut 1977). A number of variants on this definition have appeared over the years, but all of them embrace much of Truhaut’s original concept (Newman 1998).

The ultimate goal of ecotoxicology is to understand the effects of chemical toxicants, or other biotic and abiotic stressors such as temperature, UV light, predation, etc., on ecologically relevant species in order to predict the effects of pollution, so that the most efficient and effective action to prevent or remediate any detrimental effect can be identified. Classically, this has been carried out by evaluating the effects of such stressors at the physiologic, behavioural or community levels. Effects on survival and

growth, molting, behavior, feeding rates, reproduction parameters, sex ratio (namely for endocrine disruptor compounds), shell morphology, morphological abnormalities and many others have been broadly used as endpoints for the effects of exogenous compounds on a wide variety of organisms (Levy et al. 2004; Pestana et al. 2007, 2009; Drobne et al. 2008; Loureiro et al. 2009).

1.2 Application of omics technologies in ecotoxicology

Ecotoxicological studies are related to the result of interactions at many levels: genes, proteins, biochemical reactions, exposure routes, contaminants, trophic level, habitat, and eco system. Classic ecotoxicology has focused upon a bottom-up approach to understand stressor effects in which a few genes, proteins, or biochemical reactions are studied. Top-down approaches have quickly evolved since technology has developed the ability for rapid and broad characterization of many levels of biology through genome sequencing, transcriptome sequencing, whole genome arrays, proteomics, and metabolomics. These omics technologies (Figure 1.1) have resulted in whole systems approaches in the field of systems biology (Garcia-Reyero and Perkins 2011).

Rapid progress of omics technologies during the last years has revolutionized biology, providing a clear increase of knowledge and development of new methods for measuring families of cellular molecules, such as genes, mRNA, proteins and intermediary metabolites. During the last decade omic technologies (genomics, transcriptomics, proteomics and

metabolomics) have been widely implemented in the field of farm animals with a very positive impact in areas such as aquaculture (Rodrigues et al. 2012). Genomics, proteomics and metabolomics are complementary approaches that all contribute to unique information.

These technologies allow researchers to more deeply investigate and better understand how pollutants cause toxicity, as well as the cause of adverse effects. The focus is now on the evaluation of the effects of contaminants by measuring the unbalance of specific gene, protein or metabolita expressions in exposed organisms

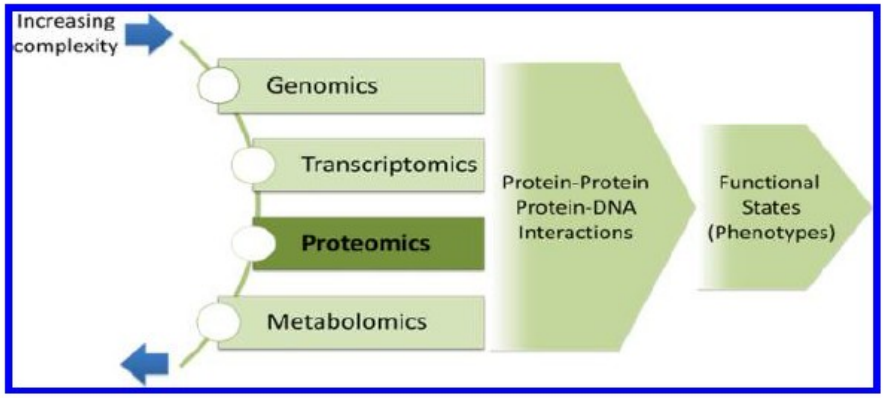


Figure 1.1 Overview of "omics" related research approaches. Proteomics is one of the most complex fields of science, as it represents the interface between genotypic and phenotypic variation, and it provides broad information regarding eco-physiological responses and adaptations, including important functional information related to biosynthesis and natural products expression.

Genomics refers to the study of the genome of an organism, either at the DNA or at the mRNA (transcriptomics) level. Recent advances in human

and molecular genetics provide an opportunity to understand how genes and genetic changes interact with environment (Schwartz et al. 2004). The fields of environmental genomics has enormous potential to affect our ability to accurately assess the risk of developing disease, identify and undestand basic pathogenic mechanisms that are critical to disease progression (Niederreither et al. 2002).

Gene expression analysis has been much less employed in ecotoxicology than in mammalian toxicology, mainly because genome and transcriptome sequencing for ecologically relevant aquatic species is still in its early stages. Only a few aquatic species, such as the green spotted puffer fish (*Tetraodon nigroviridis)*, the zebrafish (*Danio rerio*), and the water flea (*Daphnia pulex*), have completely sequenced genomes (Larkin et al. 2007; Garcia-Reyero et al. 2009).

While sequence information for ecologically relevant species has become more accessible, functional annotation of genes in non-model species remains a challenge, not only due to the complexity of the aquatic animals analyzed but also to the complexity of the chemicals and mixtures to which they are exposed. Functional annotations, essential to transcriptome analysis, identify genes with known functional characteristics such as molecular function, pathways, or biological processes. It can help identify what genes are responsive to a toxicant by grouping the changes in multiple genes associated with specific pathways and function (Tarca et al. 2009; Rawat et al. 2010) leading to do hypotheses regarding molecular mechanisms underlying potential adverse effects (Hook et al. 2006; Martyniuk et al. 2007).

Metabolomics is the analysis of the complete metabolome (all the metabolites that one organism produces) at one moment (Fiehn 2002), so that

it can be considered as the systematic study of the unique fingerprints that specific cellular processes leave behind (Sardans et al. 2011). Recent rapid improvements in analytical methods and in the ability of computer hardware and software to interpret and visualize large data sets have multiplied the possibilities of rapidly identifying and simultaneously quantifying an increasing number of compounds (e.g., carbohydrates, amino acids and peptides, lipids, phenolics and terpenoids).

The use of metabolomics to characterize the interactions of organisms with their environment has also been increasingly applied in ecotoxicology Therefore ecometabolomics, aimed to analyze the metabolome, the total number of metabolites and its shifts in response to environmental changes, is gaining importance in ecological studies (Michaud and Delinger 2007; Viant 2007; Bundy et al. 2008). Ecological metabolomics can thus serve as a powerful indication for defining organism lifestyle. It achieves a dynamic view of the metabolism and health of an organism, a population or an ecosystem. Even if metabolomics is not yet as well established as genomics in the ecotoxicological field, it might be more useful due to the limited number of possible metabolites, eliminating the need for a sequenced genome.

1.3 Ecotoxiproteomics (Environmental proteomics)

Proteomics is the study of the entire complement of proteins (including protein variants and posttranslational modifications such as phosphorylation, ubiquitination, methylation, or acetylation) also known as proteome (Wilkins et al. 1996), produced by an organism or system.

Post-translational modifications, that have critical effects on their function, and protein turn-over imply that the cellular phenotype may differ considerably from the one predicted by the analysis of the transcriptome (Barret et al. 2005). In fact the transcriptome does not account for the posttranscriptional and post-translational regulation of protein expression. Over 300 different types of protein modifications have been described in the literature and it has been reported that on average every eukaryote protein has eight to ten post-translational variants (Garavelli 2004). An added complexity is that many genes can generate different proteins or protein isoforms through alternative splicing. For example, some genes, such as neurexins, can have more than 1000 protein isoforms (Belle et al. 2006).

The proteome is more complex and larger than the genome. Whereas the genome of an organism is apparently stable throughout life, with few exceptions (Abdel-Rahman 2008), the proteome is highly dynamic and continuously changes as a response to numerous intra and extracellular signaling (Barrett et al. 2005).

Proteomics is a very challenging but promising technology because it can give a very good understanding of what functional components are currently present in an organism so that it can provide relevant information of an organism physiological state that is missed by the transcriptome.

It is recognized that all living organisms respond to even the most subtle environmental changes through alterations in the expression of multiple proteins (Monsinjon and Knigge 2007). Since deleterious effects on aquatic and terrestrial wildlife have been reported worldwide (Depledge and Billinghurst 1999; Guillette 2000), novel approaches based on monitoring effects rather than detecting and quantifying pollutants are needed. In the

toxicological sciences, the possibility to assess the proteome is a gigantic step since the proteomic profiles embody the link between effects at both the molecular level and the whole organism level, given that proteins are the first functional stage directly or indirectly affected by toxicants (Kovacevic et al. 2009).

Due to their high complexity, proteomic-based studies are still limited in the ecotoxicological field. Therefore, more and more researchers in the ecotoxicological world are directing their efforts towards proteomic studies and have demonstrated the utility of proteomics in ecological species. Recently, environmental proteomics has been applied to laboratory and field studies in marine organisms, such as fish and molluscs (Bradley et al. 2002; Rodriguez- Ortega et al. 2003; Knigge et al. 2004).

Interpretation of proteomic data require availability of information on genomic DNA and expressed RNA, so a major limiting factor in marine species proteomics is still the lack of information at the genome level (Rodrigues et al. 2012). As a result, protomic studies on aquatic species still face some challenges at the level of protein identification.

Applied to environmental toxicology, proteomics may be used to identify chemical-specific protein expression signatures (PES) that in some cases provide useful molecular descriptions of the cell or tissue state. Since this approach involves measuring changes in hundreds of proteins simultaneously 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 (Gomiero et. al 2006).

Proteomic methodologies can be used to unravel mechanisms underlying toxicological effects of stressors helping to discover unexpected

relationships and protein responses, which by itself can lead to the development of new hypothesis, and also to identify new candidate biomarkers (Bosworth et al. 2005; De Souza et al. 2009). The possibility of measuring and identifying thousands of proteins without any prior assumption on the biomarker or mechanisms of action as well as revealing associations between proteins and toxicant exposure that have not been described earlier is indubitably the major advantage of proteomics (Frohlich et al. 2009).

Therefore, ecotoxicoproteomics allows the identification of the molecular events that are involved in toxicant responses and that are responsible for the adverse effects observed at higher levels of biological organization, earlier in time and at lower environmental stressor concentration.

1.3.1 Identification of the mode of action

Ecotoxicoproteomics is a powerful tool to generate hypotheses on the mode of action (MoA) of stressors and also to determine which components affect the survival, growth and reproduction of a given species, which may ultimately impact populations and communities (Heckmann et al. 2008). Elucidating the effects of these stressors on the molecular mechanism of the cell allows a deeper understanding of known events that happen at both the cellular and the organismal levels of organization and that were predicted by traditional toxicological approaches (Iguchi et al. 2007).

The main difference between traditional experimental biology coupled to biochemical analysis and proteomics is that the latter is not hypothesis-driven (Lemos et al. 2010). When conventional methods of toxicology are not

sufficiently sensitive, the study of proteome alterations at toxicant concentrations that do not induce significant physiological alterations can help to explain the early molecular events involved in toxicant responses (Aardema and MacGregor 2002). Proteomics not only allows validating previous genomic data but also has unravelled new pathways of toxicity never described before (Silvestre et al. 2006).

The molecular interactions of the compounds within a cell or organism are very complex so determining the complete mechanism of a stressor in environmental relevant organisms using proteomics has not yet been fully attained. The presence in the genome of genes with overlapping functions and the presence of metabolic networks, in which the loss of function of one or more enzymes is compensated by alternative metabolic pathways, increases complexity of interactions and represents great difficulty in data interpretation. Nevertheless, strong efforts have been made and alterations of cellular proteins expression induced by toxicants have been reported and linked to (partial) MoA of stressors, especially for endocrine disruptor compounds (Lemos et al. 2009) and for cadmium and other metal ions (Poynton et al. 2007; Burger 2008).

1.3.2 Biomarkers and proteomics

The scientific community has become increasingly concerned about the potential adverse effects to humans and wildlife resulting from environmental exposure to persistant industrial, pharmaceutical and natural chemicals with toxic properties such as estrogenic, androgenic or thyroid-dierupting

preperties. Since deleterious effects of environmental contaminants on both aquatic and terrestrial wildlife have been reported worldwide, novel approaches based on monitoring effects rather than detecting and quantifying pollutants are needed.

One of the goals of ecotoxicology is the identification of molecules that place in evidence the exposure of organisms to a stressor. The use of biological markers or biomarkers has been proposed as a sensitive early warning tool for biological effect measurement in environmental quality assessment (Cajarville et al. 2000).

A biomarker is a measurable indicator of a specific cellular, biochemical or molecular parameter that show whether a key organism has been exposed to a stressor (van Ommen et al. 2009). A high-quality biomarker should be inducible or repressible, the measured response should be specific to chemicals, the response should have sufficient sensitivity for routine detection, the biomarker should be highly accurate and reproducible among experiments within a laboratory and among different laboratories and the biomarker should be quantifiable so that degree of risk can be estimated (Benninghoff 2007).

A variety of molecules, such as hormones (Zaccaroni et al. 2009), enzymes such as cytocrome P450, cholinesterase, catalase, glutathione S-transferase (Menezes et al. 2006; Howcroft et al. 2009) and other proteins such as vitelogenin, Hsp, etc and even organelles (Ortiz-Zarragoita and Cajaraville 2006) have been selected as biomarkers of specific chemicals or classes of chemicals (Porte et al. 2006). The use of conventional pollution biomarkers requires a previous and deep knowledge of the processes underlying the response, which does not necessarily imply that the whole molecular mechanisms responsible for these responses are entirely understood. In fact the

interaction of a target molecule with a given xenobiotic may be well characterized and yet the downstream harmful effects still remain enigmatic.

During the last decades, a considerable effort has been made in discovering, quantifying, verifying and validating biomarkers. However, new and better biomarkers are urgently needed to improve health and pollution diagnosis. The use of ecotoxicoproteomics to biomarker discovery can be widely applied to environmental exposure to pollulants. This process aims at identifying proteins being up and down-regulated in association with a specific biological state (Kussmann and Affolter 2009).

Measurements of single molecules such as gene transcripts, proteins or other metabolites are controversial procedures since these molecules may not fulfil all the requirements that a robust biomarker should possess. Limitation of most single-molecule biomarkers is the lack of sensitivity to mixed pollutants, making risk assessment analysis related to chemicals much more uncertain. Therefore, these are currently being substituted by the use of biomarker patterns, called protein expression signatures (PES), which potentially are more specific and sensitive and therefore a more robust indicator of stress exposure (Poynton et al. 2008). In this way protein analysis may provide a specific "fingerprint" directly associated with either exposure to or effect of certain classes of chemicals. Protein patterns, composed by more than ten different protein spots, allowed distinguishing organisms from clean and polluted areas demonstrating the usefulness of PES determination (Amelina et al. 2007).

The identification of these minimal PES may overcome the lack of genome information, characteristic of many environmental relevant species (Apraiz and Cristobal 2006; Magi et al. 2008). Variations in specific protein

expression signature are commonly used as indicators of pollutant exposure and have been determined for several environmental relevant organisms exposed to metals (Shepard and Bradley 2000) polychlorinated biphenyl (Shepard et al. 2000; Rodriguez-Ortega 2003), polyaromatic hydrocarbons (Knigge et al. 2004), physico-chemical parameters (Gardestrom et al. 2007) and even to natural contaminated environments (Romero-Ruiz et al. 2006).

1.4 Aquatic animal models

Aquatic animal models have been long used in biomedical research and many of them are directly relevant to understand physiology, genetics, anatomy and pathology of human disease processes. Several fish species have been used as a model in areas like cancer, neurology, toxicology, infectious diseases and drug development (Amatruda et al. 2008). Despite their relevance, the physiology of only a low number of fish species has been addressed more or less extensively (Forné et al. 2010). These are basically some species of interest in aquaculture, and other small laboratory species established as experimental models for studies in developmental biology, genetics and environmental toxicology.

Several approaches have been used to study these fish at the molecular level, including biochemistry, molecular biology and more recently omics technologies. Proteomics has emerged as a powerful tool for the study of biological systems and their dynamics in different conditions, and therefore this technology has been increasingly used during the last years to address

different questions related to biology of fish species, specially of those with commercial interest (Pineiro et al. 2003).

Environmental proteomics has been focused mostly on vertebrates, such as the rainbow trout, zebrafish, hake and other aquatic vertebrates (Wang et al. 2007; Ling et al. 2009; Martyniuk et al. 2009).

Undoubtedly, the freshwater teleost zebrafish (*Danio rerio*) has been selected as the main model-fish organism. Small size, good availability, low cost maintenance, genome sequence coverage and an available large-scale proteome profile (De Souza et al. 2009) make zebrafish highly attractive in disciplines as genetics, developmental biology and physiology (Phelps and Neely 2005; Rodrigues et al. 2012). During the last years, this species has also been used in studies for drug discovery as a model for human diseases, and to test the effect of aquatic pollution for example due to endocrine disruptors (Shrader et al. 2003), brominated flame retardants and ethanol (Kling and Forlin 2009).

The use of fish species for environmental research is however not restricted to zebrafish. Despite the interest in the zebrafish model, no studies are available relative to the identification of biomarkers in this species by using proteomics approaches. In contrast, most proteomic studies have been carried out in Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), catfish (*Ictalurus puncta*tus), fathead minnow (*Pimephales promelas*) and cod (*Gadus morhua*), mainly due to their commercial importance (Larsen et al. 2006; Fang et al 2010; Karlsen et al. 2011). They have been selected as a model for aquatic toxicological studies with particolar reference to endocrine disruptors and hepatotoxic contaminants not only to understand the

mechanisms of toxicity but also to discover potential biomarkers for environmental monitoring and risk assessment.

1.5 Proteomics of ecotoxicologically relevant invertebrates

Ecotoxicoproteomics studies have focused mainly on vertebrate species rather than in invertebrates (Kumagai et al. 2006). Although several investigations regarding invertebrates as models for the investigation of the effects of toxicants in the proteome have appeared in the last years, the number is still rather low but gradually increasing especially if we consider the economical and ecological importance of these organisms (Lemos et al. 2010).

The lack of sequenced genomes is the main reason for this apparently disregarding of invertebrates in ecotoxicoproteomics. These techniques have been applied to well characterized species, that are inappropriate from an ecotoxicological perspective. The use of proteomics to study ecologically relevant species is not impossible, but it entails more work and money to obtain less information, becoming derogated by the lack of DNA and protein sequences in the databases increasing the complexity of a proteomic project (Barret et al. 2005).

One of the most emblematic and long-term studied organism in ecotoxicology is *Daphnia* sp. (Martins et al 2007; Costa et al. 2008) even if the number of publications that explore the proteome of daphnids is low (Schwerin et al. 2009).

Aquatic invertebrates, as bivalves, often serve as reservoirs for many environmental pollutants, due to their environment and exposure to aquatic

sediments. In fact, these organisms, specially seawater mollusks of the genus *Mytilus*, are popular sentinel species used in estuarine and coastal monitoring programs for the monitoring of marine pollution (Rodrigues-Ortega et al. 2003; Manduzio et al. 2005; Dondero et al. 2006).

Many authors have been trying to establish protein patterns of environmental contaminants exposure, as well as comparing protein profiles of bivalves residing in polluted areas to those of reference sites (Apraiz and Cristobal 2006). Environmental contaminants proteomics studies include exposure to cadmium, nonylphenol, and p,p-dichlorodiphenyldichloroethylene in the clam *Ruditapes decussates* (Chora et al. 2010), benzo(α)pyrene in zebra mussels (*D. polymorpha*) (Riva et al. 2011), polychlorinated biphenyls, polyaromatic hydrocarbons and heavy metals in *Mytilus edulis* (Knigge et al 2004), cylindrospermopsin in *Mytilus galloprovincialis* and *Corbicula fluminea* and crude oil in blue mussels (Manduzio et al. 2005).

Vertebrates and invertebrates are extremely different organisms, but fascinatingly, the use of proteomics in invertebrates has allowed to suggest that toxicants can have the same target molecules in both types of organisms. The existence of a similar MoA still remains to be proved but has been suggested (Apraiz et al. 2006; Lemos et al. 2009, 2010).

Although using whole organisms can be the first approach of invertebrate proteomics investigations, mainly due to their relative small size, it should be considered the information that can be sought by using different organs or different organism sections in order to better conceive the entire pathways of toxicity (Mi et al. 2005).

1.6 *Mytilus galloprovincialis*

Mytilus galloprovincialis is one of the three principal, closely related species in the complex of blue mussels, which collectively are widely distributed along the temperate coasts. *M. galloprovincialis* often hybridize with the closely related *Mytilus edulis* and *Mytilus trossulus*, when they are found in the same locality. *M. galloprovincialis* is considered the most warm-water-tolerant species of the three, and has the most distribution in Europe specially in the Mediterranean Sea. It is an invasive species in many parts of the world, where it was introduced from Europe by human activity (Carlton 1992; Branch and Steffani 2004), and also an object of aquaculture because it is a widespread food (Sievers et al. 2013).

M. galloprovincialis is a species of bivalve, that belongs to phylum *Mollusca*, whose shell, as suggested by the same name, is composed of two valves usually symmetric. This animal grows up to 140 mm in length; the shell is blue-violet or black, but may shade to light brown (Bayne 1976; Gosling 1992). *M. Galloprovincialis* is a filter feeders, using the gills to respire and to capture particulate food such as phytoplankton from the water (Seed 2009). Figure 1.2. shows the diagram of the internal organization of a bivalve mollusk.

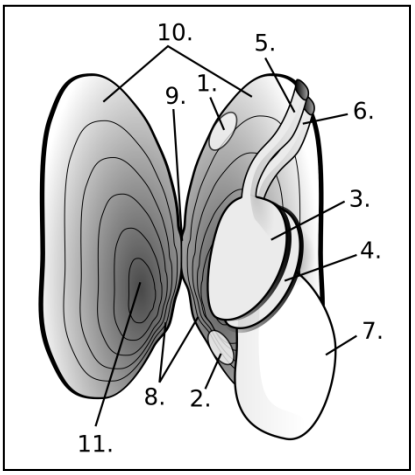


Figure 1.2 Anatomy of a bivalve:
1: posterior adductor,
2: anterior adductor,
3: outer gill demibranch,
4: inner gill demibranch,
5: excurrent siphon,
6: incurrent siphon,
7: foot,
8: teeth,
9: hinge,
10: mantle,
11: umbo.

Under normal conditions, a mussel of average size, filter approximately 4 to 5 liters of water per hour, and is capable of retaining 90% of the particles contained in it, always falling within a range of sizes filterable. The size of the retained particles are between a maximum of about 400-500 microns, and a minimum diameter of 1-5 microns. Below a few microns, only the particles adsorbed on the particles of larger size are retained. It is known that the mussel is able to efficiently capture the particles with diameter between 2 and 5 microns (Seed 2009). When they live in polluted waters, bivalve mollusks have a tendency to accumulate substances such as heavy metals and persistent organic pollutants in their tissues. This is because they ingest the chemicals as they feed but their enzyme systems are not capable of metabolising them and as a result, the levels build up. Since the standard chemical analysis methods do not provide information about the presence and accumulation of these contaminants in water, because their concentrations in water are often below

the instrument detection limit, aquatic organisms become an even more attractive method for their determination.

The criteria required for an acceptable bioindicator are that it must be able to tolerate large concentrations of pollutants and accumulate them in addition to being able to withstand changes in temperature and salinity. It should be static, representative of a location, and easy to identify, sample and handle. It should have a long enough life cycle and a sufficient amount of tissue required for chemical analysis. Being sedentary widespread and easy to collect and because they live in direct contact with substrate, the blue mussel *Mytilus galloprovincialis* fulfills most of the above-mentioned criteria, thus it was chosen to serve as the bioindicator of water pollution (Adami et al. 2002; Licata et al. 2004).

1.7 Aim of the project

The overall objective of this PhD study was to to conduct a chemical and toxicological study on *Mytilus Galloprovincialis* exposed to a polychlorinated biphenils (PCBs) mixture to evaluate changes in protein expression and to identify PESs that could characterize the exposure to these specific environmental contaminants through a proteomic approach. This could be a starting point for the definition of new biomarkers of early exposure in biomonitoring programs.

The specific aims of the study were:

- to prepare an *in vivo* experiment in which a sufficiently large number of mussels were exposed to the mixture at a suitable concentration for different time;
- to evaluate the effectiveness of the treatment through chemical analysis;
- to compare protein pattern obtained by 2-DE of the mussels exposed to environmental relevant concentration and not exposed;
- to investigate time-dependent effects on proteome of contaminated mussels detecting proteins differentally expressed;
- to identify proteins involved in the response of bivalves to these contaminants.

1.8 Contaminats object of the current study: polychlorinated biphenyls

Polychlorinated biphenyls (PCBs) cover a group of 209 different congeners, depending on the number and the position of chlorine atom substituents (Figure 1.3)

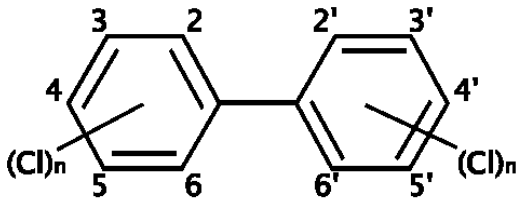


Figure 1.3. Chemical structure of PCBs.

These compounds are odourless, tasteless and colourless to pale-yellow, viscous liquids or solids. Some PCBs are volatile and may exist as a vapour in air. There are no known natural sources of PCBs in the environment (ATSDR 2000).

Due to their physico-chemical properties, such as chemical stability, low heat conductivity and high dielectric constants, PCBs were widely used in a lot of industrial and commercial applications such as hydraulic and heat transfer systems, cooling and insulating fluids in transformers and capacitors, pigments, dyes, repellents and carbonless copy paper or as plasticizers in paints, sealants, plastics and rubber products. For technical purposes, PCBs have been used as complex, technical mixtures and not as single compounds.

PCBs have been globally circulated by atmospheric transport and thus are present in environment, in particular highly chlorinated congeners adsorb strongly to sediment and soil. Because of both lipid solubility and the absence of adeguate metabolic pathway in the organisms PCBs tend to bioaccumulate along the trophic chains where they are stored in fatty tissues. They were first detected in environmental samples in 1966 (Jensen 1966).

Accordingly, the manufacture, processing and distribution of PCB has been prohibited in almost all industrial countries since the late 1980s (Council Directive 85/467/EEC); however their entry into the environment still occurs, especially due to improper disposal practices or leaks in electrical equipment and hydraulic systems still in use (Council Directive 96/59/EC). Other sources include the burning of some wastes in municipal and industrial incinerators and leakage from paint and sealants in older buildings (WHO 2001). As a consequence, PCBs are considered major components of "persistent organic pollutants" (POPs) together with polychlorodibenzodioxins and polychlorodibenzofurans (PCDD/Fs) (WHO 1993; Stockholm Convention 2001).

Based on structural characteristics and toxicological effects, PCBs can be divided into two groups. One group consists of 12 congeners that easily can adopt a coplanar structure giving them a structure similar to PCDD/Fs and allowing them to act in the same way. This group of PCBs called "dioxin-like" PCBs (DL-PCBs) show toxicological properties similar to dioxins (effects on liver, thyroid, immune function, reproduction and behaviour) being agonist of the aryl hydrocarbon receptor (AhR) in organisms. The remaining PCBs are referred to as 'non-dioxin-like PCBs' (NDL-PCBs), that do not share the dioxin toxic mechanism (Safe et al. 1985). In fact they have not been

found to activate the AhR, and are not considered part of the dioxin group; however, studies have indicated some neurotoxic and immunotoxic effects, but at levels much higher than normally associated with dioxins. NDL- and DL-PCB have to be both considered as endocrine disrupters, potentially being able to alter a number of hormone systems such as thyroid and sexual hormones (Mantovani et al 2009). Nevertheless mechanisms are still to be completely clarified (La Rocca and Mantovani 2006).

1.8.1 Indicators PCBs.

The main pathway of human exposure for the majority of the population is via food consumption (90%) with the exception of specific cases of accidental or occupational exposure (EFSA 2005). The major dietary sources of PCBs are food of animal origin (Baars et al. 2004; Arnich et al. 2009).

Due to the practical impossibility of monitoring all PCB congeners, surveillance activities target a limited number of congeners, considered as markers of the overall contamination (EFSA 2005).

Data on occurrence of NDL-PCBs in different food and environmental sample, carried by European Food Safety Authority (EFSA 2010), have been reported as the sum of six PCB congeners (PCB 28, 52, 101, 138, 153, 180 according to IUPAC) often referred to as indicator PCBs . The six PCBs were chosen as indicators not because of their toxicity but because they are easily quantified compared to the other NDL-PCBs, they represent all relevant

degrees of chlorination because, are appropriate indicators for different PCB patterns in various sample matrices and are most suitable for a risk assessment of NDL-PCBs on the basis of the available data. The sum of the six indicator PCBs represents about 50 % of the total NDL-PCBs in food (EFSA 2005, 2010).

Although there might often be different sources for dioxins/furans (PCDD/Fs), DL-PCBs, and NDL-PCBs some studies have demonstrated that there is a certain correlation between the occurrence of NDL-PCBs and DL-PCBs on the one hand, and between NDL-PCBs and total PCDD/F+DL-PCBs on the other (EFSA 2005). Maximum levels for PCDD/Fs, DL-PCBs and NDL-PCBs in food and feed have recently been laid down in Commission Regulation (EC) No. 1259/2011.

Chapter II

2. Proteomics technologies

The typical workflow for proteomics encloses three main steps (figure 2.1): a powerful protein extraction method capable to deal with the interfering compounds, the combination of different complementary protein fractionation, separation and quantification techniques to maximize the resolution and to cover the proteome as much as possible, and the usage of different complementary MS techniques and error tolerant database searches (Carpentier et al. 2008).

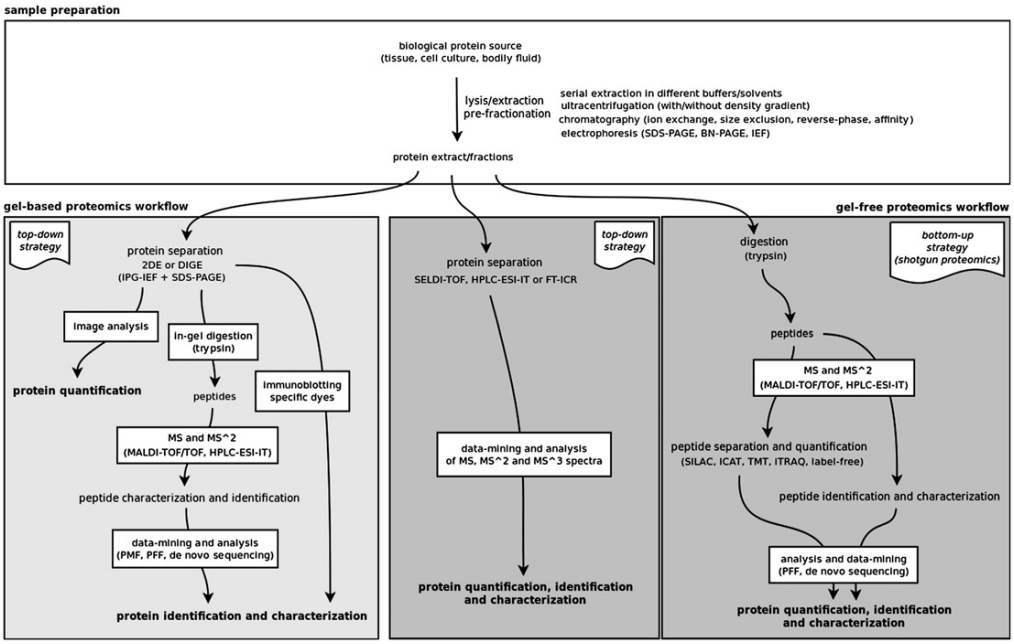


Figure 2.1 Typical proteomics workflow used in proteomics studies. The common feature is sample preparation that can then be followed by a gel-based or a gel-free proteomics Final objective is in all cases the identification and characterization of proteins of interest.

The two most important analytical techniques in proteomics are two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS). Due to the central role that these two methods have in proteomic studies, most experiments are broadly classified as either "gel-based proteomics" or "gel-free proteomics", depending on whether 2-DE is used for protein separation and quantification or not (de Roos 2009; Rodrigues et al. 2012).

The classical 2-DE proteomic approach, which is used today and is based on the concept of combining isoelectric focusing (IEF) and SDS-PAGE, was initially introduced in the pre-omics era in the mid-seventies by O'Farrell (1975) and Klose (1975), who began the mapping of proteins from *E. Coli* and mouse, respectively (Klose 2009; O'Farrell 2008). Moreover, the major breakthrough was the development of technologies in the beginning of the 1990's following the development and advances of MS and MS/MS based identification of proteins. More recently the development of Difference Gel Electrophoresis (DIGE), first described by Unlu et al., has greatly improved the quantification of proteins in 2-D gels allowing the detection of subtle changes in protein abundance (Unlu et al. 1997).

Due to the continuous improvement and cost-reduction of MS-based methods, gel-free strategies are becoming increasingly popular, since they generally allow higher analytical throughput and generally deeper proteome coverage than gel-based methods (Forné et al. 2010). These methods are based on liquid-phase chromatography procedures, which can readily be coupled to ESI-based mass spectrometers. Even multidimensional chromatographic separations are commonly used, as in the case of MudPIT, where peptides are separated by charge (SCX-HPLC) and hydrophobicity (RP-HPLC) prior to MS analysis. Most gel-free workflows rely on stable isotope labeling for

peptide quantification, either by metabolic incorporation of radioactive amino acids in proteins (SILAC) or by post-extraction chemical modification (ICAT, TMT, iTRAQ) (Rodrigues et al. 2012).

2.1 Sample preparation

The first step is usually protein extraction, since most analytical techniques used in proteomics require prior solubilization of proteins in an appropriate solvent (aqueous buffers, organic solvents). Preparation should be as simple as possible to increase reproducibility of the experiment minimizing the loss of proteins during the procedure. The solubilization process includes denaturation of the protein to break non-covalent bindings within and among the proteins, while maintaining the native charge and molecular weight of soluble proteins. Ideally, the perfect solubilization mixture should freeze all the proteins in their exact state both in terms of amino acid composition and posttranslational modifications (Rabilloud 1999).

Commonly used aqueous extraction buffers often contain detergents, chaotropes, reducing agents and protease inhibitors, ensuring that enzymatic activity is halted during extraction and that intra and inter-molecular interactions (disulfide bridges and non covalent interactions including ionic bonds, hydrogen bonds and hydrophobic interactions) between proteins are minimized, preventing aggregation. Protein denaturation achieved by addition of chaoptropic agents like urea and thiourea is widely used in sample preparation due to their efficiency for solubilizing proteins. Urea is quite efficient in disrupting hydrogen bonds while thiourea is more suitable for

breaking hydrophobic interactions. Addition of excess reducing agents is necessary for cleavage of intra and intermolecular disulfide bonds so proteins completely can unfold. Dithiothreitol (DTT) is one of the most frequently used reductants. Additional detergents such as CHAPS are commonly included in the solubilisation mixture due to its efficiency for solubilising hydrophobic proteins (Gorg et al. 2004). In addition, it has been shown that urea, thiourea and CHAPS in combination are very effective inhibitors of proteolytic activity (Castellanos-Serra and Paz-Lago 2002) in order to prevent degradation of proteins.

Although standard extraction buffers work reasonably well for a broad range of samples, these protocols can be optimised also considering other procedures based on chromatography, electrophoresis, differential solubility and ultra-centrifugation simplifying protein extracts and improving the dynamic range of a protein mixture. Finally the removal of lipids, that can interfere with the IEF current in the first dimension and the 2-DE resolution, desalting, necessary because salt ions can interfere with the focusing of proteins, and minimization of polysaccharides and nucleic acids that may interact with carrier ampholytes and proteins can be usually achieved by TCA and acetone precipitation (Gorg et al. 2004; Kim and Kim 2007).

2.2 Proteome separation and visualization

2.2.1 Two-dimensional electrophoresis

2-D electrophoresis is a powerful and widely used method for the analysis of complex protein mixtures extracted from cells, tissues, or other biological samples. This technique separates proteins according to two independent properties in two discrete steps. The first-dimension step, isoelectric focusing, separates proteins according to their isoelectric points (pI); the second-dimension step, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), separates proteins according to their molecular weights (Mr). Carrier-ampholyte (CA) generated pH gradient was originally used in the first 2-DE protocols introduced by O'Farrell and Klose in 1975 but the method suffers from several limitations including resolution, reproducibility, separation of very acidic and very alkaline proteins and sample loading capacity (Weiss et al. 2009).

The robustness of 2DE has dramatically increased improving the resolution and reproducibility of protein separation in the first dimension due to the introduction of immobilised pH gradients (IPG) developed by Bjellqvist (1982) and further improved by Gorg and colleagues (1988). The IPG gradient is immobilised due to covalent anchoring of the pH gradient to the acrylamide matrix during casting, which generates an extremely stable pH gradient in different ranges between pH 2.5-pH 12. Consequently, IPG is nowadays the current method of choice in IEF of 2-DE in proteome analysis (Gorg et al. 2009).

Following the separation of proteins by first dimensional IEF, the second dimension is carried out by SDS-PAGE in an electric field on horizontal or vertical systems. The horizontal system has limited capacity of only a single gel, whereas the vertical system permits multiple runs in parallel, particularly useful for analysis which requires simultaneous batch electrophoresis to maximise reproducibility.

The result of 2-DE analysis is a protein map of spots where each spot potentially corresponds to a single protein in the sample. Thousands of different proteins can thus be separated, and information such as the protein pI, the apparent molecular weight, and the amount of each protein can be obtained (Zimny-Arndt et al. 2009).

The visualization of proteins can be obtained by application of various staining techniques including organic dye, silver stain, reverse stain, fluorescent stain, radio-labelling and chemiluminescent stain. Coomassie Brilliant Blue is one of the most widely used dyes for 2-DE protein staining due to low cost, ease of use and good compatibility with protein identification methods such as mass spectrometry. Silver staining is more sensitive but with a restricted linear dynamic range. (Wu et al. 2005; Weiss et al. 2009). Thanks to development of DIGE, that allows several samples to be run on a single gel, fluorescence staining of proteins is gaining popularity due to high sensitivity, broad dynamic range and ease of use. (Unlu et al. 1997).

2.2.2 Image analysis

After protein staining, to facilitate rapid and accurate image analysis and to ensure high quality data from proteome experiments a number of commercial software packages are available e.g. Delta 2D, Melanie, PDQuest, Image Master 2D and Progenesis. Although they are based on different detection and matching principles and have a broad range of options, they use the same traditional workflow involved in differential image comparisons analysis: spot detection, gel-to-gel matching and spot quantification (Raman et al. 2002). Yet, manual editing (e.g. deletion of false protein spots and correction of spot shape) during the spot detection is nearly impossible to avoid due to complexity of spots on a gel as well as manual matching because dissimilarity of spot positions may be caused by variations in pH of running buffer, incomplete polymerisation, current leakage, air bubbles in gels and influence of high abundant proteins on the pH gradient (Berth et al. 2007). Moreover spot volume normalization is a necessary step to minimize the analytical variation caused by gel-to-gel variations, due to protein loadind and gel staining that can have a considerable impact on the raw spot volumes, thus is very important for quantification of spot volumes.

The main purpose of differential proteomics is to study the changes in expression level of proteins and generally, data is divided in two groups, treatment versus control group. In these circumstances spots are commonly tested individually by use of univariate statistics such as the Students t-test or analysis of variance (ANOVA) to reveal spots changing in abundance between the two groups (Biron et al. 2006). However, the normal distribution of data is often assumed and not tested (Wilkins et al. 2006) so multiple testing

correction methods can be used e.g. Bonferroni correction and False Discovery Rate (FDR) to adjust the values of protein spots based on Students t-test or ANOVA and to keep the overall rate of error as low as possible (Storey and Tibshirani 2003).

Using univariate statistics alone, it is impossible to reveal all the complex interactions in protein expression profiling, and essential data information as well as data structure is lost in the analysis. In multivariate statistical analysis e.g. principal component analysis (PCA) and Partial Least Squares Regression (PLSR), all spots are analyzed simultaneously and this method offers a strong approach for the evaluation of 2-DE maps to get an overview of the main variation and structure in data (Jensen et al. 2008).

2.2.3 OFFGEL fractionation

Most proteomics workflows from complex biological matrices require extensive sample processing at peptide or protein level to increase identification coverage. Recently, due to its separation capabilities, easy of use and relatively low cost, OFFGEL isoelectric focusing has become a popular tool to fractionate proteins and peptides by their isoelectric point prior to LCMS/MS (Michel et al. 2003; de Godoy et al. 2008). The increase in the number of peptide identifications acquired from all fractions compared to the number derived from unfractionated samples demonstrates the value of this technology (Hörth 2006; Hubner et al. 2008).

OFFGEL electrophoresis differs from conventional gel electrophoresis in that the sample components do not remain in the gel. Instead, they are

recovered from a buffer solution, making the recovery much easier than with conventional gels. After rehydration of gel strips, the immobilized pH-gradient gel seals tightly against the compartment frame. Then, the diluted sample is distributed across all wells in the strip. When a voltage is applied to the ends of the gel strip, the proteins or peptides move through the gel until the molecules reach a well covering a portion of the gel where the pH equals the pI of the molecule. After fractionation, the liquid fractions containing pI-based separated proteins or peptides can easily be removed and processed for downstream experiments (Fraterman et al 2007; Azulay et al. 2010).

2.3 Mass spectrometry based identification of proteins

Most proteomic studies (both gel-based and gel-free) attempt to identify proteins by looking at peptides, after digestion of proteins by a specific protease, usually trypsin which has high specificity for cleavage at the carboxylic side of arginine and lysine and generates peptides in the useful mass range for mass spectrometry.

The classical method is called “peptide mass fingerprinting” or PMF. Identification is performed by comparing experimentally obtained MS mass lists with theoretical obtained mass data generated in silico from an already identified protein in a database. Nonetheless, this is a reliable method when working with a model species (like zebrafish), for which there is full genome data, and for simple digests (like 2DE spot digests). On the other hand, by using tandem MS instruments, you obtain not only a peptide mass list, but also information on the fragmentation mass spectra of those peptides, providing a

fingerprint for each peptide that directly reflects its sequence. For this strategy called "peptide fragment fingerprinting" or PFF identification of peptides is then performed by comparison of experimentally obtained MS/MS spectra against all possible fragmentation spectra in the database. Since a fragmentation spectrum (unlike mass) is usually very specific for a certain peptide sequence, identification of proteins can often be attained also from a single high-quality peptide match. It is therefore important to underline that protein identifications depend not only on the quality of spectra, but also on the quality of the sequence database used (Graves and Haystead 2002).

Ionization techniques commonly used in proteomics include the soft-ionization methods of nanoelectrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI). The evolution of ionization sources has advanced to include desorption/ ionization on silicon, a matrix-free MALDI technique (Peterson 2007) and laserspray ionization, which allows for the analysis of proteins directly from tissue using atmospheric pressure ionization mass spectrometers (Inutan et al. 2011). Mass analyzers are broadly categorized into scanning and ion-beam analyzers (quadrupole, TOF) and the trapping analyzers (ion-trap, orbitrap, FT-ICR) and include a variety of instrument configurations (Yates et al. 2009; Slattery et al. 2012).

Various peptide mass search programs are available on the Internet e.g. Mascot, MS-fit and PeptideSearch (Kim et al. 2004) and the complexity of the algorithms used to search sequence databases is program dependent. The Mascot MS/MS-search (www.matrixscience.com) where different MS variables e.g. instrument, taxonomy, database, enzyme and modifications etc. are decided before the search, is the most used one for database searching.

Chapter III

3 Materials and Methods

3.1 Reagents

All the reagents for electrophoresis and proteomic analyses (agarose ammonium carbonate, ammonium persulfate, analytical standards of PCB 138, 153 and 180 bromophenol blue, BSA, CHAPS, Coomassie Brilliant Blue, DTT, formic acid, glycerol, Immobiline Dry Strips, iodoacetamide, ß-mercaptoethanol, pharmalyte 3–10, reagent of Bradford, SDS, thiourea, trichloroacetic acid, trypsin, Tris, urea,) were from GE Healthcare (Buckinghamshire, UK) and from Sigma (St Louis, MO, USA). All the reagents were molecular biology grade, if not otherwise stated.

3.2 Animals and exposure condition

Adult mussels (*Mytilus galloprovincialis* Lam) were carefully collected, taking care to avoid damaging the animals from an unpolluted acquaculture farm (Pozzuoli-NA, Italy) in June 2011. This site was chosen as reference because of its good physic-chemical quality. The mussels were transported to the Pozzuoli Fish Market laboratory and put in 100 l tanks (Fig. 3.1) for acclimation for 7 days at the same temperature and salinity as collection.

Figure 3.1. Image of 100 l tank used in the experiment.

In detail, 2 groups of 100 mussels, of approximately the same size (6-7 cm valve size), were kept in the tanks filled with continuously aerated seawater at 15.0 °C. Seawater was collected offshore and then subjected to analysis of six indicator NDL-PCBs.

After acclimation, 1 group was exposed to a mixture of three PCB indicators (PCB138, PCB153, PCB180) (Table 3.1) at the concentration of 30µg/l and the other group kept in seawater, as control. During the experiment animals were fed twice a week with a microalgae, Isocrysis Parke (56 ml per Kg of edible part), which was certified for absence of PCBs, and seawater was not changed.

Contaminants were dissolved in 1 ml of commercial peanuts oil. 10 mg of each analytical standard of three PCBs were solubilized in order to obtain a concentration of 10 mg/ml for each congener. Then 300 µl of every stock solution were added to the feed and vigorously mixed with it before feeding

mussels. This addition was carried out only once, just immediately after the time of acclimation at the beginning of exposure. The controls were fed at the same time with feed added to 900 µl of penaut oil.

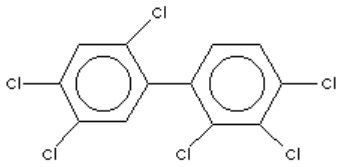
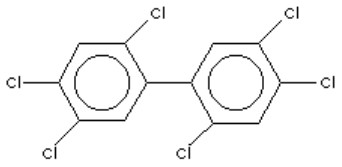
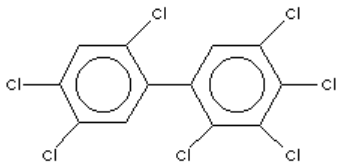
Congener Number	Chemical Structure	IUPAC name
PCB 138		2,2',3,4,4',5'-Hexachlorobiphenyl
PCB 153		2,2',4,4',5,5'-Hexachlorobiphenyl
PCB 180		2,2',3,4,4',5,5'-Heptachlorobiphenyl

Table 3.1. PCBs used in the experiment.

Sampling was performed at regular intervals (0, 7, 14, 21 days), taking 20 mussels from each tank and transferred live to our laboratories to be analysed. Finally experiment resulted in eight samples as described in the figure 3.2.

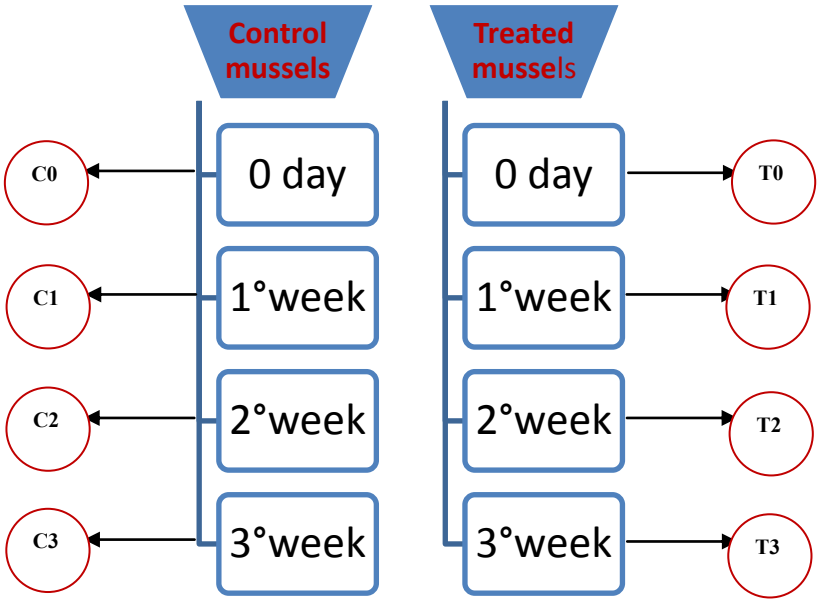


Figure 3. 2 Schematic design of *in vivo* experiment.

3.3 Experimental design strategies

Proteomics technologies have been largely employed in ecological studies regarding marine life in order to study environmental stress response and identify new protein biomarkers. Most studies have been conducted on bivalves, particularly on mussels, clams and oysters because these can be considered "sentinel species" from both environmental and seafood point of view (Meiller and Bradley 2002; Dowling et al. 2006). Protein expression patterns (PES) have been determined for exposures to some different environmental contaminants (Alves de Almeida et al. 2008; Kling and Förlin,

2009; Berg et al. 2011). However, none of these studies considered the effect of exposure for different times. On the contrary, investigation proposed in this PhD thesis was carried out following an exposure for 1, 2 and 3 weeks in order to correlate proteomic profiles of contaminated mussels to PCB concentration.

In addition, whereas PCBs are widespread environmental pollutants, never present as a single congener but always as mixtures, it was decided to use a PCB mixture of three NDL-PCBs (most chlorinated and toxic congeners) belonging to indicator PCBs (see chapter I). PCB concentration was chosen to correlate with legal limits imposed by the European Community for NDL-PCBs in shellfish (Reg. 1259/2011) and considering bioaccumulation capability of mussels.

The choice of analyzing complete edible part of mussels was addressed by two main reasons. On the one hand, because of the lacking of records in proteomics and genomics databases for some organisms, chances of a reliable identification of proteins increases considering complete proteome. On the other hand, *Mytilus galloprovincialis*, one of the main foods of Mediterranean diet, is considered of great interest in food safety because PCBs are predominant in this specie, representing a risk to human health.

3.4 Chemical analysis of PCBs

Concentrations of six indicators PCBs were measured according to recently published method by Serpe et al. (Serpe et al. 2013). PCBs concentrations, expressed in ng/g wet weight, were determined in pools of twenty organisms. Briefly, 5 g of homogenate were extracted by shaking machine for 12 h with 20 ml of diethyl ether. The extract was filtered over

Na_2SO4, dried under nitrogen flow and reconstituted with 2 ml of petroleum ether.

A diatomaceous earth solid support (Extrelut NT3, Merck, Darmstadt, Germany) was conditioned with 3 ml of H_2SO_4. The extract was loaded on the solid support and mineralization was carried out for 20 minutes at room temperature. Compounds were eluted with 20 ml of petroleum ether, passed through a solid phase 6 ml clean-up florisil cartridge (Isolute, Uppsala, Sweden), dried in the bathroom Rotavapor (Büchi, Assago, Italy) set at 40 °C and reconstituted with 1 ml of isooctane. Sample was filtered on a membrane of 0.45 μm nylon syringe (Millipore) and injected to the gas chromatograph (Perkin Elmer Autosystem XL, Waltham, Massachusetts) equipped with an electron capture detector (ECD) and a 30 m x 0.25 mm x 0.25 micron capillary column (35%-phenyl-65% dimethylpolysiloxane-fused silica).

Instrumental parameters were: 0.5 μl as injection volume, injector at 250 °C and detector at 380 °C; oven temperature was programmed as follows: 100 °C to 250 °C at a rate of 15 °C/minute, 250 °C to 300 °C at a rate of 5 °C/minute, hold for 1 minute. Chromatographic conditions were tested also for possible interference of polybrominated diphenyl ether 47 (PBDE 47, stock solution at 10 ng/ml), which is the most predominant congener in environment, at Rt of PCB 180. The retention time (Rt) window for the identification of analytes was Rt ± 0.5% and the amount of each compound was determined by external standardization with a three point curve (1.0, 10.0, 20.0 ng/g PCBs mixture in isooctane).

3.5 Preparation of samples and protein extraction

The mussels were first cleaned, dissected and washed with distilled water. After wiping dry, the edible parts were homogenized for three minutes using the Ultra-Turrax (IKA, Staufen, Germany), a high-performance dispersing machine used for the production of a variety of emulsions and suspensions in batch operations, lyophilized and then frozen at -20 ° C until analysis.

Proteins were extracted by suspending 30 mg of lyophilised tissue immediately in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS and 3% DTT) to avoid proteolysis. Proteins were solubilised for 3 h at 30 °C with vigorous shaking using a Thermomixer shaker (Eppendorf, Hamburg, Germany). The mixture was centrifuged at 12,000 g for 15 min. Supernatants were either used immediately for electrophoresis or stored at –80 °C.

The protein concentration of each extract was determined according to the method of Bradford (Bradford 1976) which is a colorimetric protein assay, based on an absorbance shift of the dye Coomassie Brilliant Blue G-250. One µl of lysate was added 1 ml of the mixture H20: Bradford reagent (8:2 v: v) and the assay was always performed in duplicate by UV-visible spectrophotometer at 595 nm. It was constructed a calibration curve with bovine serum albumin (BSA) (2 µg/ml , 4 µg/ml, 8 µg/ml and 16 µg/ml) in the mixture H_20: reagent of Bradford.

3.6 One-dimensional SDS-PAGE

SDS-PAGE was done according to Laemmli (1970). The protein extracts were diluted 1:2 with two-time SDS-PAGE Buffer (0.13 M Tris-HCl, 4.2 % (w/v) SDS, 21 % (v/v) Glycerin, 19 % (v/v) ß-Mercaptoethanol, Bromphenolblue), denaturated for 5 min at 95 °C and subsequently centrifuged for 15 min, at 9000 x g in a table centrifuge (Biofuge Pico, Heraeus, Buckinghamshire, UK). 1 µl protein solution was then loaded to a 3 % acrylamide collecting gel. For protein-separation, 12 % acrylamide separation gels were used and separation was done at constant 30mA/gel for 2 h at room temperature. Gels were stained by colloidal Blue Coomassie staining solution. For protein molecular weight estimation, 5 µl of protein standard marker (peqGOLD Protein-Marker I, Peqlab®, Erlangen, Germany) was run alongside of each gel.

3.7 Proteomic strategy

Figure 3.3 shows the working outline pattern used for this project.
After the preparation of protein extracts have been used two different strategies for the separation of proteins prior to identification by mass spectrometry: classic proteomic approach that uses two-dimensional electrophoresis and the most recent OFFGEL fractionation.

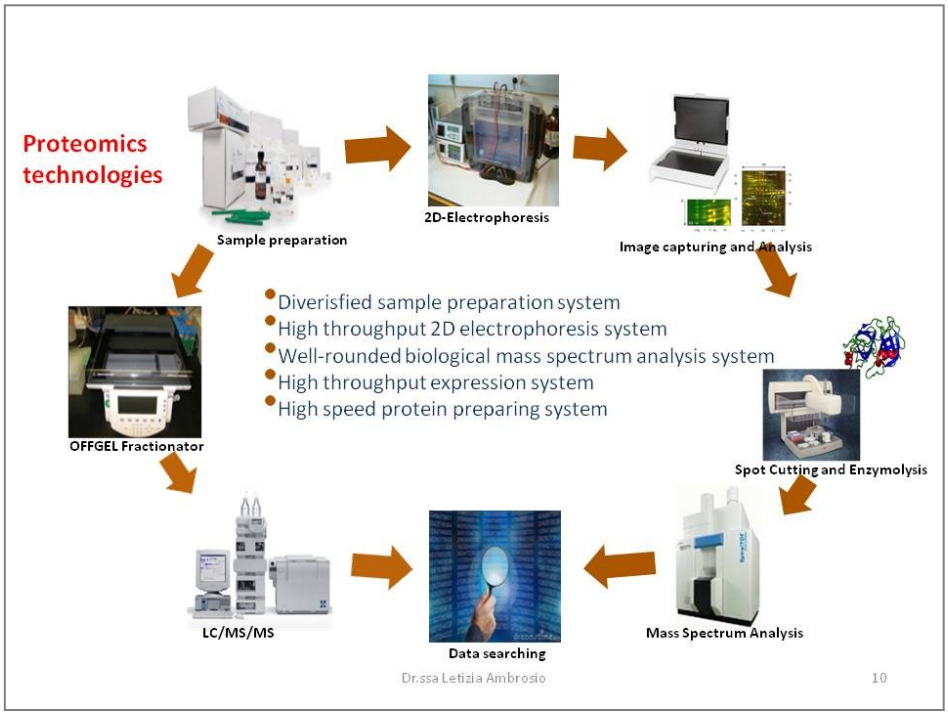


Figure 3.3 Proteomic technologies applied in PhD Study.

3.8 Separation of proteins

3.8.1 OFFGEL fractionation

An Agilent 3100 OFFgel fractionator (Agilent Technologies, Santa Clara, CA, USA) was used for all experiments. An aliquot of the peptide mixture obtained by tryptic digestion of each protein extract (220 µl, corresponding to 500 µg of proteins) was diluted with carrier ampholyte

mixture (3.6 ml final volume, 10% carrier ampholyte concentration). Each sample was then loaded on a single Immobiline Dry Strip, linear pH range 3.0–10, 18 cm length. Peptides were focused at constant temperature of 20°C, and at constant current intensity of 50 mA. During focusing, the applied voltage rose from 300 to 8000 V in 24 h. After focusing was completed, fractions were collected and an 10-µl aliquot from each fraction was injected for nano-LC-MS/MS analysis.

3.8.2 High-resolution two-dimensional electrophoresis

IPG strips (GE Healthcare) were passively rehydrated for at least 12 h with 125 µg protein in 125 µL (for 7 cm IPG strips) or 400 µg protein in 350 µL (for 18 cm IPG strips) of rehydration buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 20 mM DTT, 0.5% Ampholine 3–10, and bromophenol blue traces. IEF was carried out at 20 °C, using a Multiphor II system (GE Healthcare) (Figure 3.4). For 7 cm IPG strips (pH 3–10), IEF was started with 300 V for 1 hour, then increased gradually to 5000 V for 1.5 h, and finally risen to 5000 V until 50000 Vh was achieved. For 18 cm IPG strips (pH 3-10), IEF was started with 500 V for 8 hours, then increased gradually to 10000 V for 3 h, and finally risen to 10000 V until 80000 Vh was achieved.

Before the second dimension, the IPG strips were first soaked for 15 min in equilibration solution (50 mM Tris- HCl buffer, pH 8.8, 6 M urea, 30% v/v glycerol, 2% SDS, and bromophenol blue traces) containing 2.5 mg/ml DTT, and subsequently soaked for 15 min in equilibration solution containing 45 mg/ml iodoacetamide.

A

B

Figure 3.4

A. Image of IPG strip. **B.** Image of Multiphor II system.

Separation in SDS-PAGE was carried out at 20 °C in 12.5% polyacrylamide gels containing 375 mM Tris-HCl buffer, pH 8.8, and 0.1% SDS, using the PowerPac Basic system (Bio-Rad, Hercules, CA, USA) for 7 cm strips, at a maximum output of 30 mA/gel, or Ettan Dalt Twelve tank (GE Healthcare) for 18 cm strips, at a maximum output of 25 W/gel (Figure 3.5). Each experiment was conducted in triplicate.

Figure 3.5 Image of Ettan Dalt Twelve tank.

3.8.3 Image acquisition and analysis

Preparative 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, 10% acetic acid. Gel images were obtained using the Image Scanner III LabScan 6.0 (GE Healthcare). Images were analysed using Image Master 2D Platinum 6.0 (GE Healthcare) according to manufacturer's instructions. Image analysis included spot detection, quantification and matching.

All the 2-DE map analyses were performed with identical background subtraction directly after the spot detection. A master gel was chosen by combining the 2-DE maps (triplicates) from the control condition. The 2-DE maps from the exposed tissues were matched to the reference 2-DE maps. To accurately compare the measurements of spots in different gels, a normalization step was used. In this step, the normalized volume for a spot was obtained by dividing its volume by the total volume of the detected spots on the image. Normalized volumes from different spots on sample from the exposed tissues were compared against the corresponding spots from the reference gel.

The number of valid protein spots was determined for each gel, as well as the number of proteins matched to every gel, and qualitative and quantitative differences in the protein patterns between the treatment and control group were determined. The average ratios of expression were analyzed by one-way ANOVA ($p \leq 0.05$). Proteins showing differences in expression were further analyzed in couples comparing the different experimental exposure groups with the control groups using the Student's t test ($p \leq 0.05$).

3.9 Protein identification

Stained protein spots of interest were manually excised from the preparative gel to be identified by mass spectrometry coupled with data bank investigations. Excised gel spots were washed 3 times with water, cysteines were reduced with a 10Mm DTT solution for 45 min at 56 °C and then alkylated with 50mM iodoacetamide at room temperature in the dark. Digestion was performed overnight with 12.5ng/ml of trypsin in 100 mM ammonium carbonate buffer, pH 8.4. The resulting peptides were extracted with 0.1% formic acid in acetonitrile, dried in a vacuum centrifuge, and re-suspended in 30 ml 0.1% formic acid in water and stored at −20 °C until used.

Analyses by MALDI/TOF mass spectrometry were performed using a MALDI micro MX instrument (Waters, Milford, MA, USA), equipped with a nitrogen laser and an extended flight tube of 1.2 m operating in reflector mode. Peptide extracts (1 μl) from each tryptic digest were crystallized in 1 μl of a-cyano-4-hydroxycinnamic acid matrix solution on a 96-well Teflon target plate. Mass data acquisition was performed in the mass range of 200–3000 m/z. All MALDI-TOF spectra were calibrated for the correction of masses using peptides mixtures from human α-enolase as external standard and [Glu]-Fibrinopeptide B human as lock mass standard.

Alternatively peptides were analyzed by nano-HPLC-ESI-MS/MS (Wilm et al. 1996) on a Q-TOF mass spectrometer (Waters) coupled with pumps and auto-sampler under standard conditions: capillary temperature 90.°C; source voltage 3.5 kV. Helium was used as collision gas.

The digests were separated by reverse-phase liquid chromatography using a BEH column (0.3 x 100 mm, 3 Å) in a nano-Aquity liquid chromatography system. Mobile phase A was 0.1% formic acid in water;

mobile phase B was 0.1% formic acid in acetonitrile/water 80/20. The digest (5µl) was injected, and the organic content of the mobile phase was increased linearly from 5% B to 40.% in 60 min . In survey scan, MS spectra were acquired for 0.5 s in the m/z range between 500 and 2000.The most intense peptides ions 2+ or 3+ were sequenced. The collision-induced dissociation (CID) energy was set according to mass to charge (m/z) ratio and charge state of the precursor ion.

Raw data MS/MS spectra were converted in PKL format by ProteoLynxs data analysis software (Waters) for subsequent protein identification against the NCBI non-redundant protein database through the MS search algorithm on the Mascot search engine (Perkins et al. 1999). Search parameters were set as follow: MS tolerance 50 ppm; MS/MS tolerance 0.25 Da; fixed modifications enzyme specificity: trypsin; 1 missed cleavage permitted; fixed modification: carbamidomethylation of cysteine; variable modification: methionine oxidation; mass tolerance for precursor ions: 10 ppm; mass tolerance for fragment ions: 0.6 Da. Significance threshold $p < 0.05$ and score above 50. The taxonomy was limited to other metazoan species (Lopez 2002). The MS/MS spectra were used to search the sequence databases (NCBI) for a homology search with the program BLAST (Basic Local Alignment Tool).

Web addresses for the different programs were the following: Mascot Server, http://www.matrixscience.com/; NCBI, http:// www.ncbi.nlm.nih.gov; BLAST,http://www.ncbi.nlm.nih.gov/Education/BLASTinfo/information3.html.

Chapter IV

4. Results and Discussion

4.1 Design strategies

In order to minimize the chance that different variabilities might affect the results of analyses, sampling procedure was standardized and tissue extracts were obtained from homogenates of pooled dissected from 20 individuals. The extracts obtained were submitted to chemical and proteomic analyses, to quantify their content of PCBs and to characterize their protein patterns.

In order to standardize our comparisons, an identical number of control and contaminated samples was taken from each tank (table 4.1).

***In vivo* Experiment**

Time of exposure	Treated Groups	Control Croups	Date of sampling
Week 0	T0 (20 mussels)	C0 (20 mussels)	17/06/2011
Week 1	T1 (20 mussels)	C1 (20 mussels)	24/06/2011
Week 2	T2 (20 mussels)	C2 (20 mussels)	01/07/2011
Week 3	T3 (20 mussels)	C3 (20 mussels)	08/07/2011

Table 4.1. Schematic sampling of mussels.

Mussels sampled for each tank were grouped into four groups (T0, T1, T2 and T3 for treated ones and C0, C1, C2 and C3 for controls.) according to date of sampling.

Mortality of organisms during experiments was less than 5% for the controls and for contaminated organisms, showing an appropriate concentration selection in the range of chronic contamination.

4.2 Data analysis of sampled mussels

Data concerning mollusk length were statistically analyzed, to assess the normality of their distribution within each sampling group (treated sampling and control sampling), eventually exclude outliers and identify the boundaries of the size classes. The distribution histograms, the density graphics and the parameters values for descriptive statistics are reported in Figure 4.1, which shows that data adequately approximates the normal distribution.

	C0	T0	C1	T1	C2	T2	C3	T3
Whole weight of mussels (g)	500.92	509.22	515.66	525.39	501.83	541.17	466.18	475.38
Weight shells (g)	207.87	210.49	218.10	237.35	211.88	221.71	187.24	196.83
Weight edible part (g)	100.98	103.46	99.00	93.29	87.87	99.44	76.0	104.15
Weight lyophilized (g)	17.04	18.92	19.79	16.22	17.95	18.12	15.78	18.03
% shells	41.50	41.34	42.30	45.18	42.22	40.97	40.16	41.40
% edible part	20.16	20.32	19.20	17.76	17.51	18.38	16.32	21.91
% lyophilized	21.12	22.77	20.69	20.77	21.84	21.64	21.89	20.49

Table 4.2 Weights of treated and control groups.

Analysis of the weights was also carried out considering the whole weight of mussels, the weight of shells, the weight of edible part and lyophilized part and their relative percentages (Table 4.2) and, as can be observed, there were no significant differences throughout the exposure period.

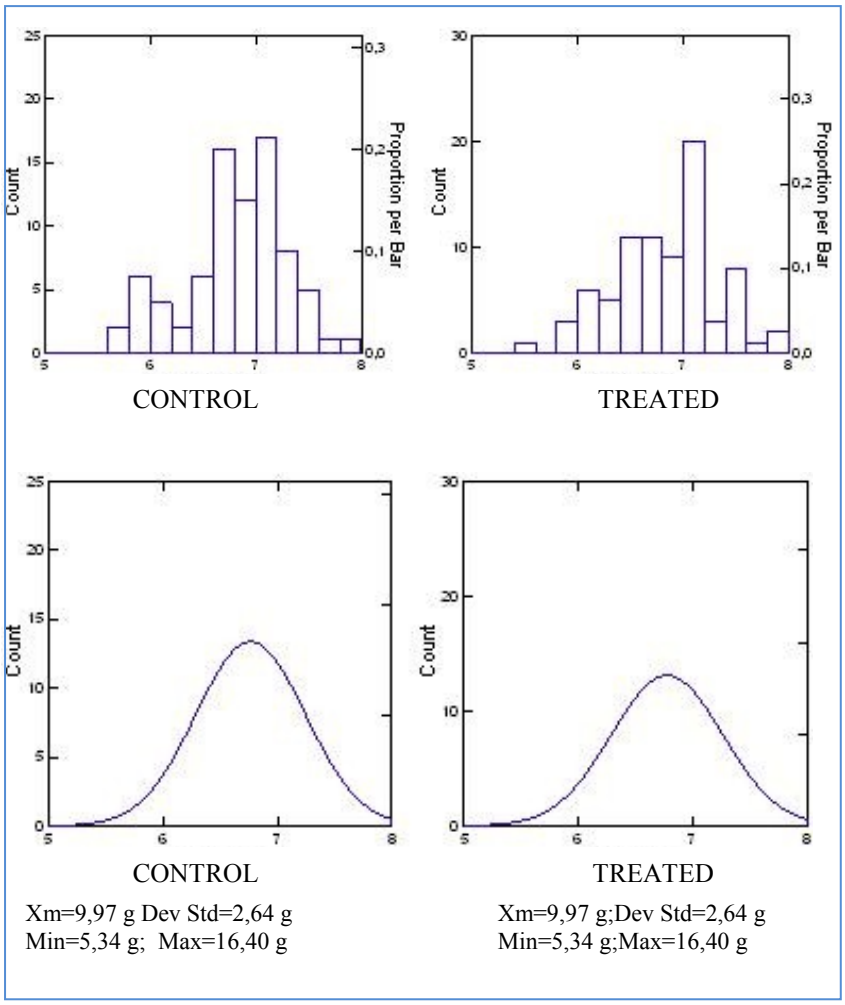


Figure 4.1 Exploratory data Analysis of mollusk lengths for the sampled mussels.

4.3 Chemical analysis of PCBs

To confirm uptake of the PCBs 138, 153 and 180 in exposed mussels, chemical analyses were performed. The concentration values obtained by GC-ECD in all treated groups are reported in Table 4.3. These values were higher than those measured for the water (30μg/l), thus indicating that the compounds were effectively bioaccumulatted.

Bioconcentration factor (BCF) is the concentration of a particular chemical in a biological tissue per concentration of that chemical in water surrounding that tissue. That is a dimensionless number representing how much of a chemical is in a tissue relative to how much of that chemical exists in the environment:

$$\mathrm{BCF}=\frac{\text{Concentration Organism}}{\text{Concentration Environment}}$$

This physical property characterizes the accumulation of chemicals, including pollutants, through chemical partitioning from the aqueous phase into an organic phase.

All PCBs concentrations were below the concentration limits defined by the European Commission (Reg. 1259/2011/CE) after one week of treatment and over legal limits after at least two weeks, except for PCB180 whose concentration is always lower. However, PCBs concentration values differently characterize the mussels after a week of exposure and after two or three weeks, having smallest concentrations of all three congeners in the first week and substantially similar concentrations after two and three weeks.

Bioconcentration factors (BCFs) were calculated in contaminated organisms (table 4.4). Concentrations in organisms were lower for PCB 180 than for PCB138 and PCB153, whose concentrations were similar considering one, two or three weeks of exposure. In fact congener 180 showed always a lower BCF. Bioconcentration was significant for all congeners used in thus study, despite the different concentrations measured. This indicates that animals were sufficiently exposed to PCBs and that a suitable experimental approach was chose to perform contamination .

Chemical analyses were conducted at the "Istituto Zooprofilattico Sperimentale del Mezzogiorno –Laboratory of Portici (IZS-Portici)" , determining all six indicators PCBs using a new method developed in this IZS laboratory (Serpe et al. 2013).

Congener	C0 (ppb)	T0 (ppb)	T1 (ppb)	T2 (ppb)	T3 (ppb)	Recovery 10ppb (%)
PCB 28	<LOD	< LOD	< LOD	< LOD	< LOD	66.0
PCB 52	< LOD	< LOD	< LOD	< LOD	< LOD	74.1
PCB 101	< LOD	< LOD	< LOD	< LOD	< LOD	85.9
PCB 138	< LOD	< LOD	62.96	78.53	76.41	75.3
PCB 153	< LOD	< LOD	66.13	81.86	80.42	77.8
PCB 180	< LOD	< LOD	44.66	54.69	50.86	91.1

Table 4.3 six indicator PCBs concentration (ppb) values determined by GC-ECD with corresponding recovery value.

	PCB 138	PCB 153	PCB 180
BCF (T1)	2.1	2.2	1.5
BCF (T2)	2.6	2.7	1.8
BCF (T3)	2.5	2.7	1.7

Table 4.4 Bioconcentration factors.

In particular, this method was optimized in the sample preparation step, that was particularly laborious, using diatomaceous earth sorbents for SLE instead of overnight acid hydrolysis of the lipid extracts and allowing to perform the analysis in a single day. This method has proved to be selective and specific for each indicator PCB, as can be seen by the absence of any interfering matrix signal at the retention time of analytes (Figure 4.2). The detection limit of the method resulted to be equal or lower to 1.0 ng/g (wet weight) for each compound, thus fulfilling the requirements recommended by the Commission Decision 657/2002/EC. Moreover, according to ISO/IEC 17025:2005 quality of the results was verified inserting spiked samples, whose recovery resulted compatible with general requirements of the ISO standard.

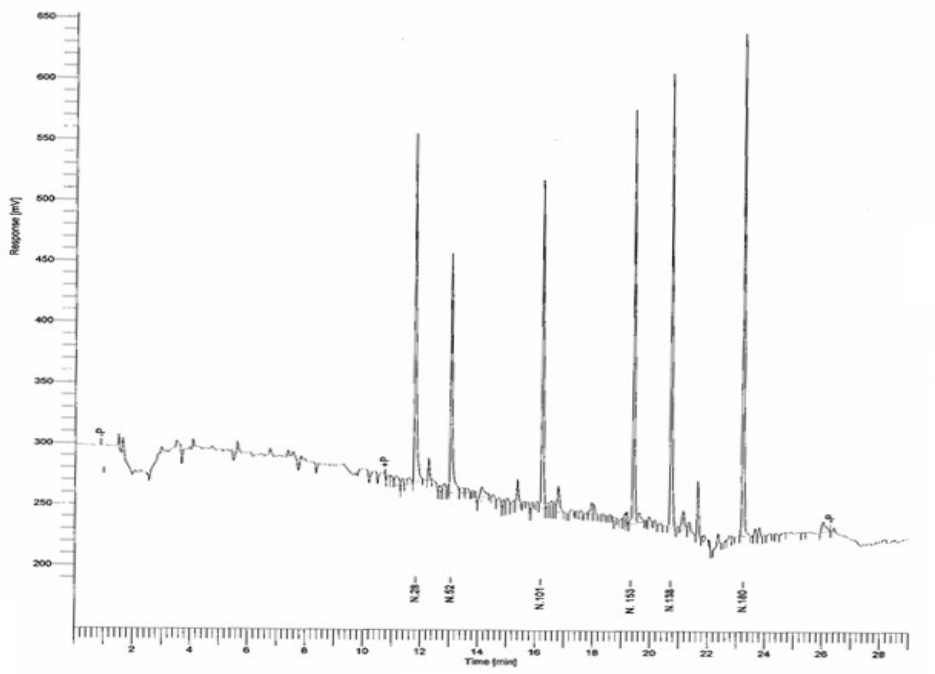


Figure 4.2 PCB-free mussel sample spiked at 10 ng/g with mix of six indicator PCBs.

4.4 Protein extraction and quantitative analysis

Mussel protein extracts were obtained using the procedure previously described. Protein concentration in each extract (Table 4.5) was assessed using the modified Bradford method previously cited and the quality of the extracts was checked by SDS-PAGE (Figure 4.3). Protein concentrations obtained in the different extracts ranged from 18,95 ±0,06 µg/µl to 24,27 ± 1,90 µg/µl . The narrow distribution of the protein amounts achieved for each sample demonstrated low variability in the extraction procedure.

Group	Mean Value µg/µl	Dev St µg/µl
C0	19,93	0,79
C1	18,95	0,06
C2	21,84	1,22
C3	20,17	0,99
T0	21,68	1,36
T1	22,50	1,61
T2	24,27	1,90
T3	22,10	1,17

Table 4.5. Concentration of protein extracts.

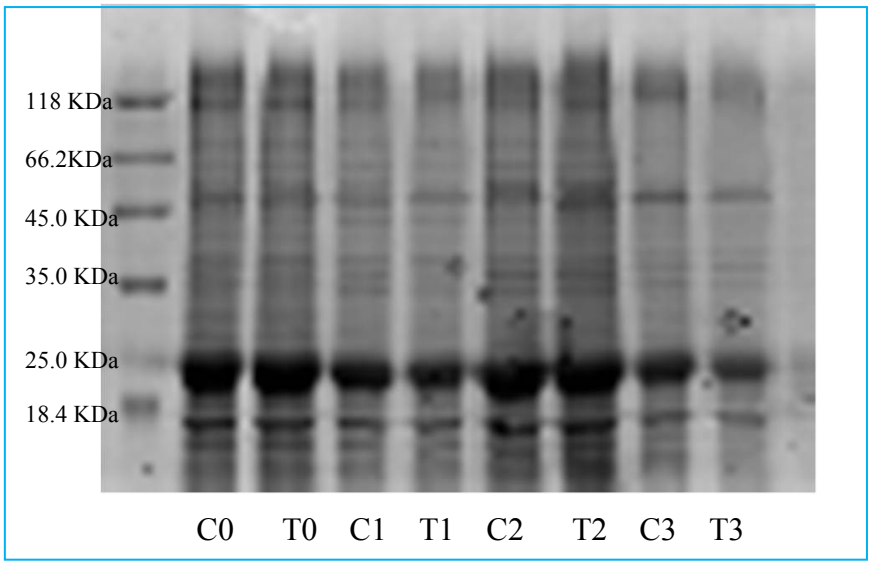


Figure 4.3. A coomassie stained 1-D 12% SDS-PAGE showing protein compositions of eight different protein extracts. 1 µl of protein extracts was loaded on each lane.

4.5 OFFGEL approach

A variety of methods exist for fractionation of protein or peptide samples before MS analysis to increase the number identified proteins and, in particular, to facilitate detection of low-abundance proteins; in that end, the use of OFFGEL separation is becoming one of the more common as a reliable method for separating peptides.

Peptides separation after trypsin digestion was performed for samples T2 and C2 which corresponded to mussels treated for two weeks and their controls, respectively, as described previously.

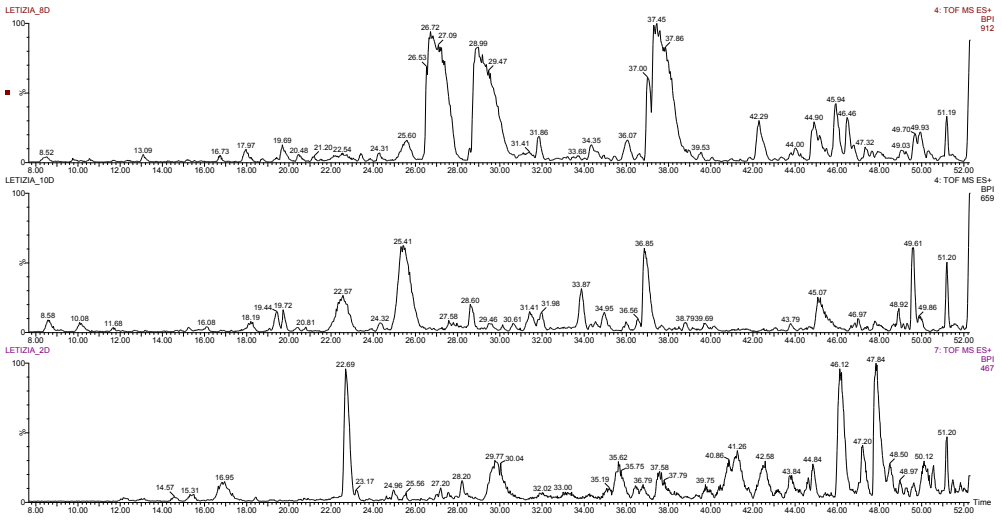


Figure 4.4 Chromatograms of fraction 2 (A), 10 (B) and 18 (C) of peptide mixture obtained from protein extract of sample T2 and separated with OFFGEL fractionator.

A total of 24 fractions were collected from OFFGEL separation and than analysed on a nanoHPLC/ESI/QTOF mass spectrometer. The pIs of the peptides recovered from each filter pad overall were consistent with the pI range of the underlying part of the IPG strip, demonstrating successful fractionation of a complex mixture of peptides generally according to pI. In particular, as expected, this method recovered acidic peptides from the acidic end (fraction 1), basic peptides from the basic end (fraction 20), and peptides with intermediate pIs from the middle of the gel (fraction 10) (Figure 4.4 A , B and C).

The chromatographic profiles of treated and control samples were very similar for all collected fractions; however, overlapping chromatograms (eg Figure 4.5) significant differences were observed for several peptides for almost all the collected fractions (Table 4.6).

N.Fraction	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
N.Peptides Down	1	5	4	6	4	3	4	2	4	5	6	1
N.Peptides Up	0	2	7	2	2	3	2	5	2	5	1	0
N.Fraction	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
N.Peptides Down	2	1	2	0	10	6	1	0	0	3	3	5
N.Peptides Up	0	2	1	0	1	2	1	0	0	1	1	0

Table 4.6 Number of peptides different between T2 and C2 for 24 fractions.

In Table 4.5 the number of peptides showing a significantly higher or lower concentration in sample T2 than in the control are listed. Peptides showing a lower amount in treated samples compared to the control were almost twice than those showing an higher amount one (78 versus 40). No clear relationship between different peptides and pH values were detected, but the peptides with significant differences were observed along the whole pH range (pH 3-10).

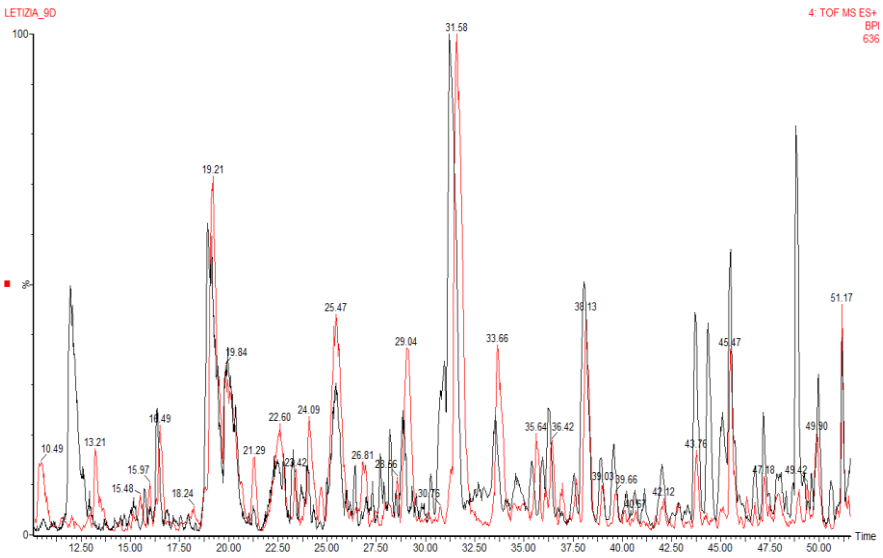


Figure 4.5 Overlayed chromatograms of fraction n.8: T2 (red line) and C2 (black line).

Peptides showing different concentration in treated and control samples were subjected to MS/MS analysis and the spectra of fragmentation were used for the search with Mascot in order to identify the corresponding proteins.

Unfortunately, when Mascot was used to analyze this data set no protein was identified, possibly because proteomic and genomic information on *Mytilus galloprovincialis* actually disposable in the protein data banks are insufficient.

4.6 2D-electrophoresis-based proteomic approach

4.6.1 Assessment of technical variability

Replication is central to experimental design and allows more robust data analysis. Technical replicates, also called repeated measures, address the technical error, or noise, in the experiment. In electrophoresis the technical noise may be caused by factors such as dust, irreproducibility of sample preparation and variation in gel running parameters (Karp et al. 2005). The technical replicates were obtained for 2-DE gels running gels in triplicate with the same sample; the uncertainty about the true reading for a given sample is reduced by taking multiple measurements. Scatter plots were used to represent the repeatability of the experimental technique (Figure 4.6). In these graphs, each protein spot has been plotted according to its volume in the first gel (x-axis) versus its volume in the second gel (y-axis). Protein intensity values were reported as normalized values. The values of the correlation coefficient r^2 between 0,874 and 0,988 shows a good accordance between replicates.

The 20 mussels selected for 2-DE analysis were considered numerous enough to adopted for the comparative analysis of protein expression among the groups considering exclusively quantitative differences in the expressed proteins that are common to all studied groups.

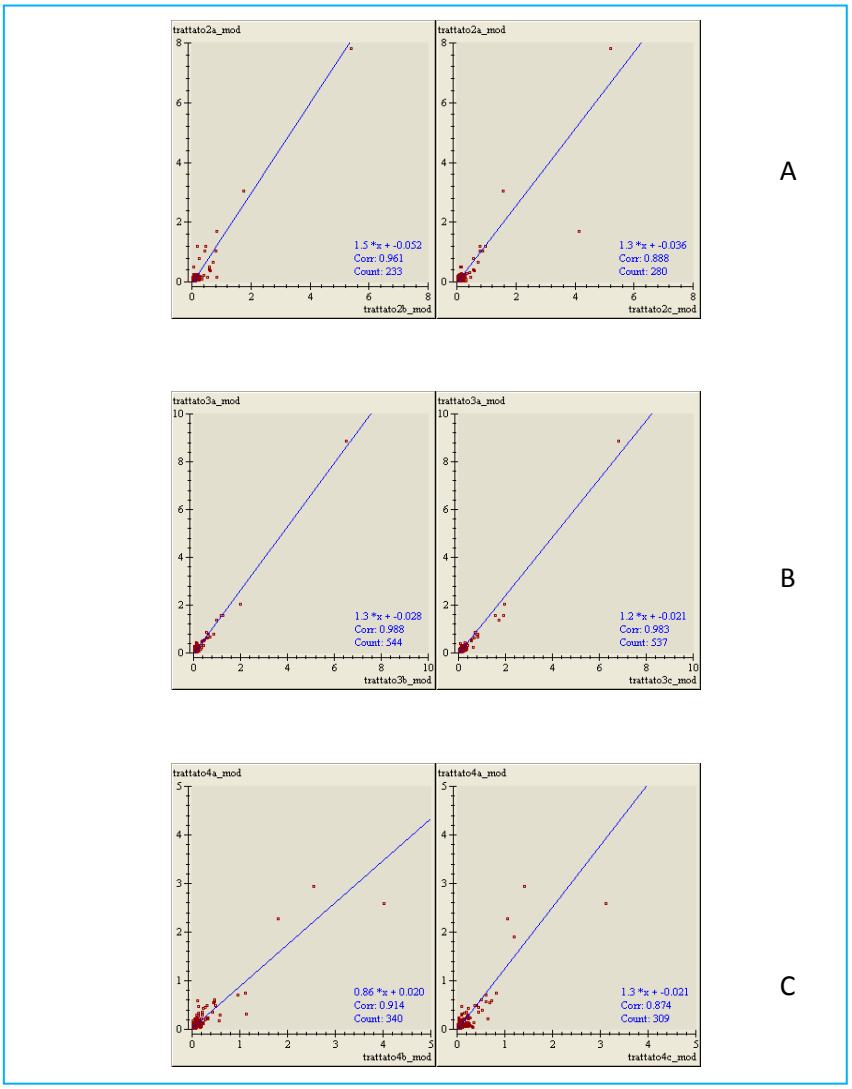


Figure 4.6 Repeatability scatter plots. A: sample T1; B: sample T2; C: sample T3.

4.6.2 Analysis of 2-DE protein maps

In this work, proteomics were applied to investigate the protein composition from *Mytilus galloprovincialis* exposed to a mixture of three PCBs. This study was based on the use of 2-DE and needed a preliminary step aimed to the optimization of the conditions for proteins extraction and separation.

High-resolution two-dimensional electrophoresis allows to separate complex mixtures of proteins, resolving thousands of genetic products in a single gel, and detecting changes in the order of 0.1 pH units in the first dimension and of 1 kDa in the second dimension (Hanash et al. 1986). To date, a few groups have applied proteomics to obtain chemical-specific protein expression signatures in mollusks. (Shepard et al 2000, Rodrigues-Ortega et al 2003). Moreover, all of the previously published researches analyzed changes in expression associated with exposure to one or more compounds in laboratory experiments considering only a single exposure time. In contrast, our is the first study trying to evaluate effects of chemical pollulants on proteome on a time course basis.

Protein expression profiles were studied for four groups of mussels: T0, T1, T2, T3 (Table 4.1) compared with the expression of the same protein spots resolved by 2DE from control bivalves (C0, C1, C2, C3).

The protein extracts were separated in well defined colloidal Coomassie Blue stained spots within isoelectric point pI and molecular mass intervals of 3 – 10 pH units and 10 – 250 kDa, respectively; therefore a bidimensional map for each treated and control group was obtained, and the normalized average ratio of expression for the spots present in the maps were calculated.

When proteomics analysis aimed to compare different patterns of protein expression are performed, an optimization of the protein maps resolution is required. In the present study, optimization of the electrophoresis separation was first carried out performing preliminary analysis on 7 cm strips.

Analytic experiments were then performed using longer strips (18 cm) thus achieving 2-D gels with resolution close to 1 pH unit. In order to detect the larger number of proteins, gels were loaded with 400 μg of proteins. As a preliminary study to verify similarity of the organism as a starting point of the two tank the proteomic profile of groups T0 and C0 were analyzed and no statistical significant differences between the two samples were observed. Figure 4.7 shows a typical two-dimensional gel of proteins from *Mytilus galloprovincialis* in the broad pH range of 3–10. This gel is representative of all groups given that upon visual inspection no quantitative differences were observed.

The quality of the achieved gels was determined by considering the parameters: resolution, definition, homogeneous distribution, morphology and clarity of the spots, minimum background, streaks, or veined bands, and no overlapping of proteins. Spots were quite homogeneously distributed across the entire pI range, clear, well-defined and their morphology appeared uniformly circular to oval, although some significant protein clusters of high molecular weight proteins appear. Ambiguous regions, such as crowded areas or areas containing high-molecular-weight proteins that were not well defined, were discarded. Some of the spots detected at the boundaries of the gels and therefore not satisfactorily resolved were also excluded from further analysis.

Moreover, qualitative (presence–absence) differences were discarded: only spots that were detected in all mussels were used to avoid expression variation artefacts due to allelic variation that modifies protein charge.

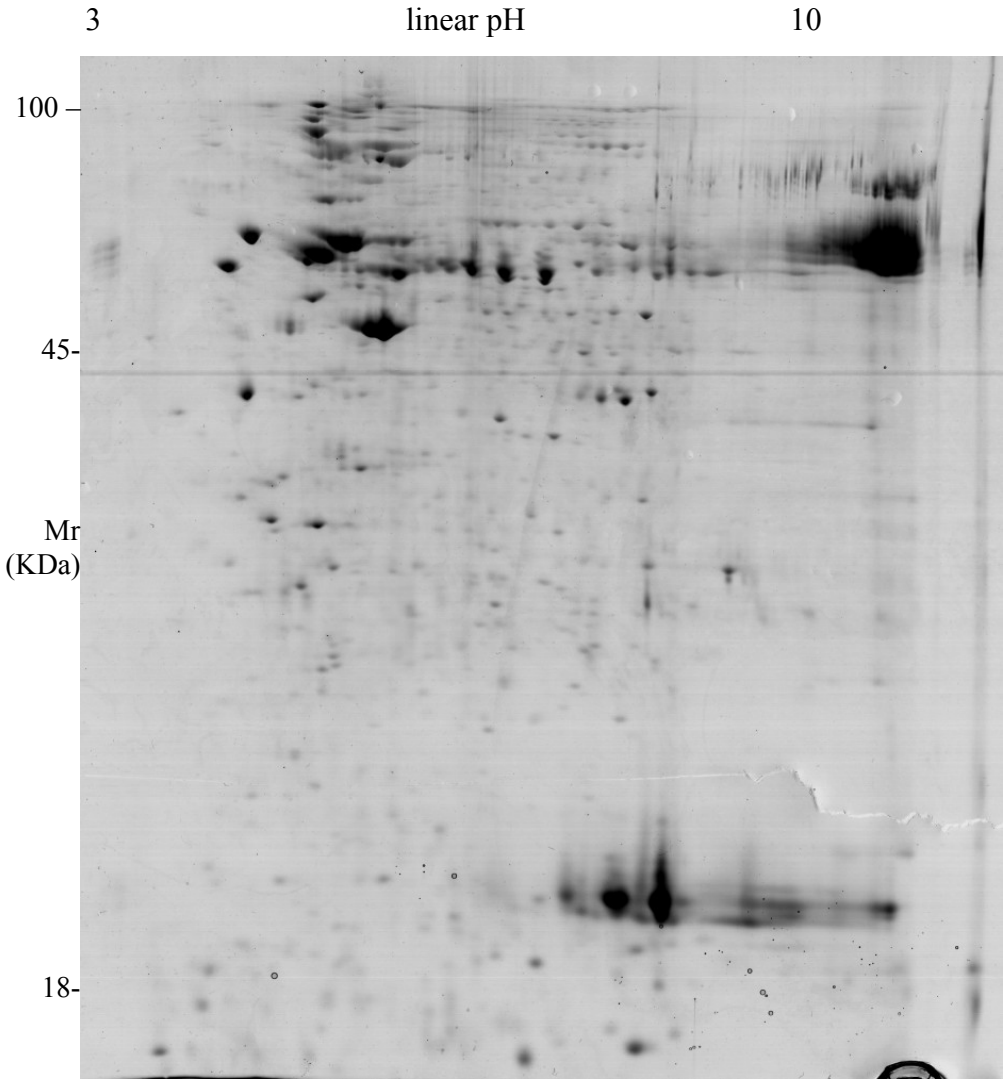


Figure 4.7 Typical colloidal Coomassie Blue image of two-dimensional gel of M. Galloprovincialis. The map is oriented with acidic pI to the left, basic pI to the right, low Mr at the bottom, and high Mr at the top of the figure. Relative positions of the molecular mass marker proteins are indicated on the left.

Some spots overlap or are associated with streaking; however, these spots usually appear close to well-shaped spots, and this may indicate that streaks such as these were due to individual properties of the streaking proteins rather than to problems of the 2-DE technique employed (Klose and Kobalz 1995). Proteins with a molecular weight above 200 kDa could not be seen because of the limited capacity of large proteins to be introduced into the gel of the first dimension (Tsuji et al. 1999).

Figure 4.7 shows that most of the proteins were focused in the 4-7 pI range and molecular mass between 40 and 80 kDa .

Using the spot-finding protocol of the Master Image Platinum software, the number of spots detected on gels varied between 963 and 1195. The number of spots detected was always below that expected from the resolution capability of the technique, which has the potential of separating thousands of proteins (Klose and Kobalz 1995); this result is related to our choose to use a conservative approach for the analysis of the gels, counting only clearly defined spots and not considering outermost and confusing areas.

In order to identify proteins whose levels were significantly affected by PCBs treatment, we have used Student's t test in order to check whether the levels of some proteins in mussels might be specifically correlated with PCB contamination comprised between 44 and 81 ppb (Table 4.3).

According to our statistical threshold and considering the three comparisons between treatments, statistically significant differences ($P<0.05$) in the intensity levels of 96 protein spots were observed. The number of spots up- or down-regulated by each treatment is summarized in Table 4.7.

Treatment Time	Up-regulated proteins	Down-regulated proteins
1° week	**7**	**15**
2° week	**12**	**32**
3° week	**8**	**22**

Table 4.7 . Number of differentially expressed protein in exposed mussels.

PCBs had a predominantly decreasing effect on protein expression profiles considering all exposure times. In fact, exposure to 30µg/l PCB 135, 153 and 180 for one week resulted in the up-regulation of seven proteins and down-regulation of fifteen. The intermediate period of exposure (2 weeks) induced up-regulation of twelve proteins and down-regulation of thirtytwo polypeptides. Mussels treated with PCB mixture for three weeks exhibited eight up-regulated and twentytwo downregulated protein spots.

Nine proteins (4 up and down 5) were differentially expressed only following the first week of exposure, 28 proteins (10 up and 18 down) only following the second week and 12 (5 up and 7 down) only following the third week. Moreover 4 proteins (1 up and down 3) were in common between the first and the second week of exposure, 6 (2 up and 4 down) between the first and third weeks and 9 (1 up and 8 down) between the second and third week. Only three of the down-regulated proteins and no up-regulated ones were common to the three tested groups (Figure 4.8).

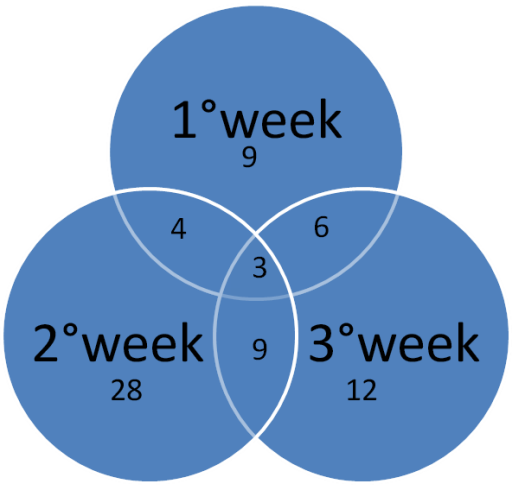


Figure 4.8 Venn diagram representing differentially expressed spots in common for the different experimental groups.

Three master image showing the statistically relevant spots were therefore obtained (Fig.4.9, 4.10 and 4.11) for groups T1, T2 and T3. In all three groups, both up-regulated and down-regulated spots were situated in the central part of the 2-DE map with acid or neutral pI e well distributed, between 80 and 15 kDa. Spots were numbered in order of appearance for low to high pI (left to right) and high to low Mr (top to bottom).

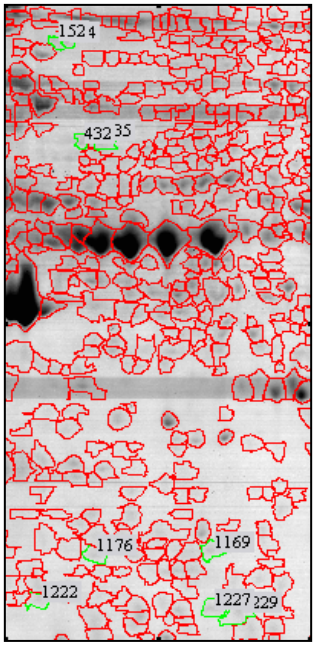


Figure 4.9 2-DE gel maps showing the 9 up/down regulated spot protein related only to group T1.

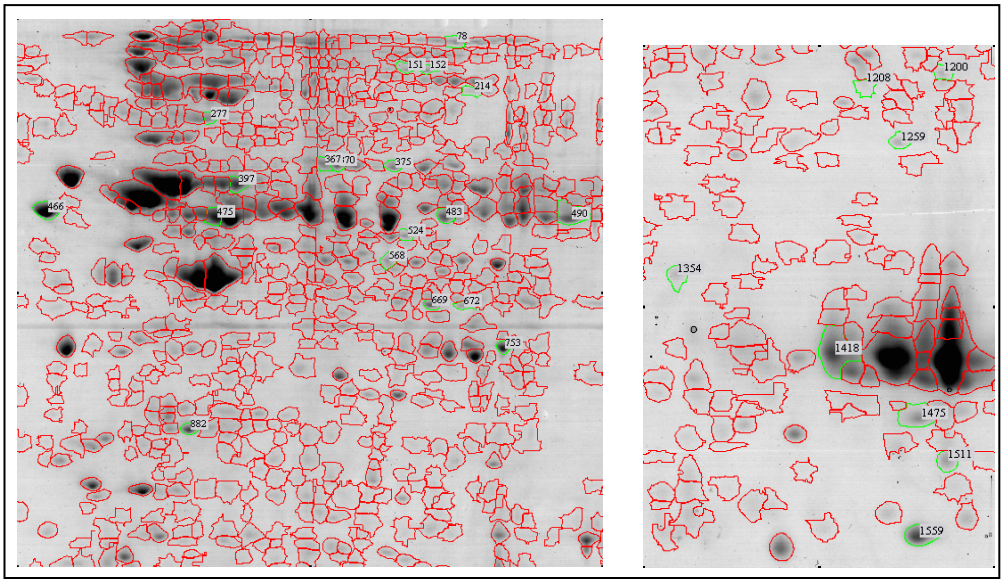


Figure 4.10 2-DE gel maps showing the 28 up/down regulated spot protein related only to group T2.

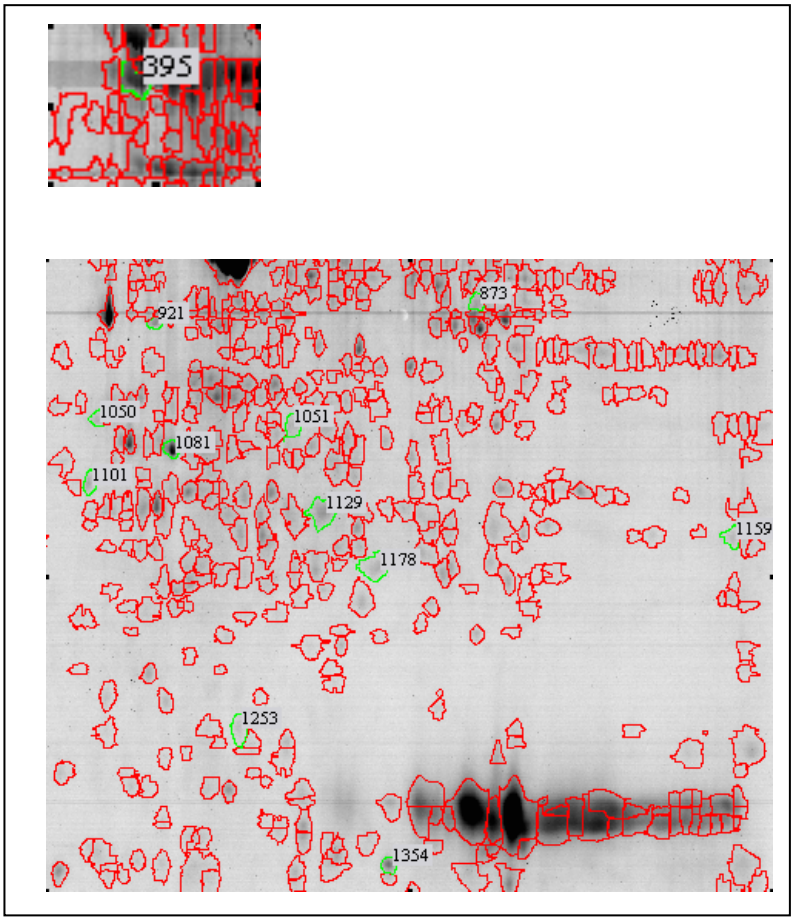


Figure 4.11 2-DE gel maps showing the 12 up/down regulated spot protein related only to group T3.

Figure 4.12 shows some examples of detailed view of spots taken from different high-resolution gels of PCB-exposed and control mussels while figure 4.13 gives a 3D view of same spots.

Mussels exposed for two weeks showed the highest number of statistically significant differentially expressed spot.

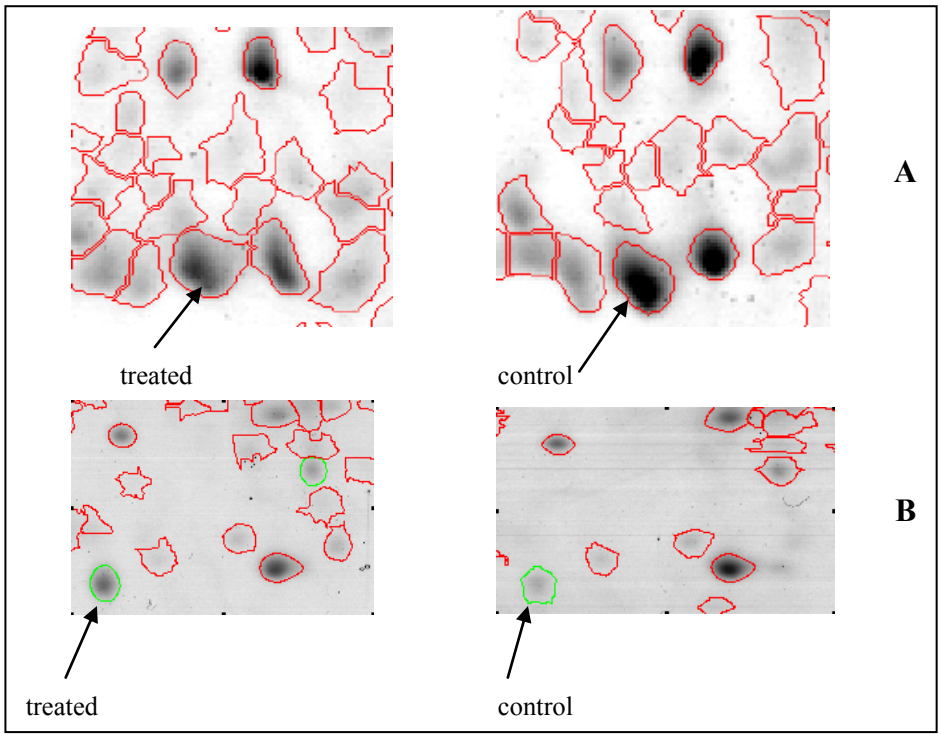
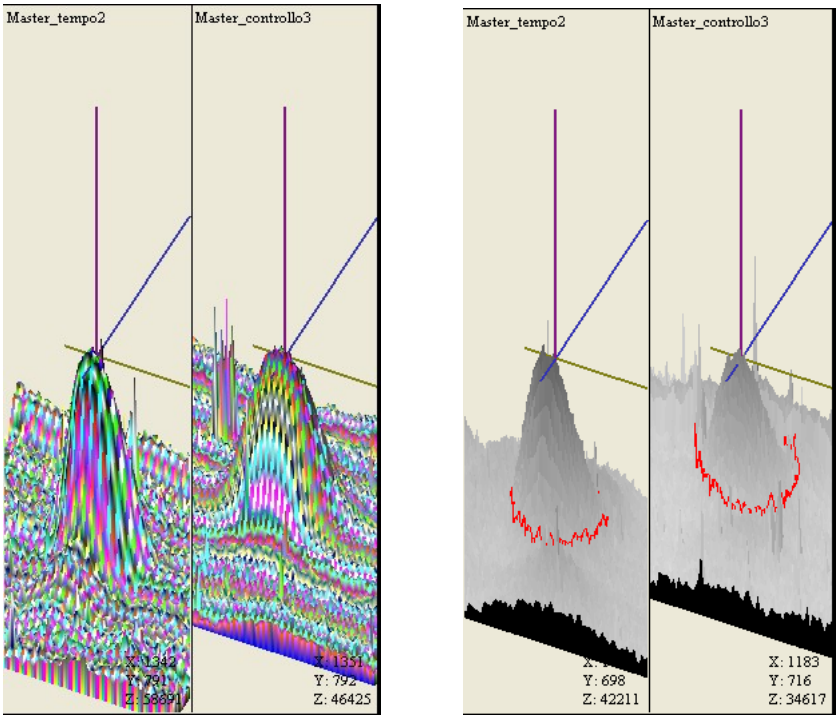


Figure 4.12 Details of protein spots that have been found affected by PCB treatment. **A.** Spot number 669 from Master gel T2 and its correspondent control. **B.** Spot number 1418 from Master gel T2and its correspondent control.

In the first group , the increase in abundance ranged between 1.45 and 2.76, while the ratio decrease reached 3.61 (Figure 4.14). In the second experimental exposed group, the highest ratio increase and decrease were 1.29 and 3.10, respectively (Figure 4.15). In the third group a low increase in protein expression (between one- and two fold) was observed in 5 spots, moderate increase (between two and four fold) in 3 spots and no high increase (more than fourfold). Decrease in protein expression between one- and twofold was detected in 15 spots, between two- and fourfold in 7 (Figure 4.16).



A **B**

Figure 4.13 3D view for the comparision of corresponding spots in different gels **A.** Spot number 669 from Master gel T2 and its correspondent control. **B.** Spot number 1418 from Master gel T2 and its correspondent control.

It was also remarkable that values of increases and decreases protein levels were all in the same range and in no case values higher than 4-fold were observed.

Only the spots with altered expression confirmed at low and high resolution underwent further studies aimed to identify the corresponding proteins. Therefore these spots were excised from stained 2D gels and submitted to in gel trypsin digestion; resulting peptide mixtures were then analyzed by high resolution mass spectrometry techniques.

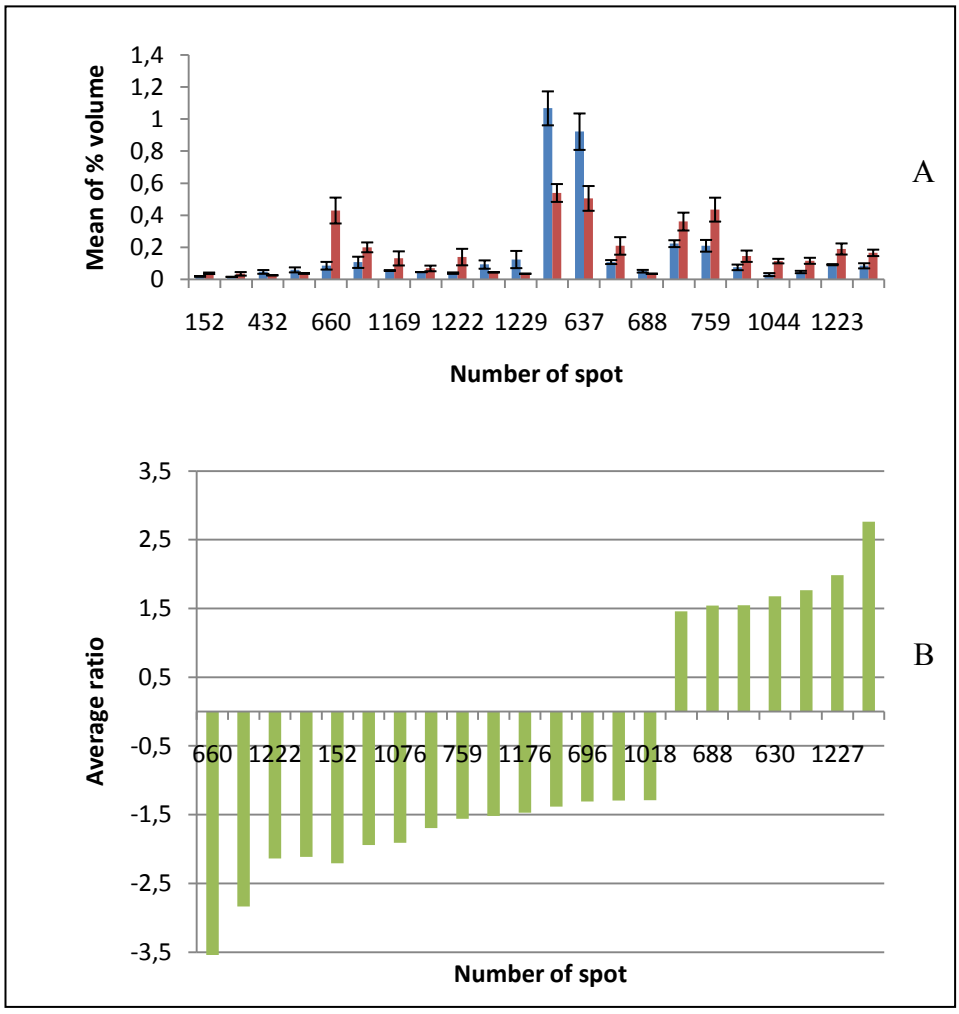


Figure 4.14 Proteins differentially expressed in T1 group against C1 group.
A. The vertical axis corresponds to the average % volume. In the horizontal axis spots are organized in increasing order.
B. The vertical axis corresponds to the average ratio of expression, above the 0 value for the up-regulated proteins and below the 0 value for the down-regulated proteins. In the horizontal axis the up-regulated proteins are organized with the highest values on the right side , and the down-regulated proteins show the highest values on the left side.

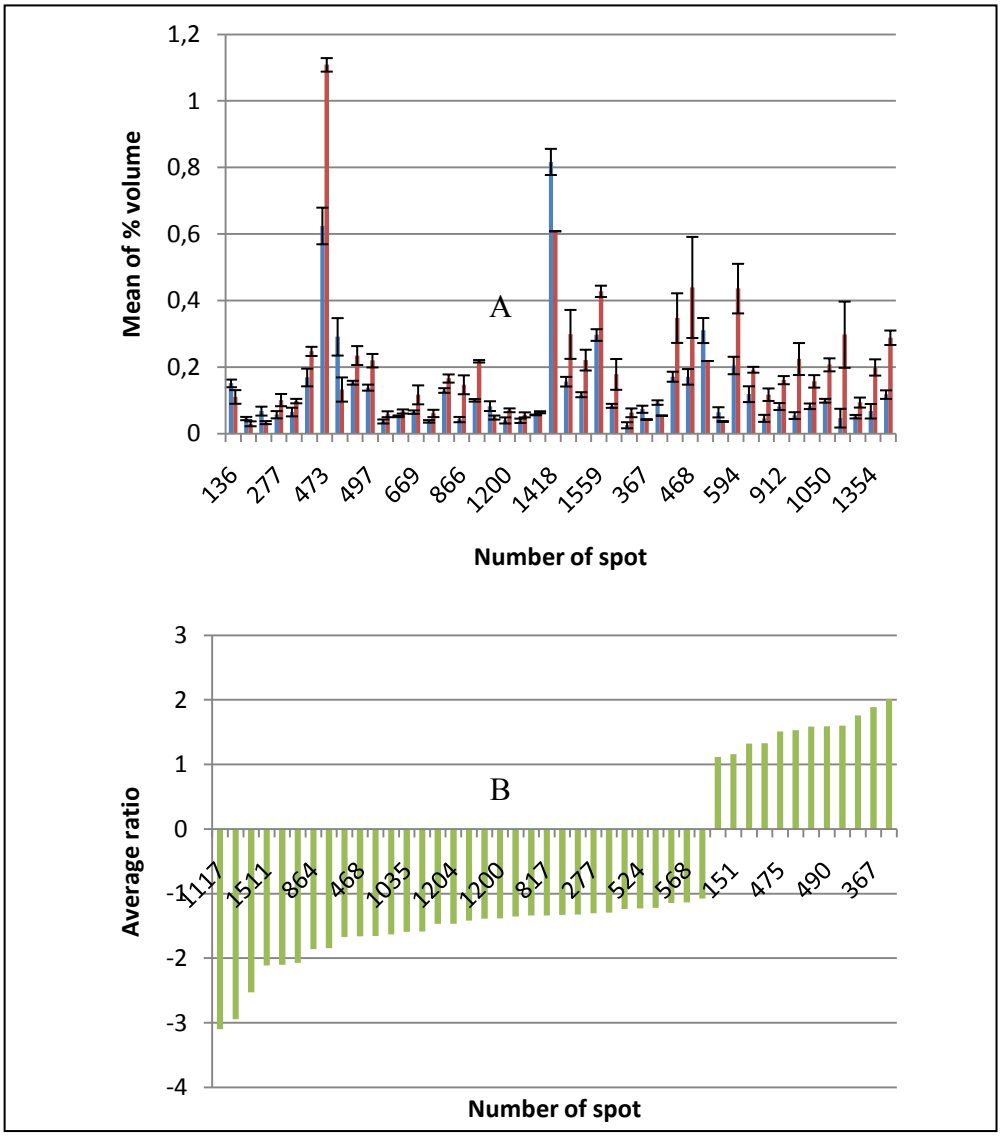


Figure 4.15 Proteins differentially expressed in T2 group against C2 group.
A. The vertical axis corresponds to the average % volume. In the horizontal axis spots are organized in increasing order.
B. The vertical axis corresponds to the average ratio of expression, above the 0 value for the up-regulated proteins and below the 0 value for the down-regulated proteins. In the horizontal axis the up-regulated proteins are organized with the highest values on the right side , and the down-regulated proteins show the highest values on the left side.

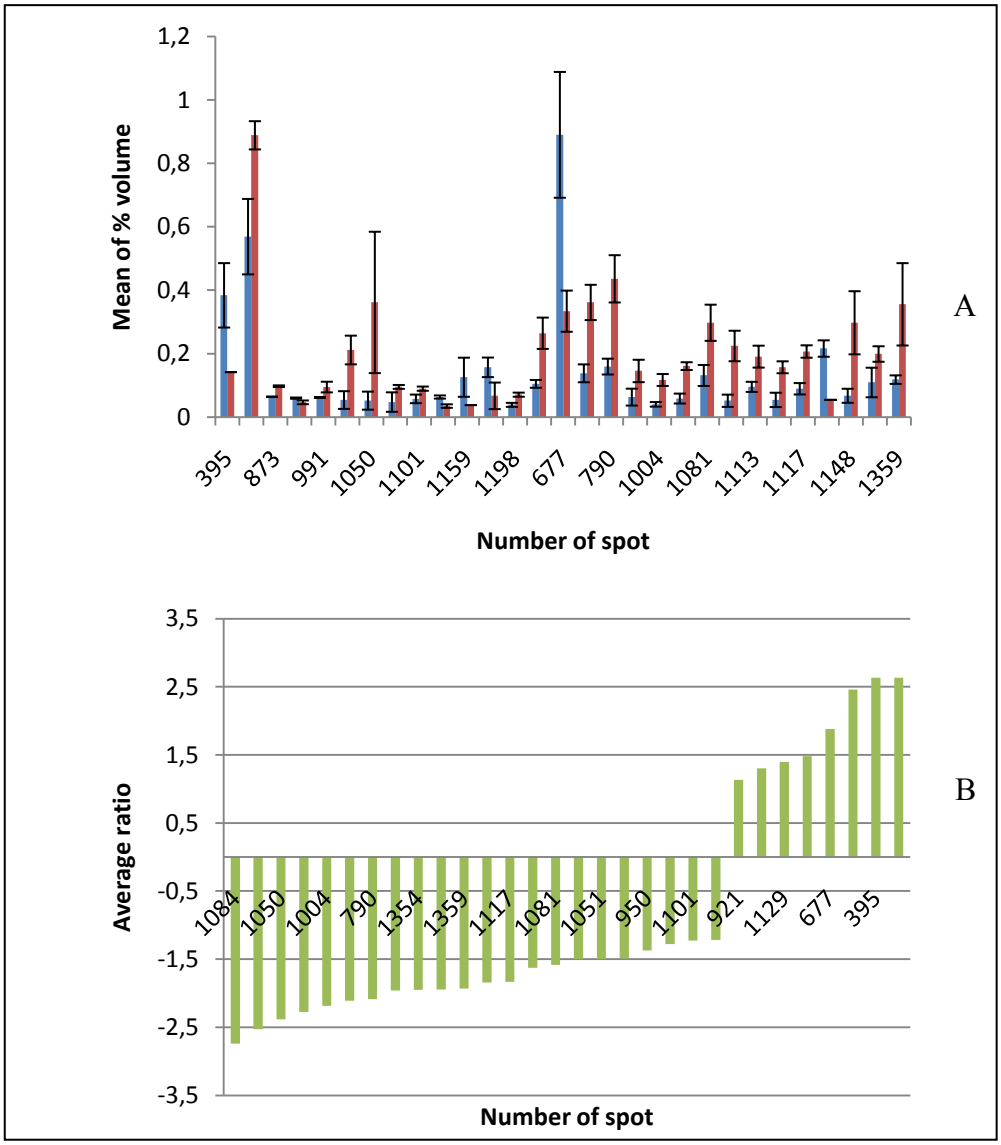


Figure 4.16 Proteins differentially expressed in T3 group against C3 group.
A. The vertical axis corresponds to the average % volume. In the horizontal axis spots are organized in increasing order.
B. The vertical axis corresponds to the average ratio of expression, above the 0 value for the up-regulated proteins and below the 0 value for the down-regulated proteins. In the horizontal axis the up-regulated proteins are organized with the highest values on the right side , and the down-regulated proteins show the highest values on the left side.

4.6.3 Mass spectrometric protein identification

We identified a group of differentially expressed proteins. Evident up- or down-regulation suggests that those proteins could become biomarkers of exposure to a PCB mixture. Protein identification was accomplished by either MALDI-TOF or ESI-MS/MS analysis of peptides produced by proteolytic digestion of spots excised from the 2-DE maps. However, the MALDI-TOF mass spectra (peptide mass fingerprinting) of the proteins didn't allow to achieve a reliable identification due to the limited applicability of conventional protein identification methods to the proteomes of organisms with non-sequenced genomes such as *M. galloprovincialis*, Thus this technique was used only to evaluate the efficiency of trypsin digestion process.

Applying nanoHPLC-nanoESI-MS/MS analyses (e.g. figure 4.17) 36 proteins, representing about 50% of the total number of spots considered of interest, were identified. Sequence information of several peptides were obtained and protein searches were performed in available databases.

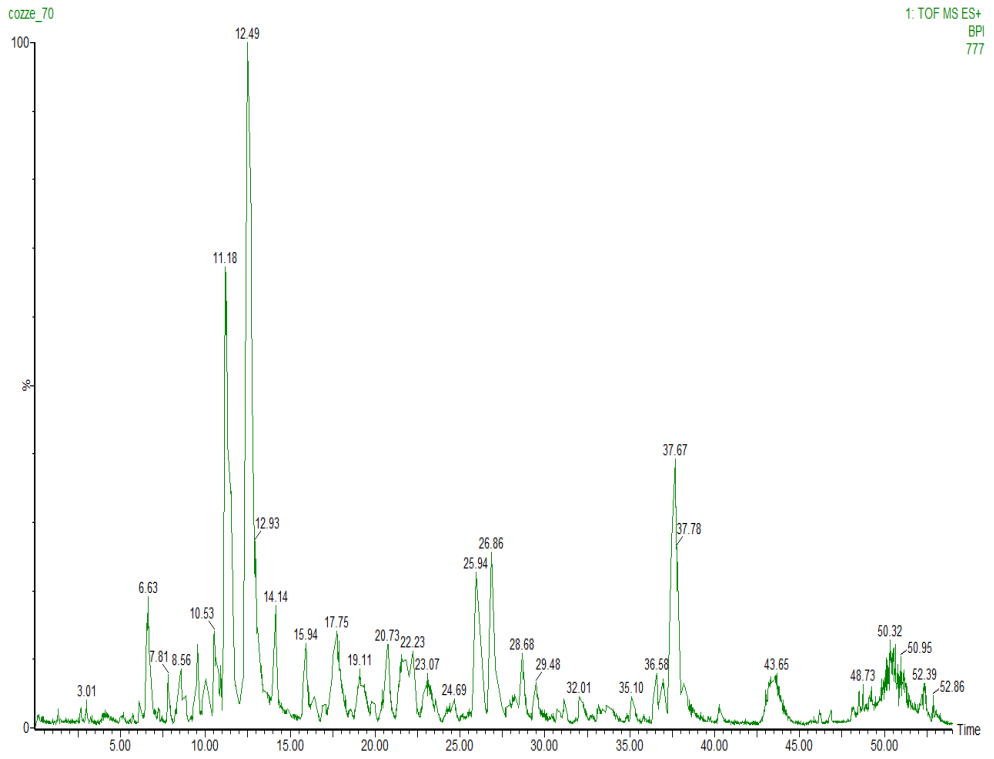


Figure 4.17 Chromatographic profile of peptides from spot n.70 by nanoHPLC-nanoESI-Q-TOF.

Since *M. galloprovincialis* is a non-model organism, most of the protein sequences are absent from databases. Consequently, protein identification was subjected to BLAST analysis, to identify possible protein homologues. In the case of same amino acid sequence and same access number, NCBI blast gives the species with the best score and homology. The remaining proteins were not identified, in spite of having these two methods available for our use and the fact that the spectra obtained were of good quality.

Proteins identified in this work were divided depending on time of exposure into several classes. The first class comprised proteins differentially expressed only after one week of treatment, the second class only after two weeks and the third class only after three weeks (Tables 4.8, 4.9 and 4.10). Other classes comprised proteins differentially expressed in all the groups (T1, T2 and T3), or were formed by proteins common to two groups (T2 an T3 or T1 and T3 or T1 and T2) are summarized in tables 4.11, 4.12, 4.13 and 4.14.

All these tables include spot number, accession number, name of specie and principal functional pathways.

Spot number	Up/Down regulation	Identification	Accession number	Organism	Functional pathway
1169	Down	putative TyrA protein	gi|145896447	*Schistosoma japonicum*	amino acid metabolism
1176	Down	small heat shock protein 24.1	gi|347545633	*Mytilus galloprovincialis*	response to stress
1222	Down	prohibitin	gi|238643256	*Oreochromis niloticus*	respiratory chain
1227	Up	dihydropteridine reductase	gi|21282726	*Acromyrmex echinatior*	protein folding
1229	Up	enoyl-CoA hydratase	gi|223027768	*Danio rerio*	peroxisomal β-oxidation

Table 4.8 List of identified proteins differentially expressed after only 1 week of exposure.

Spot number	Up/Down regulation	Identification	Accession number	Organism	Functional pathway
370	Up	fascin	gi|145902263	*Haliotis diversicolor*	cytoskeleton
375	Down	protein disulfide isomerase	gi|149382436	*Amblyomma variegatum*	protein folding
475	Up	beta-tubulin, partial	gi|32967412	*Paracentrotus lividus*	cytoskeleton
669	Down	calponin	gi|14422379	*Mytilus galloprovincialis*	cytoskeleton
753	Down	arginine kinase	gi|301341836	*Conus novaehollandiae*	amino acid metabolism
1200	Down	hillarin	gi|145902263	*Haliotis diversicolor*	zinc ion binding
1259	Down	small heat shock protein 22	gi|347545631	*Mytilus galloprovincialis*	response to stress
1511	Down	C1q-like protein	gi|343455230	*Mytilus edulis*	cytoskeleton

Table 4.9 List of identified proteins differentially expressed after only 2 weeks of exposure.

Spot number	Up/Down regulation	Identification	Accession number	Organism	Functional pathway
921	up	raminin receptor	gi|126697324	*Haliotis discus discus*	strucrural
1050	down	proliferating cell nuclear antigen	gi|145895072	*Litopenaeus vannamei*	DNA synthesis
1051	down	electron transfer flavoprotein subunit alpha	gi|58307490	*Megachile rotundata*	electron transport
1081	down	14-3-3 protein	gi|310706696	*Chlamys farreri*	regulatory
1101	down	cathepsin L	gi|145883962	*Pinctada fucata*	proteolysis
1129	up	rootletin-like	gi|291239961	*Saccoglossus kowalevskii*	cytoskeleton
1178	up	glutathione S-transferase	gi|22094809	*Mytilus galloprovincialis*	detoxication of xenobiotics

Table 4.10 List of identified proteins differentially expressed after only 3 weeks of exposure.

In the first group, five of a total of nine spots forming the minimal PES were identified, in the second group, only eight of twenty-eight, in the third grouo seven of twelve. For proteins common to at least two groups, sixteen of a total of twenty-two proteins were identified.

Spot number	Up/Down regulation	Identification	Accession number	Organism	Functional pathway
759 594 1004	down	myosinase-I	gi|238638946	*Todarodes pacificus*	zinc ion binding
1076 864 1116	down	guanine nucleotide-binding protein subunit beta	gi|121014	*Euprymna scolopes*	energy
1211 1035 790	down	actin	gi|33469507	*Strongylocentrotus purpuratus*	cytoskeleton

Table 4.11 List of identified proteins differentially expressed after 1, 2 and 3 weeks of exposure.

Spot number	Up/Down regulation	Identification	Accession number	Organism	Functional pathway
1020 817	down	glyceraldehyde-3-phosphate dehydrogenase	gi|29423699	*Lytechinus variegatus*	energy
660 497	down	matrilin4	gi|58307379	*Danio rerio*	cytoskeleton

Table 4.12 List of identified proteins differentially expressed after 1 and 2 weeks of exposure.

Spot number	Up/Down regulation	Identification	Accession number	Organism	Functional pathway
1018 950	down	cytosolic malate dehydrogenase	gi|73656269	*Mytilus trossulus*	energy
1223 1113	down	voltage-dependent anion channel 2	gi|145896447	*Haliotis diversicolor*	energy
637 677	up	enolase	gi|14161517	*Cryphalus abietis*	energy
696 746	down	gelsolin	gi|72089178	*Strongylocentrotus purpuratus*	structural

Table 4.13 List of identified proteins differentially expressed after 1 and 3 weeks of exposure.

Spot number	Up/Down regulation	Identification	Accession number	Organism	Functional pathway
473 700	down	collagen alpha-1(XII) chain-like	gi|58306751	*Anolis carolinensis*	structural
866 999	down	EP protein precursor	gi|34304719	*Mytilus edulis*	calcium ion binding
912 1030	down	tropomyosin	gi|212815279	*Mytilus galloprovincialis*	cytoskeleton
1050 1117	down	paramyosin	gi|42559342	*Mytilus galloprovincialis*	cytoskeleton
1094 1135	up	elongation factor 1-beta	gi|223027747	*Danio rerio*	cytoskeleton
1204 1198	down	Rho GDP dissociation inhibitor	gi|149382257	*Schistocerca gregaria*	GDP/GTP exchange
1528 1359	down	myosin regulatory light chain A	gi|127163	*Mizuhopecten yessoensis*	calcium ion binding

Table 4.14 List of identified proteins differentially expressed after 2 and 3 weeks of exposure.

The identified differentially expressed proteins following PCB exposure represented a heterogeneous group and although it is difficult to classify them into perfectly defined groups, basically, they can be grouped into four broad functional classes :

- cytoskeletal and myofibrillar proteins:
  fascin, beta-tubulin, calponin, C1q-like protein (group T2); raminin, rootletin-like (group T3); matrilin4 (T1 and T2); gelsolin (T1 and T3); collagen alpha-1(XII) chain-like, tropomyosin, paramyosin, elongation factor 1-beta, myosin regulatory light chain A (T2 and T3); actin, myosinase-I (T1, T2 and T3).
- proteins associated with oxidative stress response:
  small heat shock protein 24.1, prohibitin (group T1); small heat shock protein 22 (group T2); glutathione S-transferase (group T3).
- proteins associated with energy metabolism :
  enoyl-CoA hydratase (group T1); arginine kinase, hillarin (group T2); proliferating cell nuclear antigen, electron transfer flavoprotein subunit alpha, 14-3-3 protein (group T3); glyceraldehyde-3-phosphate dehydrogenase (T1 and T2); malate dehydrogenase, voltage-dependent anion channel 2, enolase (T1 and T3); EP protein precursor (T2 and T3); guanine nucleotide-binding protein subunit beta (T1, T2 and T3).
- proteins associated with rearrangement and synthesis of native structures:
  putative TyrA protein, dihydropteridine reductase (group T1), disulfide isomerase (group T2); cathepsin L (group T3); Rho GDP dissociation inhibitor, (T2 and T3).

Some of these proteins are represented in figure 4.18.

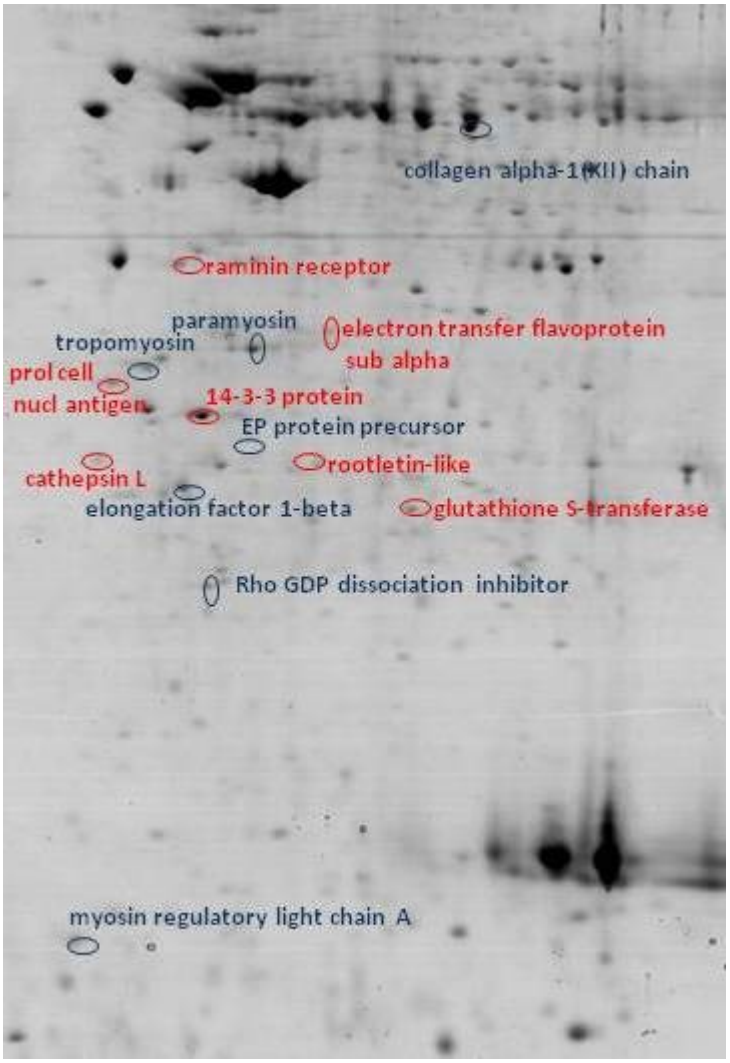


Figure 4.18 Identified proteins for 2 and 3 weeks of exposure to PCBs (blu ones) and only for 3 weeks of treatment (red ones) represented on Master gel T2.

4. 7 Discussion

As with other biological stress, the adaptation to environmental pollution involves changes in protein expression, that can be produced specifically in response to a particular contaminant, and also in a concentration-dependent manner (Shepard et al. 2000). To date, few reports have described changes in protein expression profiles in marine organism exposed to pollutants (Rodrigues et al. 2012). The present work was aimed to provide preliminary qualitative studies of the changes in protein expression induced by polychlorinated pollutants in the bivalve *Mytilus Galloprovincialis* to identify new putative protein biomarkers.

Our results indicate that exposure of the mussel *M. Galloprovincialis* to PCB 138, 153, 180, three non-dioxin-like PCBs, had a mainly downregulating effect on protein expression/level, probably reflecting the potent inhibitory action of these compounds towards several bio-transforming and detoxifying cellular systems (Alzieu 2000).

The limited presence in available databases of proteins from mollusks or other invertebrate species used as bioindicators in pollution monitoring programs, generally restricted to cytoskeletal proteins, caused the identification of only 36 of the 71 studied proteins. Significantly, the proteins with altered expression profiles identified in the present work turned out to be related to structure/function of cytoskeleton, which has been proposed as one of the first targets of oxidative stress. (Rodriguez-Ortega et al. 2003; Miura et al. 2005). This can reflect the abundance of these proteins in all cell types, but also indicate that they could be major targets of pollutant-related damages. Cytoskeletal proteins are related to plasma membranes, through which pollutants enter the cell, possibly explaining membrane labilization as a major

cellular biomarker of environmental pollution (Gómez-Mendikute et al. 2002). Alterations in actin cytoskeleton and microfilaments have long been reported in situations of cellular stress and apoptosis (Alvarez and Sztul 1999; Bursch et al. 2000). Taking in consideration this knowledge we can infer that the changes in these proteins observed in this work translate to a condition of physiological stress and cellular injury in both bivalve species exposed to PCB. Actin is one of the most abundant proteins in the cell, being a fundamental component of cytoskeleton in muscle and non-muscle cells. It represents 12–15% of total protein in most of non-muscle cells, and about 30% in muscle cells (Kekic and dos Remedios 1999).

The wide range of proteins affected suggests that PCBs have profound effects on various biological processes, such as the general stress response, energy metabolism, in addition to the already described cytoskeleton. The function of these proteins can provide new clues on the molecular mechanisms by which PCBs induce toxicity in mussels. Within the cells, proteins can be exposed to highly reactive molecules and to conditions that favour denaturation. Additionally, protein damage can be induced by xenobiotic exposure. A critical function of molecular chaperones and intracellular protein degradation is to serve as a quality control system that eliminates misfolded or damaged proteins to avoid their interference with normal cell function and viability (Goldberg 2003). Damaged proteins are generally either rescued by chaperones, or degraded by proteases, or they form insoluble aggregates, in particular when the chaperone/protease machinery is overwhelmed (Yerbury et al. 2005). In this study some chaperones such as small heat shock protein 24 and small heat shock protein 22 have been identified.

Heat shock proteins (HSPs) are evolutionarily ancient and highly conserved intracellular molecular chaperones that non covalently bind to

exposed hydrophobic surfaces of un/misfolded proteins. Enhanced synthesis of HSPs occurs in almost all animals as a response to heat shock, but also to environmental, chemical and physiological stresses. Hsp inductions are markers of multiple stress exposures, and the Hsp70 family comprises the most important proteins responsive to toxic compounds (Goksoyr 1995; Solé et al. 2000). However, other families of these proteins, less characterized in mollusks, such as Hsp60, Hsp20–30, and Hsp90, have been reported to be induced in mussels exposed to hydrocarbons and copper (Snyder et al. 200).

Disruption of energy metabolism has often been associated with exposure to xenobiotics. To date, toxicological studies dealing with the responses of metabolic enzymes activities to chlorinated compounds remain largely limited. Nevertheless, exposure of common some aquatic species to different pollulants led to the down-regulation of genes encoding proteins that were mainly involved in energy metabolism and oxidative phosphorylation (Dorts et al. 2011).

With this work we demonstrated the high degree of sensitivity of proteomic approach and their utility in toxicological studies, as we have shown that protein expression varies significantly between examined groups. A challenge of proteomics would be to correlate biological response to environmental quality conditions. The evaluation of the results suggests that analysis can be used to identify specific PES in response to pollutants. This proteomics approach can be considered to be a valuable and promising tool for the development of environmental research.

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 proteome after exposure to those three pollutants was adaptive responses to oxidative stress. Moreover this could represent a starting point in the search of new specific molecular markers and can serve as the basis of future investigations that will further characterize the biology of the genus *Mytilus*.

5 Conclusion

The present thesis aimed to make *Mytilus galloprovincialis* accessible to ecotoxicoproteomics applications and to investigate the potential of proteomic approaches with this mussel for identifying protein expression signatures and discovering new molecular biomarkers. These challenges were addressed in different consecutive steps.

A large number of mussels were exposed to PCB mixture at a suitable concentration for different times (1, 2 and 3 weeks) and we investigated the effects of bivalve exposure to these environmental contaminants. Chemical analysis of PCBs were conducted and demonstrated effectiveness of the treatment.

Another step included the establishment of appropriate 2-DE conditions for *Mytilus galloprovincialis* protein samples and protein identification success with MS based methods after 2-DE separation.

Well separated protein patterns could be achieved for all investigated mussel groups and subsequent MS based identification of the proteins was possible. The identification of candidate biomarkers was based on the finding that toxicant exposure induced changes in protein patterns. The 2-DE analysis revealed specific protein patterns for each exposed sample.

Therefore proteins specificly or unspecificly responding to toxic stress were found. The patterns of significantly changed proteins were substance and time of exposure dependent. Additionally, some proteins unspecifically changed in expression during all three weeks. Hence, it was concluded that a toxicoproteomics approach in *Mytilus Galloprovincialis* enables the detection

of substance specific effects and more general stress responses in the cellular protein pattern and allows the identification of candidate protein biomarkers.

Most of differentially expressed proteins were identified despite the lack of genomic and proteomic available information for *Mytilus* spp. and other invertebrates species.

Identification of the proteins of interest is necessary to relate these proteins to biochemical pathways, to obtain insights in the molecular mechanisms of toxicity and to establish them as novel biomarkers.

Based on their function it can be assumed that PCBs alter several important metabolic pathways, namely the cytoskeleton and related genetic information (e.g. tubulin, tropomyosin and actin) oxidative stress response (e.g. small heat shock proteins and glutathione S-transferase), the energy metabolism (e.g. glyceraldehyde-3-phosphate dehydrogenase, malate dehydrogenase and enolase) and the metabolism of native structures (e.g. dihydropteridine reductase, disulfide isomerase).

Alterations in the expression of cytoskeleton proteins were reported in the bivalve's organs in result of animal exposure to PCBs, translating a condition of physiological stress, moreover it was suspected that toxicant exposure costs energy and leads to an altered energy metabolism.

The results demonstrate that exposure to PCB mixture induces significant changes in proteomic profiles in mussels. The protein expression profiles resulting from our research could be used to distinguish control from exposed organisms. Preliminary proteomic results obtained in our laboratory support the capability of PBCs to alter proteome expression in a specific compound-response pattern signature.

This work demonstrates the importance of proteomics to assess the biochemical changes and the physiological conditions of the organisms. Moreover it is a promising approach towards the elucidation of the underlying mechanisms of toxicity in bivalves induced by polychlorinated biphenyls.

In conclusion, the results of this thesis demonstrate and confirm the high potential of ecotoxicoproteomics with mussels for sensitive effect detection at low toxicant concentrations, for obtaining insights in molecular processes underlying toxicity, and for discovery of novel biochemical biomarkers. Of course, some research is still necessary to elaborate the powerful tool of toxicoproteomics to identify new toxicity targets, discover novel biochemical biomarkers or for hazard characterization. Although the aim of this project was to probe the utility of proteomics to assess marine pollution, additional field experiments are required to confirm if this minimal PES is robust for high throughput performance.

Future studies could include:

- a deeper and quantitative analysis of the effect of pollutant exposure on the proteins identified in this work, including the characterization of possible post-translational modifications;
- their validation with established conventional biomarkers;
- assessment of the performance of the new proteins in field studies;
- investigation of the true mechanism of toxicity of these environmental pollutants.

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