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Proteomics, Ecotoxicology and Seafood Safety: the *Mytilus galloprovincialis* in Sardinia

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A Mamma e Papà,

per avermi dato

quello che ho e quello che sono.

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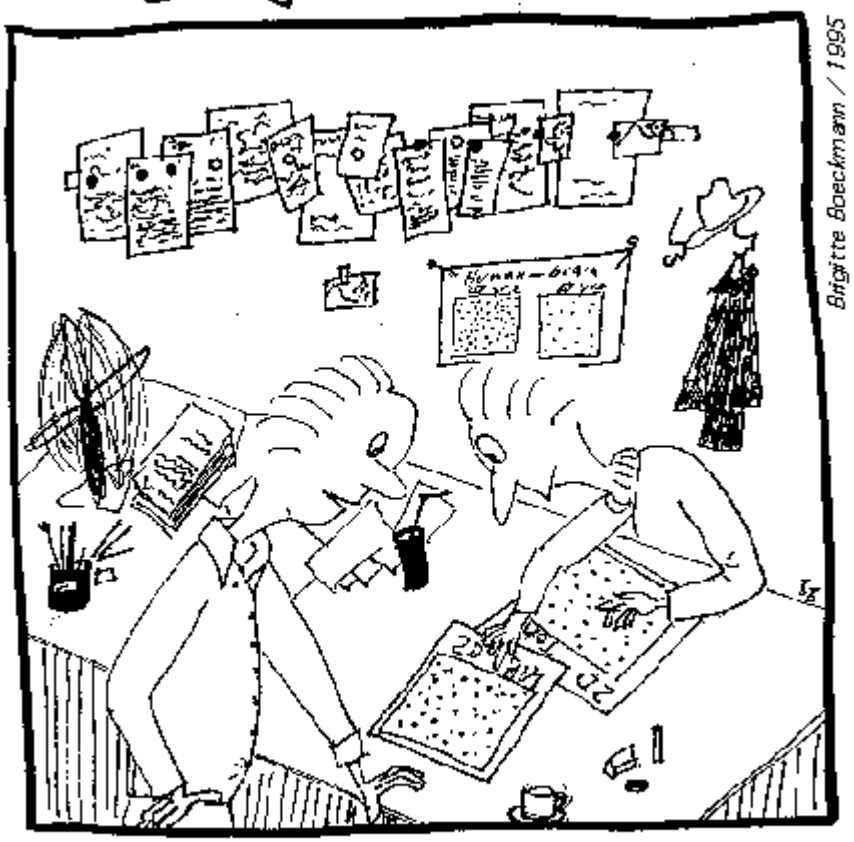
2D gels



"You've got one protein missing..."
"No, you've one extra protein!"

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Abstract

As a consequence for the irrational exploiting of marine resources, their contamination produced by the persistent anthropogenic activity and the effects of climate changes, nowadays there is great concern about the environmental sustainability of aquaculture practices and seafood quality. Actual regulations ensure that environmental and food resources are exploited in a rational way and that, accordingly, the hazards are adequately monitored both at environmental and food chain level.

Proteomics allows the identification of biomarkers on many biological matrices for a wide range of general applications in ecotoxicity and food science. In the present work a proteomic approach has adopted for the study of the mussel *Mytilus galloprovincialis*, which is the most important aquaculture resource of Sardinia, in different rearing environments, with the aim of accomplishing a systematic characterization of its foot muscle proteome, gathering insights on the protein expression variability that may be correlated to growth and environmental factors. Lastly, also the application of proteomics in the development of biosensors to monitor toxicant hazards in aquatic environments and control seafood-related risks has been reviewed.

Chapter 1

Proteomics

1.1 What is Proteomics?

At first, Proteomics could be defined as the scientific discipline that includes all the methodologies to analyse and study proteins on a large scale, including not only their identification and quantification, but also the study of their cellular location, their modifications, their interactions and functions. However, Proteomics could be defined also as the scientific discipline that studies the proteome.

The term "proteome" was first introduced for the first time in 1994 by Marc Williams at a conference on two-dimensional electrophoresis in Siena (Italy). He defined the proteome as "the protein equivalent of a genome", in the meaning of the protein analogue expressed by genome. One year later, the same authors refined the definition as "the set of protein expressed by the genome of a cell, a tissue or an entire organism" (Wasinger et al., 1995, Wilkins et al., 1996). Unlike the genome, the term proteome refers to a dynamic state that is subjected to a multitude of changes of diverse nature, that may be brought about by growth, differentiation, senescence, disease and treatment with drugs, genetic manipulation or changes in the environment (Lottspeich, 1999). Thus, the proteome analysis is defined as the characterization of protein content inside cells, tissues, organs or body fluids which are expressed by a genome at a given moment and under precise conditions (Wilkins, 1996). The potential number of proteomes is essentially infinite, especially for complex organisms.

1.2 The role of Proteomics in the Systems Biology

"This structure has novel features which are of considerable biological interest."
(Watson J. and Crick F., Nature, April 25th, 1953)

In 1990s the Human Genome Project, the greatest scientific challenge of recent times, began. The project was coordinated by Francis Collins and aimed at completely sequencing the human genome within 15 years. With the help of Celera Genomics Corporation directed by Craig Venter, also the private sector joined the project. The cooperation within public and private careers within HUGO, the Human Genome Organization that involved nearly 20 research groups from different countries, and the relevant technological advances made in genomics helped reduce by five years the initial project time estimate. On 26th June 2000, the scientists Francis Collins and Craig Venter announced to the world the completion of the human genome sequencing, so that the first draft was complete after ten years. On February 2001, the public part of the project was published on the journal Nature (International Human Genome Sequencing Consortium, 2001) and the private part was published on Science (Venter et al., 2001). More than 90% of the human genome, had been sequenced and the entire sequencing would have been completed two years later, in April 2003, on the 50th anniversary of the discovery of the double helix of DNA by James Watson and Francis Crick.

The whole human genome sequencing triggered off a revolution inside the global scientific community. The completion of the project landmarked the beginning of a new paradigm, the one ruled by –omics disciplines. In Greek the suffix -oma (-ομα), in english –ome, means "whole", "entire", so it refers to something that in its wholeness is related to an organism. As an example, proteome refers to the entire proteins of an organism and

genome to the whole genes of an organism. Under this heading, a number of emerging high-performance, or high-throughput technologies have emerged, each one with the purpose of efficiently generating and analyzing all the possible information about a particular aspect of biology. A competition has started for the large-scale study of different biological aspects or –omas and consequently a number of disciplines with high efficiency and productivity have born: genomics, transcriptomics, proteomics, metabolomics, modificomics, interactomics and so on.

The conclusion of the Human Genome Project also marked a milestone in how to study and understand the biology (Ideker et al., 2001). The XX Century, famous as "The Molecular Era", elapsed under the great influence of the empirical reductionism. In a mechanistic conception, the reductionism defines the whole as the sum of its parts. Thus, the dynamics of metabolic pathways and cellular functions were explained under the central dogma of molecular biology, by which DNA is mould and reference of everything, RNA and proteins included. Accordingly, the traditional approach of biology investigation was a "bottom-up" approach, based on the study of a particular biologic system by analyzing its many constituting parts. However, biological systems are extremely complex and their dynamics are multidirectional, because everything depends on everyone and everything is in close relation to the surroundings. Biological systems cannot be explained or predicted by studying only their individual parts. After the Human Genome project was finished, the international scientific community has been trying to understand how the complex network of biological information that comes from genome regulates cellular functions and biological processes. A new era called "Post-genomic Era" rapidly started that is aimed at explaining and understanding the awful amount of information popping

out from –omics technologies work. The new XXI Century biology marks a new qualitative and quantitative step towards understanding living systems and the ruling paradigm is that of complexity (Oltvai and Barabasi, 2002).

Since ever, in human knowledge history, the change from the current ideological paradigm to another one that will substitute it, has been always landmarked by the contribution of various disciplines and by different innovative investigation techniques. Although challenges still remain the same, thanks to technological progress what does change in the new paradigm is the methodological approach by which facing challenges. According to the paradigm of complexity, "the whole cannot be determined or explained as the sum of its parts", thus a holistic view is necessary to build a new complete and deep knowledge of complex living systems. The biological revolution in the “Post-genomic Era” is driven by a new interdisciplinary paradigm, whose theoretical framework is the Systems Biology (Kitano, 2002).

The Systems Biology may be considered as a new integral branch of scientific discipline that is concerned with studying biological processes using a collective and multidisciplinary approach. According to this approach, metabolic pathways and molecular interactions are mapped, cellular connections and tissues networks inside an organisms are studied, all integrated into a computer model, under the leading of a number of systemic laws (Network Biology, Barabasi and Oltvai, 2004). Thus, Systems Biology belongs to a multidisciplinary area where biochemists and biologist gather together with a number of different professional, such as computer scientist and experts, mathematics, system theory scientists, bio-informatics and so on. Clearly, three different scientific disciplines, the Information Science, the System Science and the Life Science,

contribute to the theoretical framework of System Biology, all governed and harmonized by the theoretical framework of the General Systems Theory or Systems Theory (Bertalanffy, 1950). Within this framework, –omics technologies on the same level, would cooperate to glean information in the attempt to give shape and understanding to life. Proteomics is one of them.

1.3 What does Proteomics study?

Once the Human genome Project had finished, the rapid technologic advances in genomics allowed the systematic sequencing of a growing number of genomes of diverse organisms belonging to different kingdoms. According to ERGO database by Integrated Genomics Tm. (www.integratedgenomics.com), at present the sequences of 1937 microbial genomes have been completed and published, belonging to 1347 Bacteria, 248 Viruses, 149 Eukarya, and 99 Archaea, to which the genetic sequences of other 74 plasmids and 20 organelles must be added. Nowadays a number of genome sequencing projects are still under development: a complete list of finished and ongoing gene sequencing projects and their state is available at the NCBI web site (www.ncbi.nlm.nih.gov/Genomes/index.html).

Nevertheless, no matter how big the genome of an organism is, yet it is still limited and static if compared to the huge number of expressed proteins that take part in the metabolism of a living being. Even if each gene encodes for an amino acid sequence, the expressed proteome presents a large structural, functional and quantitative variability, is involved in intricate networks and is governed by systemic laws, thus is extremely dynamic and determines the high variability of the observed changes at the phenotypic level. The central dogma of molecular biology driving the second half of XX Century, that

"the physiological development and functioning of an organism consist essentially of an integrated system of chemical reactions controlled by genes, either by acting directly as enzymes or by determining the specificities of enzymes" (Beadle and Tatum, 1941), does not truly adhere to reality. In respect to DNA, protein variability is boosted by various different biochemical mechanisms. One of these is the alternative splicing of primary mRNA transcripts. In eukaryotes, coding regions of genes, called exons, are interrupted by non-coding intervening sequences, or introns. By means of mRNA splicing, introns are recognized in transcripts and excised, thus obtaining a codifying mRNA. The rearrangement of the mRNA transcript occurs in a differential way, thus resulting in the production of more than one protein from the same gene (Newman, 1998). In eukaryotes, mRNA splicing interests more than 40% of proteins in an organism. On the basis of the expression and alternative splicing of the mRNAs, it is estimated that human cells may be able to produce 10^6 different proteins starting from the about 32,000 genes of the human genome; however, this number, although quite relevant, yet represents a limited size for the human proteome Oh et al., 2004; Goodacre, 2007). Beside splicing, other biological mechanisms violate the well-documented principle that each gene encodes a single polypeptide. As an example, the diversification of antibody repertoire is generated through somatic hypermutation. In all vertebrates, the variable regions of each light and heavy chain of immunoglobulins (Ig) are encoded by genes containing numerous variable segments. During B-cells development, different segments are rearranged and assembled through a site-specific DNA recombination process, producing a unique gene that is then transcribed and translated into a single polypeptide, either a heavy or light Ig chain. (Dryer and Bennett, 1965; Rajewsky, 1986). Also, the single nucleotide

polymorphisms (SNPs) increases to some extent this versatility. But what is more interesting are the modifications that proteins undergo after being syntehtized. By post-translational modification (PTMs), such as acetylation, phosphorylation, glycosylations and methylation, sulfation, nitrosilation, deamidation and ubiquitination, proteins undergo other structural changes which modulate their biological function and once more enhance protein variability. More than 300 different types of PTMs are currently known and new ones are regularly discovered (Jensen, 2004) and it is estimated that about 10% of genes encode proteins whose function is to modify other proteins. Moreover, each conversion process of genetic information into proteins is regulated by different mechanisms of regulation, mediated by different transcription and translation factors, each one adjusting the relative amount of individual proteins inside a cell (Jansen et al., 1995). Similarly, variability in protein expression depends on the lifetime of each protein, in its time of staying inside the cell or cell compartment and the translocation processes inside and outside cell (Colledge and Scott, 1999; Kirschner, 1999). Furthermore, this complex process is modulated by exogenous factors. Both gene expression and PTMs are influenced by the phenomena of epigenesis, that is, by mechanisms by which each individual organism change certain aspects of its internal or external structure in response to the interaction with the environment. Post-transcriptional changes such as alternative gene splicing and post-translational modifications of proteins, such as glycosylation or phosphorylation, are differently induced in cells by epigenetic phenomena. Moreover, post-translational modifications can determine in a protein the activity state, localization, turnover, and interactions with other proteins. Therefore, what takes place inside living beings is a complex dynamic process, both temporally and spatially, that gives rise to an

infinity of protein expressions that cooperate with each other in a specific and complex physiological process. Epigenesis significantly increases the number of different proteins above that predicted by DNA or mRNA analysis: that is the reason for the poor correlation between mRNA amounts and protein amounts in cells, according to which the abundance and presence of translated proteins cannot be proportionally deduced with reliability from measuring expressed mRNA levels, as was first demonstrated by Anderson and Seilhammer (1997).

In conclusion, the word Proteomics is comprehensive of all the numerous issues that are or may be investigated inside the complex protein world. This includes the identification of protein repertoire of a particular biological system, the structural and functional knowledge of these proteins, the study of their modifications, the interactions between them, their intra-cellular localization and compartmentalization and the quantification of their expression levels, as well as the knowledge of the changes induced by external stimuli (Graves and Haystead, 2002). All this in a certain time and under certain conditions.

This is Proteomics.

1.4 An historical approach to Proteomics: from Electrophoresis to Mass Spectrometry

In 1970, the molecular biologist U.K. Laemmli used an improved method of gel electrophoresis, the sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) to separate some structural proteins expressed by the bacteriophage T4 during the process of assembly of its head and identify them with specific gene products. The monodimensional electrophoresis method separated proteins according to their

charge/mass ratio in denaturing conditions (Laemmli, 1970). Since then, various electrophoretic technologies have been developed to study protein patterns, based upon different electrophoretic separation principles, such as vertical polyacrylamide electrophoresis of native proteins (Hoh et al., 1976; d'Albis et al., 1979), isoelectrofocusing (IEF), where native (Mackie, 1980, Rehbein et al., 1995) or denaturated (Hochstrasser et al., 1988, Rehbein et al., 1999) proteins can be separated in relation to their isoelectric point (pI), and lastly, modified SDS-PAGE methods (Carraro and Catani, 1983).

More or less in the same years, various researchers tried to improve the separation of thousand of proteins with better resolution and sensitivity by coupling IEF and SDS-PAGE techniques (Kendrick and Margolis, 1970; O'Farrell, 1975; Klose, 1975) and soon it was clear that, apart the difficulties related to improving the reproducibility, the resulting two-dimensional electrophoresis (2-DE or 2D-PAGE) could prove to be a powerful tool for the analysis and detection of proteins from complex biological sources (O'Farrell, 1975). In 2D-PAGE proteins are independently separated according to two parameters (pH and charge/mass ratio), each one corresponding to one dimension; consequently, two-dimensional electrophoresis permits visualize the protein complement of a biological matrix through the separation of individual proteins in proportion to their isoelectric point (pI) along the X axis and their molecular weight (Mw) along the Y axis (O'Farrell, 1975). Reference maps can be created that precisely define the proteins and the polypeptides expressed by a living being at a given moment of his life. Therefore, two-dimensional electrophoresis is a powerful tool to study both qualitative and quantitative changes in protein expression. Protein spots can be visualized by means of different

staining methods, either with Coomassie staining (Fazekas de St. Groth et al., 1963; Neuhoff et al., 1988; Candiano et al., 2004) or silver staining (Merril et al., 1979; Merril et al., 1981) and then analyzed for their identification.

In a proteomics work identifying the polypeptides is extremely relevant, because it means that the amino acidic sequence can be linked to the nucleotide sequence, that is the proteome with the genome. With the identification of protein spots, reference maps are created that precisely define the polypeptides expressed in a cell and help understand which proteins are cell-specific and which are common to different cell types, providing a tool to verify the accuracy of the information obtained from DNA sequencing.

Several methods of protein identification are based on the definition of one or more characteristics of a protein and the comparison with results in different protein and/or genomics databases. Traditional identification methods arise from co-electrophoresis of purified known proteins, to recognition by immunoblotting with antibodies (Bini et al., 1996), internal peptide sequence or “over-expression" of interesting homologous genes of the organism that is object of study. Another identification method is the N-terminal amino acid automated sequencing, based on the protein degradation reaction (Edman and Begg, 1967). The automated stepwise chemical degradation had been traditionally employed for the *de novo* amino acid sequencing of proteins or isolated peptide fragments isolated from 2-DE gels (Hewick et al., 1981). However, these methods are often expensive and time-consuming: the N-terminal amino acid automated sequencing can sequence one amino acid every 40 minutes each; so they cannot be employed as a “high-throughput screening” (HTS) approach when simultaneously separate and study a relevant number of proteins is necessary. Moreover, proteins that have been separated

on a gel cannot be analyzed by Edman degradation since their N-terminal end is blocked. Yet this technique can be employed to design PCR primers that will be used in gene libraries to search the specific gene and get back to the corresponding amino acid sequence. Other alternative methods for protein identification had been developed, such as the use of short N and C terminal sequence tags of 4 amino acids for genomic database research, that is particularly useful with small size organisms whose genome is completely sequenced, such as bacteria (Wilkins et al., 1998). However, for living beings with larger genomes the probability of finding proteins having the same sequence for the first four amino acids is too high to efficiently apply this technique. Another identification methods for high-throughput protein analysis based on the amino acid composition had been developed (Wilkins et al., 1999); however, an ambiguous identification by the amino acid composition alone cannot be always achieved, so that it has to be coupled with secondary database search constraints such as isoelectric point (pI) and molecular weight (Mw) values, that can be experimentally derived from the gel. All these methods for protein sequencing had been soon substituted by mass spectrometry analysis techniques as alternative methods for the *de novo* sequencing of proteins (Aebersold et al., 1987).

The high resolving power of two-dimensional electrophoresis is limited by its lack in reproducibility: comparing proteins spots is complicated by variations between gels. Reproducibility of electrophoresis technique had been greatly increased by the development of immobilized pH gradient (IPG) technology (Görg et al., 2000) and pre-cast polyacrylamide gel electrophoresis (PAGE) technology (Fey and Larsen, 2001). These solutions allowed to have good reproducibility among proteomics laboratories and to obtain reference or standard electrophoretic maps to record in specific protein

databases. Various databases specialized in different aspects of protein molecular biology and in proteomics had been created, like Swiss-2DPage (Appel et al., 1993) and many others (Appel et al., 1996); the ExPASy bioinformatics resource portal (Gasteiger et al., 2003) hosts World 2D-PAGE, a list of available 2D-PAGE databases and services (http://world-2dpage.expasy.org/list). By means of specific softwares for gel image analysis, researchers can compare protein maps obtained in their laboratory with those in databases and identify the known proteins that are common to both maps. This is possible because about 80% of the proteins expressed inside cells of an organism, the so-called housekeeping proteins, are preserved while only the remaining 20% are cell-specific (Hughes et al., 1993). Coupling high-throughput proteomics techniques with bioinformatic tools afford a robust and powerful strategy for both protein identification and characterization purposes.

But the very advance in proteomics comes from mass spectrometry (MS). Mass spectrometry is a comprehensive and versatile analytical technique that, since its development in the early of the 19th century, has been extensively employed in coupling with gas and liquid chromatographic systems for the qualitative and quantitative analysis with very low detection limits of any kind of chemical compound, even in complex matrices. Briefly, MS analysis is based on the analyte ionization under high vacuum to generate in the gas phase charged chemical species (molecular and fragmented ions) that are separated and identified on the basis of their mass-charge ratio (*m/z*). However, ionizing and putting into gas phase big, non volatile molecules without decomposing or fragmenting them has limited its applicability to protein analysis. In the late 1980s the development of two “soft” ionization methods electro-spray ionization (ESI) (Fenn et al.,

1989) and matrix-assisted laser desorption/ionization (MALDI) (Karas and Hillenkamp, 1988) overcame the difficulty of conservative ionization, representing two breakthroughs that paved the way to rapid and sensitive protein characterization and revolutionized proteomics, making MS the technique of election for protein identification (Aebersold and Goodlett, 2001). MALDI ionization source are most commonly used with time-of-flight (TOF) mass analyzers that are robust, simple, sensitive and have a large mass range; the single-charged ions generated by MALDI can be easily detected with TOF detectors at amounts lower than 1 picomol, generating easy-to-interpret spectra; moreover, the method is relatively resistant to interferences with the matrices most used in proteomics. Conversely, ESI produces multiple charged ions that can be detected by quadrupole mass analyzers and other types of analyzers with limited *m/z* detection range (Aebersold and Goodlett, 2001). ESI can be easily interfaced with liquid-phase separation techniques and it rapidly gained popularity in MS instruments coupled on-line with high-performance separation techniques such as capillary electrophoresis (Wahl et al., 1992) and HPLC (Cole, 1997). Other advances in MS are related to the implementation of miniaturized formats both for ion sources (Wilm and Mann, 1994 and 1996) and for separation techniques (Wahl et al., 1993) that significantly reduce the amount of peptide mixture required for a complete characterization from several picomoles to femtomoles.

In the traditional proteomic approach (Figure 1.1), proteins of interest identified by 2-DE are cleaved into smaller peptides by enzymatic digestion and the corresponding masses are analyzed in a mass spectrometer. Protein identification may be achieved through two different methodologies: one is the Peptide Mass Fingerprinting (PMF) and the other is the Tandem Mass Spectrometry (TMS).

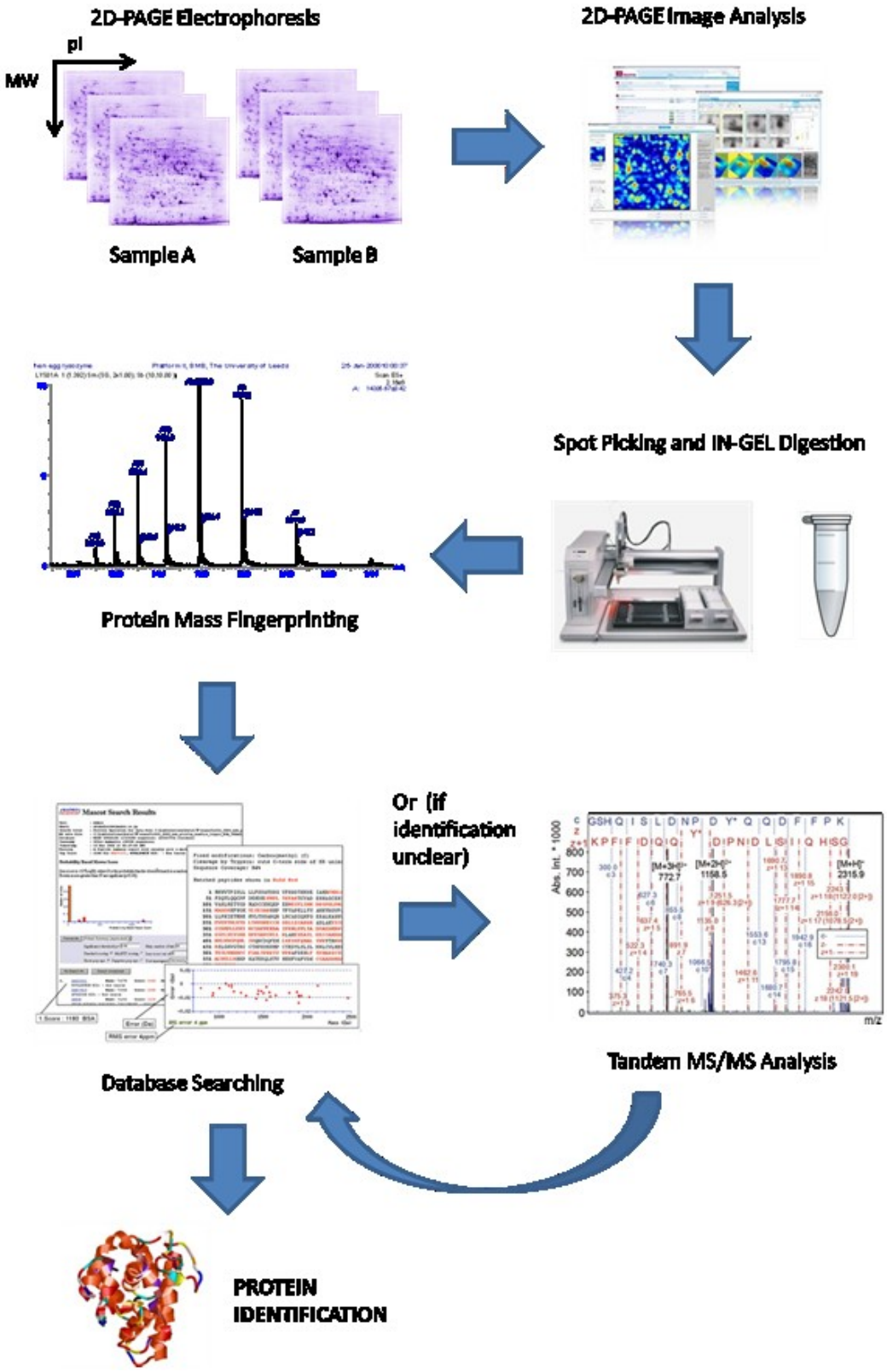


Figure 1.1 Classical 2D-PAGE workflow.

The principle of mass fingerprinting or mass mapping has been developed by several groups at more or less contemporaneously (Yates et al., 1993; Patterson and Aebersold,

1995; Pappin, 1997; Wilkins et al., 1999). The assumption of peptide mass fingerprinting is that the group of peptides deriving from a protein by a sequence-specific cleavage is unique to a protein, so the masses values are a highly effective means of protein identification (Pappin et al., 1993; Henzel et al., 1993; Mann et al., 1993; Patterson and Aebersold, 1995). Proteins are identified y matching a list of experimental peptide masses with the calculated list of all peptides of entry in a database. Peptide mass mapping can be accurately carried out with MALDI-TOF or ESI-TOF mass spectrometers and the masses values of the peptide ions are used to search in protein or genomic databases. The accuracy of protein identification by mass mapping depends on the correlation between the experimental mass value and the corresponding data calculated from the databases, so unambiguous determination can be achieved only if peptides come from highly purified proteins and if the protein sequence exists in databases. For this reason, mass mapping is very useful for protein identification of microbial species, for which the complete genome has been completed, and to identify proteins separated by 2D-PAGE, whose information on the molecular weight and isoelectric point of protein can be used as ancillary search constraints to aid identification (Aebersold and Goodlett, 2001).

In the Tandem Mass Spectrometry peptides ions can be selectively protonated and fragmented in gas-phase in a predictable way to produce reproducible series of product ions (Wells and McLuckey, 2005) (Figure 1.2). Collision-induced dissociation (CID) occurs inside the collision cell at low energy in a multiple step process and the resulting fragment ions are produced by cleavage at the amide bonds, while very little fragmentation at the amino acid side chains is observed; by consequence, the obtained CID spectrum depends on the sequence location of amino acids in the protein. Therefore, in addition to the

peptide mass, the peak pattern resulting in the CID spectra generated by Tandem Mass Spectrometry also provides information about the peptide sequence. The “Peptide Sequence Tag” approach of Tandem MS extracts a short, unambiguous amino acid sequence from the CID peak pattern that, when combined with the information of the peptides mass values, is a specific probe to determine the origin of the peptide (Mann and Wilm, 1994). The CID spectra can, in principle, contain a sufficient amount of information for unambiguous protein identification (Ducret et al., 1998) and is suitable also for the searching in Expressed Sequence Tags databases, which are stretches of about 300-500 nucleotides representing partial gene sequences and are generated by systematic sequencing of clones in cDNA libraries. Peptide fragmentation is very often carried out by CID in mass spectrometers with ion trap (IT) (Davis and Lee, 1997) or triple quadrupole (TQ) (Lee et al., 1998) or coupled quadrupole and time-of-flight (Q-TOF) (Borchers et al., 2000) mass analyzers (Figure 1.2). An advance has been brought by ESI nanospray (Wilm et al., 1996), that introduces the peptide sample inside the mass spectrometer at very low rates, usually at nanoliters per minute, allowing an adequate accumulation of the ion signal and producing high quality CID spectra. CID spectra contain peaks coming from overlapping series of ion products and other peaks of diagnostic ions, that may at first sound redundant but is extremely rich in sequence-specific information to use in sequence database search or *de novo* sequencing in the case of those species for which no genomic or expressed sequence tags is available (Mørtz et al., 1996).

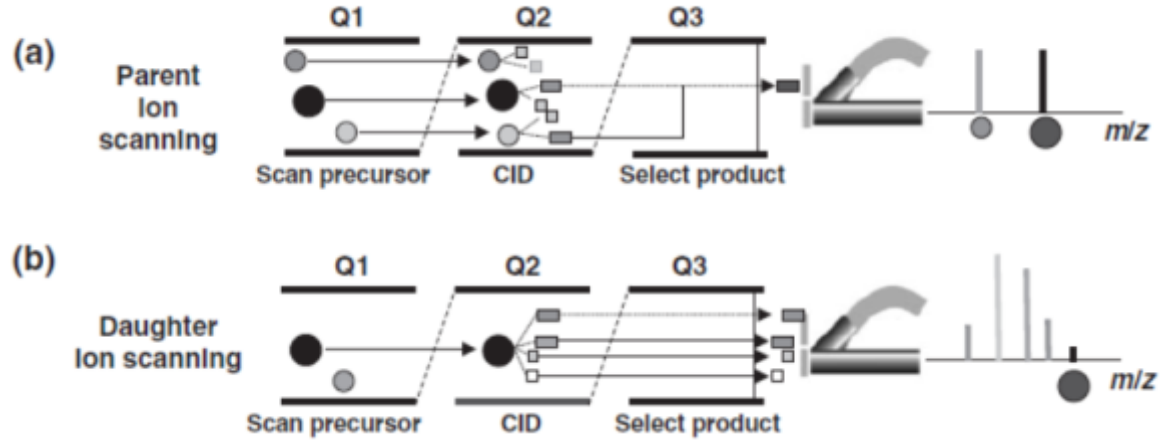


Figure 1.2 Mechanism of ion generation in Tandem MS by Collision Induced Dissociation (From: Cañas et al., 2006).

Different protein or genomic databases have been developed to search for peptide mass fingerprinting and tandem mass spectrometry data, like SwissProt (Bairoch, 2000), TrEMBL (O'Donovan et al., 2002) and Protein Sequence (Wu et al., 2003), GenPept and GenBank (Benton, 1990). SwissProt and TrEMBL databases have been managed by the European Bioinformatics Institute (EBI) and the Swiss Institute of Bioinformatics (SIB), while Protein Sequence Database (PSD) by the Protein Information Resource (PIR). Different softwares and algorithms have been developed to translate the genomic sequences into proteins, then theoretically cut the proteins into the expected pool of peptides on the basis of the enzyme used in the chemical cleavage, calculate the theoretical masses of peptides and, lastly, compare these values with the experimental values, giving possible matches with the corresponding statistical significance (Shadforth et al., 2005). BLAST (Altschul et al., 1990) and FASTA (Lipman and Pearson, 1985) softwares align and compare genetic and protein sequences. Mascot (Perkins et al., 1999) and ProFound (Zhang and Chait, 2000) are search engines that use mass spectrometry peptide data to identify proteins from primary sequence databases, while Sequest (Eng et al., 1994) is a search engine that identifies collections of tandem mass spectra generated

from peptide sequences with those present in protein sequences databases. Since 1993, the proteomic server ExPASy (Expert Protein Analysis System), created and managed by the Swiss Institute of Bioinformatics, has allowed researchers to search at the same time on the entries of many interlinked databases according to different relevant search constraints: sequence similarity, pattern and profile, post-translational modification prediction, topology prediction, primary, secondary and tertiary structure analysis and sequence alignment (Gasteiger et al., 2003). In 2002 the three institutes EBI, SIB and PIR joined in the UniProt Consortium and one year later launched UniProt, a comprehensive and freely accessible database of protein sequences and functional information derived from genome sequencing projects, that have resulted from the integration of SwissProt, TrEMBL and PIR-PSD (Apweiler et al., 2004). The growing need to have global access to protein and gene sequences databases pushed towards the creation of open source bioinformatics tools for large scale in proteomics and systems biology, like the open source search engine X!Tandem for MS database search (Craig and Beavis, 2004).

It is necessary to make some considerations concerning protein identification. The mass values of a group of peptides derived from a protein by sequence-specific proteolysis are a highly effective mean of protein identification by mass fingerprinting. But this technique does not ensures the obtainment of unambiguous matching, since that isobaric peptides, that is peptides with the same mass, can differ for their amino acid composition or for permutations in the amino acid sequence. By contrast, the amino acid sequence of a peptide is more constraining than its mass for protein identification by sequence database search. The CID fragmentation used in the Tandem MS provides a high number of amino acid sequences from short peptides derived from the same protein

(Protein Sequence Tags), so the CID spectrum obtained for a single peptide could be sufficient for the unambiguous identification of a protein. Therefore, providing that at least one CID spectrum per protein is generated, if in principle a mixture of proteins is digested, its components can be identified on the basis of their CID spectrum without any need to separate proteins prior to digestion (Link et al., 1999). This is the concept that has driven the change towards the employment of chromatographic techniques in integration or substitution of classical proteomics approaches. Two-dimensional electrophoresis has been a mature technique for more than 25 years and is the most popular analytical technique employed for protein separation. Apart from being expensive and time consuming and requiring highly skilled operators to get good levels of reproducibility, two-dimensional electrophoresis (2D-PAGE) suffers from the technical limitation of having a limited dynamic range of analysis (usually 10^3-10^5): largely the same proteins were identified repeatedly in the many works employing this technology, revealing that only the most abundant proteins can be observed by 2D-PAGE. In the last ten years several attempts have been made to improve 2D-PAGE technology with more sensitive staining methods (Rabilloud, 2002), sample fractionation techniques and the development of the Difference Gel Electrophoresis (DIGE) technique (Ünlü et al., 1997). Also, 2D-PAGE has been joined with good results by other analytical strategies to improve the separation of complex protein mixtures, like coupling 2-DE with a pre-fractionation step in ion-exchange liquid chromatographic (Amelina et al., 2007), or using capillary electrophoresis as an alternative to 2-DE, followed by ESI-MS/MS protein analysis (Doman et al., 2010).

However, the “Second Generation Proteomics” (Figure 1.3) is characterized by the different approach of analyzing complex peptide mixtures deriving from protein specific site cleavage without prior protein separation. After the pioneering work of Hunt and colleagues, who used LC-MS/MS methods to analyze the complex peptide mixtures associated to the histocompatibility complex class-I proteins (Hunt et al., 1992), the employment of LC-MS/MS at the basis of gel-free proteomics has gained rapid development.

Basically, in the second generation proteomics the complexity of the protein complement of the sample is increased by its enzymatic digestion, so adopting two orthogonal separating system is compulsory. The most popular non gel-based approach is the Multidimensional Identification Technology (MuDPIT) (Washburn et al., 2001), a chromatographic methodology to perform rapid simultaneous separation of hundreds of proteins that had developed from the original idea of Yates and colleagues (Link et al., 1999). In this technique, protein mixtures are digested in solution and the peptides produced are separated through a 2-dimensional liquid chromatography on the basis of an orthogonal separation criteria exploiting peptide physical properties of charge and hydrophobicity. Then the eluted peptides are sequenced by tandem MS in electrospray MS systems. Usually the first separation step is a strong cation exchange (SCX) and the second is a reverse-phase (RP) chromatography coupled with a MS/MS system, but also three-dimensional chromatographic separation combining strong cation exchange and reverse phase separations to affinity chromatography with avidin are employed (Han et al., 2001). The separation can be in on-line systems, where the two separation steps are

interfaced, or in the off-line approach, where the first separation fractions are collected, dried out, reconstituted in an aqueous solvent and analyzed in RP-LC-MS/MS runs.

In the separation of a complex peptide mixture deriving from the site-specific digestion of protein mixtures, an alternative to dual chromatographic separation exploited in MudPIT is the coupling of SDS-PAGE to a chromatographic method. This strategy, called GeLC (Pflieger et al., 2000; Schirle et al., 2003) is derived from an original idea of Shevchenko and colleagues (1996). In this method, proteins are separated by SDS-PAGE, the lanes cut and subjected to *in situ* digestion with trypsin; peptides are extracted from the gel, separated and analyzed in a RP-nano LC-MS/MS. Either the use of different coupled chromatographic techniques or the coupling of electrophoretic and chromatographic methods, the new strategy of the so-called "Shotgun Proteomics" offered the opportunity to directly analyze complex protein mixtures to rapidly generate a profile of the protein complement, thus revolutionizing the approach commonly used in classical proteomics (Wu and MacCos, 2002). Many work have compared the efficiency of both techniques in relation to the number of identified proteins (Breci et al., 2005; de Souza et al., 2006; Wang et al., 2010). Generally, GeLC analytic strategy is more robust than MudPIT and has a larger dynamic concentration range, since it can identify five-fold the number of proteins separated by MudPIT (de Godoy et al., 2006) and is more efficient in the study of less-abundant proteins. One drawback of GeLC and MudPIT is their globality: both techniques are based on the "scanning" of big parts of the protein mixture before identifying the proteins of interest, so these techniques are not preferable to 2-DE in the identification of changes in PES that can be targeted with further MS analysis,

unless the proteins of interest can be readily visualized on the 1-DE gel or in a blotting membrane (Nesatyy and Suter, 2008).

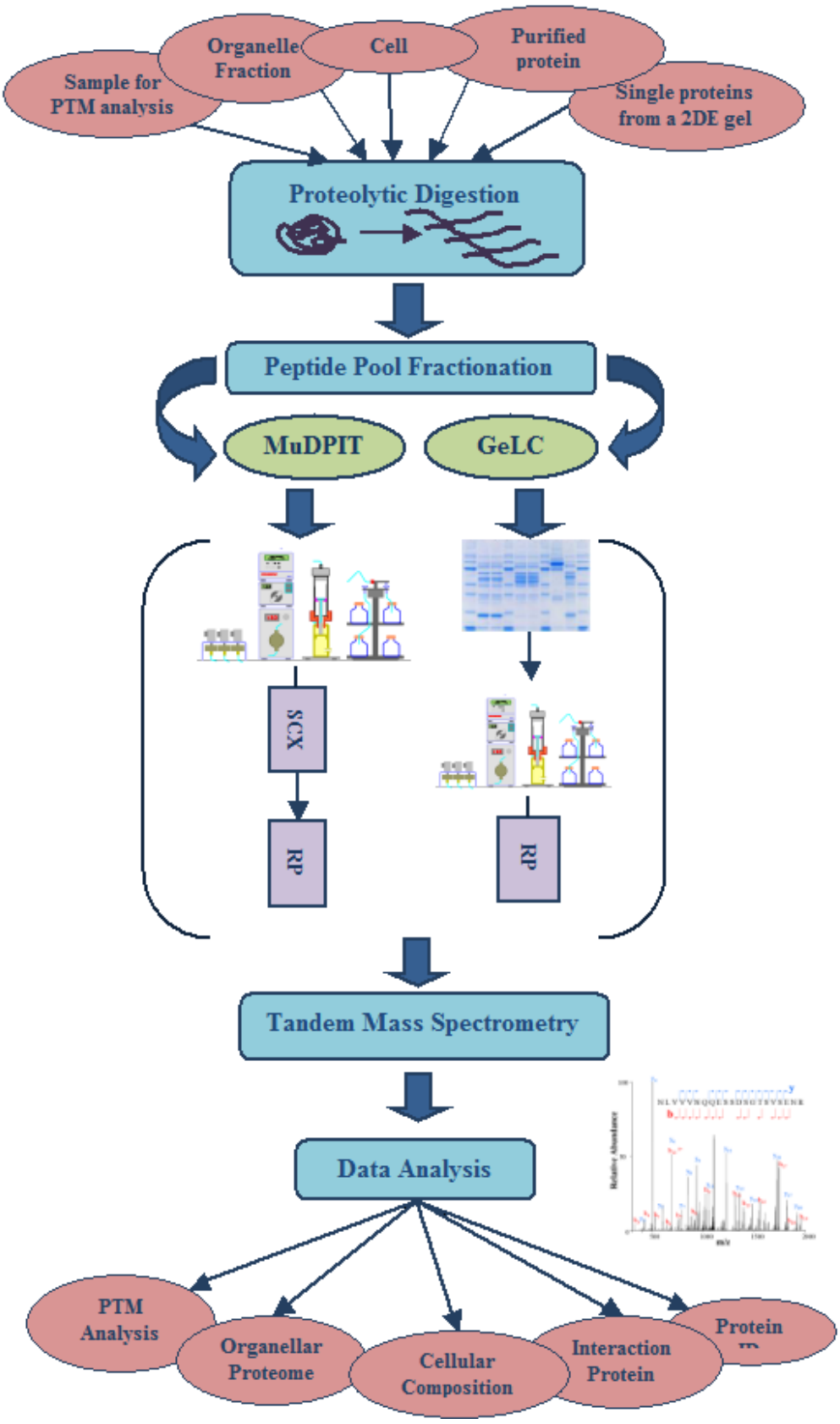


Figure 1.3 Second Generation Proteomics workflow

Nowadays, mass spectrometry is not only an established tool for classical proteomics research, it is also at the first line of differential expression proteomics. In fact, it is more important to determine how much a protein changes in its expression levels from one condition to another, rather than to know if it is present or not. Mass spectrometry is the first election tool for protein quantification, whether relative or absolute, since isotopic labelling is the widest used methodology for quantification. Basically, the strategy is based on the differential labelling of proteins or their derived peptides with two or more labelled molecules that differ only for their mass due to the presence of light or heavy isotopes ($^{12}C/^{13}C$, $^{14}N/^{15}N$, $^{1}H/^{2}H$) and that, for this feature, can be detected in mass spectrometry. Labelled proteins or peptides are mixed (or eventually mixed to peptides), then separated and identified in LC-MS/MS. Up to now, several isotope tagging methodologies have been developed: Isotope-Coded Affinity Tags (ICAT) (Gygi et al., 1999), isobaric Tags for Relative and Absolute Quantitative (iTRAQ) (Ross et al., 2004), Stable Isotope Labelling with Amino acids in Cell culture (SILAC) (Ong et al., 2002) and enzymatic $^{16}O/^{18}O$ labelling (Yao et al., 2001). They mainly differ in the type of strategy adopted to introduce the stable isotope tag into the protein or the peptide (Figure 1.4). The label can be introduced by chemical reaction of a functional group with an isotopically labelled reagent, (typically the biotin moiety that allows purification of cysteine-rich peptides) (ICAT) or labelling the N-terminus of all peptides generated by proteases with isobaric compounds (iTRAQ). In SILAC, the labelling occurs metabolically, by introduction of ^{15}N-enriched ammonium sulphate or amino acids or ^{2}H- or ^{13}C-labelled leucine or arginine in the culture medium, so it can be employed only with cultured cells. The $^{16}O/^{18}O$ labelling is based on the oxygen exchange reaction that enzymatically-

assisted occurs at the free C-terminus when a peptide is incubated with heavy water and trypsin.

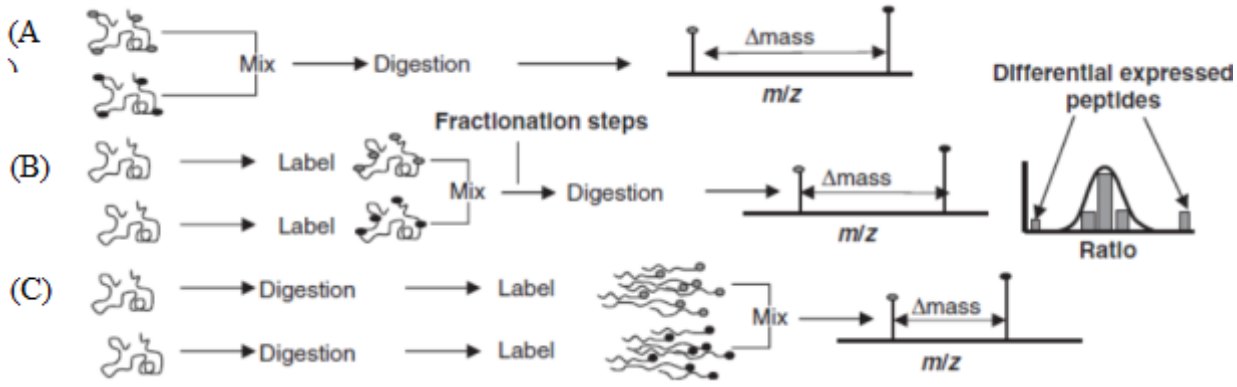


Figure 1.4 Isotope tagging strategies for MS relative quantification. A: Metabolic labeling (SILAC); B: Chemical labeling (ICAT, iTRAQ); C: Enzymatic 16O/18O labeling. (From: Cañas et al., 2006).

Beside stable isotope labelling, also label-free quantification is increasingly used (Old et al., 2005; Zhu et al., 2010). These methods consist in looking for statistical differences in the intensities of ion signals that occur when aligning separate LC-MS/MS runs of peptide mixtures: the relative quantification of a peptide directly depends on the differences in intensity between the run spectra. Attempts have been made also to achieve absolute quantification, that is of great relevance since it allows direct comparison between experiment conducted in different laboratories and enables the compilation of large data set necessary for diagnostic and toxicological studies. The use of isotope-enriched peptides (AQuA) or artificial proteins (concatamers; QconCAT) as internal standards that mimic the digested peptides is a recent development (Kirkpatrick et al., 2005; Pratt et al., 2006). However, going into a deep dissertation of these quantification methodologies is out of the scope of the present work.

Another innovative technology, the retentate chromatography-MS (RC-MS) combines SELDI-TOF mass spectrometry with protein microarray technology (Weinberg et al., 2002).

The protein array technology is a modification of the DNA array technology. In this case, a chemically-treated support with specific chromatographic features (cationic, anionic, hydrophobic, hydrophilic, ion metal chelating) acts as a bait and binds to specific proteins, thus isolating a portion of the sample proteome. After adsorption, the bound proteins are crystallized on a MS matrix and analyzed in surface-enhanced laser desorption ionization mass spectrometry (SELDI-TOF MS) (Figure 1.5). This technique can efficiently identify small proteins (<20 kDa) with a low detection limit ranging the femtomoles. A modification of this technology is based on protein separation by affinity chromatography and employs chips with specific antibodies (Hess et al., 2005). The SELDI ProteinChip technology have already been used in human medicine (Issaq et al., 2003; Wulfkuhle et al., 2003), as well as ecotoxicology studies in marine species (Knigge et al., 2004; Bjørnstad et al., 2006).

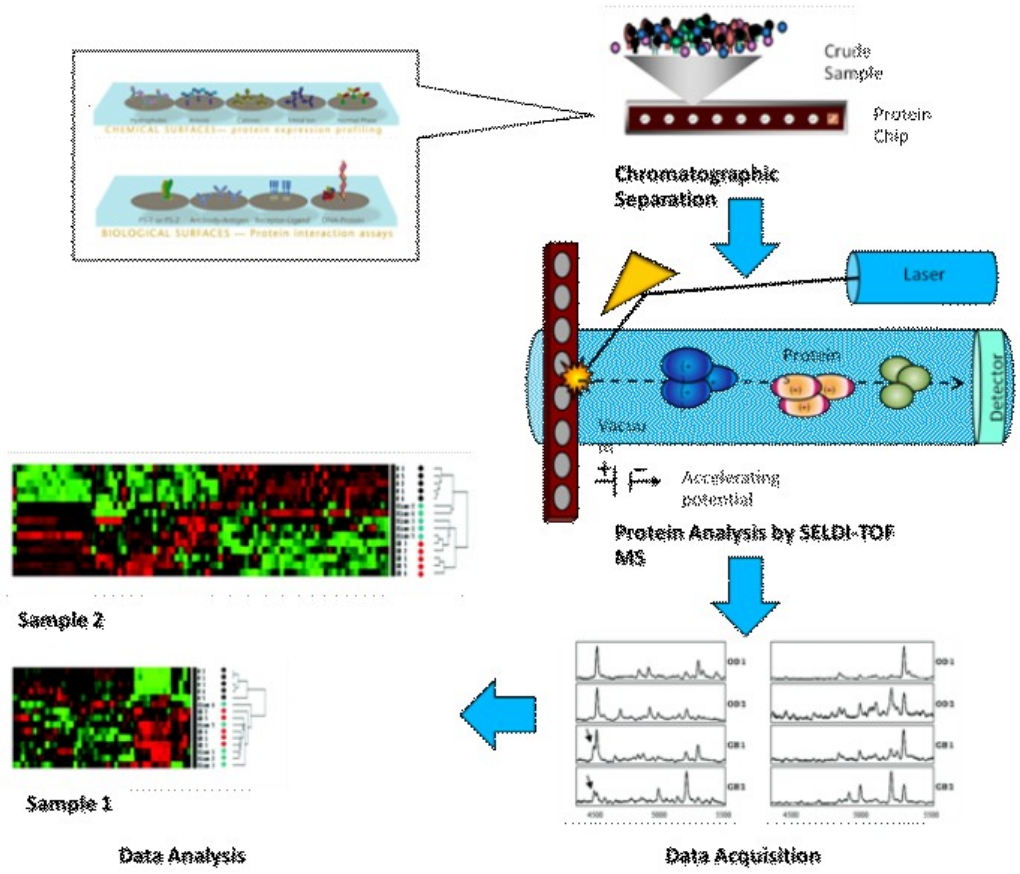


Figure 1.5 Differential protein expression profiling by SELDI-TOF and Protein chips

1.5 Proteomics: Approaches and Strategies

1.5.1 Proteomics Approaches

Proteomics studies protein profiles and protein properties such as expression levels and interactions that occur on a large scale in biological systems, thus obtaining a global view of biological processes and their modifications, such as cellular, metabolic or disease processes (Wilkins et al., 1996). Thanks to many advances in the development of new, high resolution detection methodologies for protein analysis, the holistic conception of proteomics as the large-scale study of a proteome could be translated into a new conceptualization of proteomics, from which several perspectives of proteome analysis have derived with relative different approaches and analysis strategies.

A. Expression Proteomics

The traditional use of 2-dimensional electrophoresis is mainly focused on the large-scale characterization and quantification of the whole proteome expressed by a cell, a tissue or an organism under determinate conditions. In other words, all proteins species within a single sample are catalogued and then compared with the catalogue of another sample and so on; this methodology approach is called "Expression Proteomics". Expression proteomics is focused on large-scale characterization and quantification of all components of a particular proteome, expressed by an organism, a tissue, a cell or a sub-cellular component under precise and determinate conditions. Just as a photography, expression proteomics adequately reflects, both qualitatively and quantitatively, the real protein profile, without any need to infer protein composition from their genes expression. This work allows direct characterization of proteomes and database construction. Thus, expression proteomics studies are very often not driven by a research hypothesis, but hypotheses are generated on the basis of obtained results.

B. Differential Expression Proteomics

A different approach in proteome analysis is based on the perspective of differential detection. In this case, proteome differences between two or more samples are found only by comparing dynamic changes and identifying the few proteins that differ between the samples being compared; that is, only the dynamic changes in the expressed proteome of two or more samples are detected. This kind of approach is called "Differential Expression Proteomics" or "comparative proteomics" (Minden, 2007) and allows the identification of proteins intervening in or affected by the process, so that their expression changes can be characteristic and the identified proteins can be used as diagnostic or prognostic markers of that process (Banks et al., 2000). The differential

expression proteomics approach has taken great advance from the development of the Difference Gel Electrophoresis (DIGE) technique. In DIGE, two different protein samples are labelled with different fluorescent dyes and co-electrophoresed on the same gel (Ünlü et al., 1997). Co-electrophoresis and the use of an internal control, made of a pooling of the same samples, is sufficient to neutralize the effects of gel-to-gel variations; the result is that the three images coming from the differential fluorescence scanning of the same gel are in perfect register, so experimental variations are overridden and the resulting differences are related only to protein dynamic differences (Viswanathan et al., 2006). Thanks to the employment of DIGE technique also the detection of isoforms changes such as post-translational modifications or alternative splicing could be detected (Minden, 2007).

C. Functional Proteomics

The main challenge in cell biology is revealing the biochemical mechanisms at the basis of cell working. Cells are complex systems in which a large number of components dynamically interplay, determining the outputs of many biological processes that occur in parallel. All cellular processes involves proteins and their characterization has drawn much interest over the years. Different molecular and histochemical approaches using antibodies or epitope-tags have been employed over the years to detect proteins by Western blot and microscopy, in the attempt to determine the localization and the abundance of cellular proteins and understand their role. However, despite the hours wasted applying workhorse techniques in the hope of accomplishing the extensive characterization of cellular processes, only a small window has been opened into the complex world of cell organization, and large parts of the interconnected network of

interacting proteins yet remain to be discovered. Accordingly to the concept that proteins are not isolated but are part of dynamic molecular complexes, a proteomics branch studies the relations among proteins in a determined biological system with the aim of building a sort of physical map of the interactions among all proteins in a cell (Blackstock and Weir, 1999), thus being named “Interaction Proteomics” or “Cell-map Proteomics”. The most common expression “Functional Proteomics” is used to refer to this kind of proteomic approach (Graves and Haystead, 2002) that, in addition to the amino acid sequence, studies protein structure and functionality by means of studying interactions within proteins. To understand its functionality, a protein has to be studied within the global network of its mutually interacting components; by consequence, the approach of functional proteomics is focused on the study and characterization not of a single protein, but of a group of proteins and it is realized through the isolation of protein complexes and proteasomes. Several attempts have been made for the mapping of different cellular components and cellular organisms (Rout et al., 2000; Verma et al., 2000; Gavin et al., 2002), and the first ones have already confirmed the huge complexity of the resulting maps. All cells, particularly the eukaryotic ones, are characterized by a high level of subcellular organization, so it is important to define not only protein composition and dynamics but also their localization inside the cell or the subcellular organelles. Fractionation techniques are increasingly combined with MS-based proteomics to determine the organellar localization of proteins (Yates et al., 2005). A key aspect related to the determination of organelle proteome is the detection of proteins in complex mixtures, so MS-based functional proteomics increasingly combines the shotgun proteomics approach to reduce the complexity of the analytical sample. The strategies

adopted are based on the use of two orthogonal separating systems, either both chromatographic like in the MudPIT (Washburn et al., 2001), or combining electrophoretic and chromatographic separating steps like in the GeLC (Schirle et al., 2003).

However, the most challenging question in functional proteomics is how the composition of an organelle or a proteasome changes under different conditions or in different cell types. Cellular signalling is based on the changes in the levels of post-translational modifications that proteins undergo during the process of signal transduction.

D. Post-Translational Modifications Proteomics or Modificomics

Post-translational modifications (PTMs) play a relevant role in the regulation of various prokaryote and eukaryote cellular processes, such as enzyme activity and gene expression, protein interactions and stability, but also in cellular metabolites localization and viability and in signal transduction. Depending on their intra-cellular or extra-cellular destination, proteins can be modified in their structure after translation. It is estimated that about 10% of genes encode proteins whose function is to modify other proteins and more than 300 different types of PTMs have been reported (Aebersold and Goodlett, 2001; Mann and Jensen, 2003; Jensen, 2004). Phosphorilation and glycosilation are the most occurring PTMs, but addition of lipidic groups, sulfonation, nitrosylation, methylation, ubiquitination and various oxidative modifications are made on proteins on the basis of their final function (Reinders and Sickmann, 2007). Their study is very complex, since PTMs are site-specific and polymorphic, are reversible and very often transient, time and location specific (Reinders and Sickmann, 2007). Moreover, they

cannot be predicted on the basis of the corresponding encoding genetic sequences and of mRNA levels, and very often the biological information carried by the cell transcriptome does not correlate with that of cell proteome. Nevertheless, PTMs play a pivotal role in biological function and their characterization is fundamental for understanding most biological processes. Mass spectrometry has proven to be a robust investigation tool not only in the characterization of the amino acid sequence of a proteins, but also in the definition of protein architecture, for PTMs recognition and the location of specific PTMs residues where they occur. A new MS-based proteomics branch have recently developed for the study of post-translational modifications, called Modificomics (Reinders and Sickmann, 2007).

It is at this point where Functional Proteomics and Modificomics approaches merge together. The elucidation of signal response requires measuring changes in the levels of phosphoproteins, and accordingly, very often these challenges require the quantification of the relative abundance of proteins in different samples or preparations. The MS-based approaches for the relative quantification of proteins by isotope labeling (SILAC, iTRAQ, ICAT) and free-label quantification and their coupling with the high-resolution MS technique of Multiple Reaction Monitoring (MSM), that allows to exclusively monitor a targeted precursor-to-fragment transition (Unwin et al., 2009), are being employed in the quantitative analysis of cellular and organellar proteomes, permitting the quantification of selected subsets of proteins (Forner et al., 2009; Picotti et al., 2009; Geiger et al., 2010; Luber et al., 2010). A reservoir of high-resolution and specific MS-based techniques are at disposal to determine the protein composition of a cell, to identify the members of

protein complexes, design their architecture, define protein localization and abundance inside organelles and, finally, understand the dynamic changes that proteins undergo during their biochemical role-acting. MS-based proteomics is closing the gap to gene expression analysis and thanks to MS-based proteomics, complete proteomes can be obtained (de Godoy et al., 2008).

1.5.2 Proteomics Strategies

In the proteomics methods illustrated until now, the analytical strategy adopted is focused on the analysis of peptide fragments obtained by protein enzymatic digestion. This kind of approach is commonly referred to as Bottom-up Proteomics (Figure 1.6). The classical proteomics workflow involves two steps: protein fractionation and protein identification. Firstly, proteins are fractionated or separated by two-dimensional electrophoresis or another non gel-based method, like column chromatography or capillary electrophoresis; then, the resolved proteins are identified and quantified by mass spectrometry, via mass fingerprinting of the trypsin-digested peptides (Protein Mass Fingerprinting, PMF) (Patterson and Aebersold, 1995), or tandem mass spectrometry (MS/MS) (Link et al., 1999). Alternatively, the complex protein sample is digested without any previous separation, and peptides are separated by high pressure-liquid chromatography (HPLC) coupled with tandem MS/MS. The so-called Shotgun Proteomics (Figure 1.7) has superior throughput and sensitivity than gel-based methods and has emerged as the technique of choice for the large-scale protein study. In both cases, proteins are cleaved into peptide to produce shorter segments that are more amenable to sequencing in the MS than whole proteins, so working occurs at peptide level. Since peptides are unique to specific proteins, the mass values of a group of peptides deriving

from a protein, or alternatively, the amino acid sequence of peptide tags are used to search the databases for the parent protein. Accordingly, bottom-up analyses excel at protein identification when combined with database searches.

A major problem with bottom-up proteomics and particularly with shotgun proteomics, is that tryptic cleavage generates multiple peptides from a protein, so proteomic samples consist of hundreds to thousands peptides, that no separation method can resolve in a single analytical dimension. This problem is currently being solved through the development of multi-dimensional liquid chromatographic systems with higher peptide separation power (Motoyama and Yates, 2008) However, the number of peptides is too elevated neither for a direct spectral analysis, so today it is not possible to achieve the full coverage of a whole protein sequence. Moreover, the same peptide sequence can be present in different isoforms of the same protein if not in different proteins, leading to ambiguities in the protein identity. A newer proteomics strategy that has been attracting attention during the very last years is Top-down Proteomics (Liu et al., 2009) (Figure 1.6). According to this alternative approach, intact proteins are introduced into a high resolution mass spectrometer, that can be a Fourier transform ion cyclotron resonance MS (Marshall et al., 1998; He et al., 2001) or an Orbitrap MS (Makarov, 2000), without prior digestion and the entire molecules are subjected to gas-phase ionization and fragmentation (McLafferty et al., 2007). Prior to mass analysis, proteins can be purified by various chromatographic procedures, but the protein digestion steps are eliminated. A key to the top-down strategy is the ability to fragment intact proteins and to place the entire protein sequence under examination, thus enabling a more complete protein characterization than the bottom-up strategy. Thanks to the ability of these high

resolution MS instruments to determine protein exact masses, the top-down strategy permits to identify large fractions of protein sequences and is suitable for the analysis of protein modifications such as PTMs (Bossio and Marshall, 2002; Scigelova and Makarov, 2006): modifications like phosphorylation may be deduced from the difference between experimental mass values and the theoretical values predicted from the DNA sequence, while the exact localization can be obtained from fragmentation studies (Garcia et al., 2007), as well as protein relative quantification by isotope labeling (Collier et al., 2010).

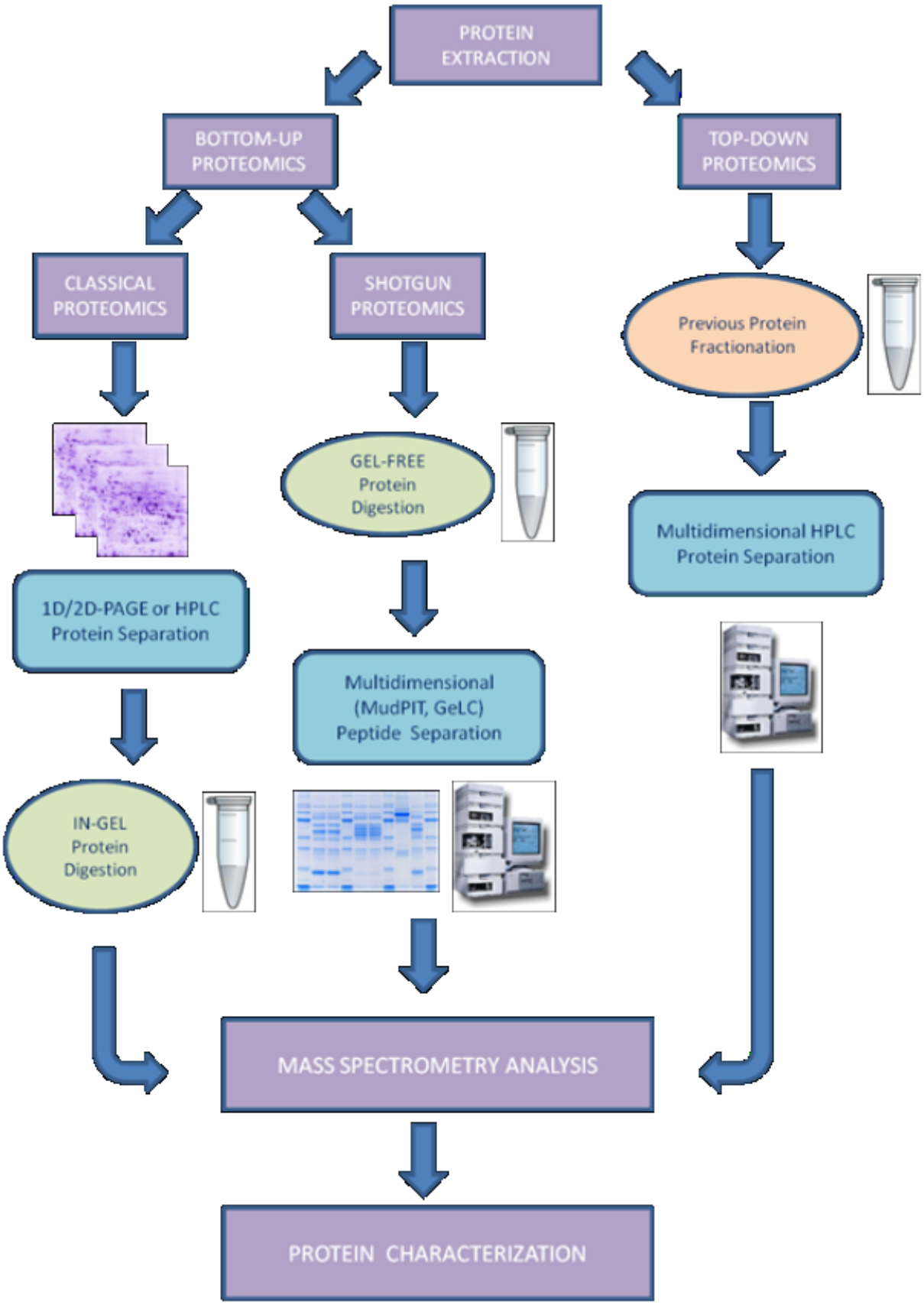


Figure 1.6 Proteomics strategies workflow

1.6 Proteomics issues in Aquaculture and Seafood.

Proteomics represent a robust analytical strategy for the phenotypical investigation of marine organisms. The early cases of proteomics application to marine biotechnology have been pioneered by the group of Martinez and colleagues during the '90s years, mainly in the form of electrophoretic studies of the physiological composition of miofibrillar proteins in fish muscles (Martinez et al., 1990a, b and c; Martinez et al., 1991; Martinez et al., 1993; Martinez and Christiansen, 1994). In the last decade proteomics has been widely used in fish biology and aquaculture to investigate the physiology, the developmental biology and the eco-toxicology of fish, crustaceans and molluscs. Some fish model organisms like zebrafish (*Danio rerio*) and mussels species (*M. edulis* and *M. galloprovincialis*) were studied, as well as some teleosts species of relevant interest in aquaculture, such as salmonids, sparids, moronids and cyprinids.

1.6.1 Physiology and Biomedicine

Several aquatic animals have been used as models of human disease processes in the areas of toxicology and chemical carcinogenesis, such as the zebra fish *Danio rerio* and some poeciliid fishes like platyfish and swordtails belonging to *Xiphophorous* species (Schmale, 2004). The freshwater teleost zebrafish (*Danio rerio*) is a well-known established animal model; its genome sequence is fully available and it has been employed as an experimental vertebrate model in development and organogenesis studies (Love et al., 2004) or for drug discovery studies in human diseases like cancer (Amatruda et al., 2008) and neurodegenerative disorders (Best and Alderton, 2008). The proteomics studies that have been carried out on zebrafish up to now are mainly related to embryogenesis and developmental biology. The proteome of zebrafish embryos at

different development stages was studied and many insights in the expression profiles of many proteins involved in morphogenesis were reported (Tay et al., 2006; Link et al., 2006; Lucitt et al., 2008). Other studies investigated phosphorylation events occurring in the proteome during embryonic development (Lemeer et al., 2008 a and b). Zebrafish has been studied also to test the effects of stress caused by aquatic pollution during embryo development (Gündel et al., 2007). Also the proteome of ovarian follicles in zebrafish at different growth stages from vitellogenesis to maturation has been studied (Knoll-Gellida et al., 2006) and compared to proteomic profiles in gilthead seabream developing oocytes, to shed light on the molecular variability between phenotypically similar oocytes (Ziv et al., 2008). Proteomics was employed in zebrafish also for more general physiological studies, related to establish the composition of cytosolic proteome in adult fish liver (Wang et al., 2006) and gills (De Souza et al., 2009) or the changes of skeletal muscle proteome under hypoxic exposure (BosworthI et al., 2005). Zebrafish was employed also in proteome toxicology studies focused on the effects of drugs administration like ethanol on brain cells (Damodaran et al., 2006), and in environmental pollution studies, to assess the effects of brominated flame retardants on liver cells (Kling and Förlin, 2009). Other aquatic animals like teleosts species have been used in proteomics studies towards biomedical research as experimental models to understand the anatomy, physiology and pathology of human disease. As an example, a proteomic study on Atlantic salmon (*Salmo salar*) identified novel proteins involved in neuronal tissue regeneration after injury (Zupanc et al., 2006). Similarly, the organ of the electrical ray (*Torpedo californica*) is used as a model to understand the proteomics of synapse transmission (Nazarian et al., 2007). Today proteomic approach is directed also toward

the identification of biomarkers for diseases like cancer and other infectious diseases. The proteome of the freshwater green swordtail (*Xiphophorus spp.*) has been analyzed towards the search of proteins involved in the malignancy of tumors like human melanoma, for which this fish species is a well-established model (Meierjohann et al., 2004; Lokaj et al., 2009).

Proteomics tools have been successfully applied in physiology studies on mussels, particularly on *Mytilus galloprovincialis* Lamarck, for the isolation and characterization from the mussel *M. galloprovincialis* of a calponin-like protein (Funabara et al., 2001). The protein was isolated from the retractor muscle of the mussel and was separated from actin by means of 2D-PAGE and is a polypeptide involved in muscle contraction processes. Moreover, proteomics may also play a significant role in the investigation of mussels adhesive proteins, that harbour an extraordinary biotechnological potential as a biodegradable material and may be readily obtained from mussels with wide availability and low price (van der Wielen and Cabatingan, 1999). Another study has investigated on the biochemical composition and structure of the byssal threads in *M. galloprovincialis*, gaining insights into the physiological process concerning its stability (Sagert and Waite, 2009).

1.6.2 Aquaculture issues

Consequently to the high growth of world food demand, seafood is getting one of the most relevant sources of food. However, the great depletion of natural fish resources still available, aquaculture is gaining increasing importance as an alternative source of fish and other seafood products. This situation has underlined the need to guarantee the safety, traceability and authenticity of seafood products. In the European Union, new

regulations on the authentication of seafood origin and quality are compulsory and the specie name of the fish, the geographical origin and the production method are obligatory in labeling fish product since January 2002 (Council Reg. (EC) No. 104/2000). In the last years seafood safety has become a major challenge for food science and proteomics has afforded valuable tools for the development of protocols for seafood safety assurance and control. Today proteomics studies represent around 35% of aquaculture-related works (Forné et al., 2010), and the high number of proteomic studies that have been performed on commercial fish species in the recent years reflects the increasing importance of aquaculture as productive sector. Recently, the application of proteomics for the investigation of seafood and other marine products has been extensively reviewed covering different issues (Piñeiro, et al., 2003). Salmonids, especially atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorynchus mykiss*) are the most important aquaculture products. Research on these species has been carried out to improve culture conditions, diminish infections and increase production yield. A similar study has been very recently conducted on the muscle proteome of seabream in Sardinia, with the aim of gathering information on its variability in physiological conditions occurring in both farmed and wild fish (Addis et al., 2010a). Proteomics-assisted research works reported the effects on fish health and food quality caused by X-ray exposition (Smith et al., 2005), viral infections (Martin et al., 2007) or stress induced by rearing conditions, such as density (Provan et al., 2006), anoxia (Wulff et al., 2008), or excessive handling (Liu et al., 2008). Similarly, some metabolic indicators of chronic stress were identified by comparative proteomics in farmed sea bream (*Sparus aurata*) subjected to repeated handling and crowding at high stocking density (Alves et al., 2010). Using

proteomics, also the effects of dietary manipulations on metabolism and health of farmed fish were studied, like the protein changes occurring in the livers of rainbow trout (*Oncorynchus mykiss*) that underwent feeding and starvation (Martin et al., 2001) or subjected to changes in dietary protein sources (Martin et al., 2003). Another study reported the effects on enhancing immune response determined by the prolonged administration of probiotic bacteria in the diet of cultured rainbow trout as an alternative of the use of antibiotics, and serum protein levels during the acute phase response of the inflammatory process were assessed using proteomics (Brunt et al., 2008). A proteomic approach was adopted also to investigate the effects of bacterial colonization on fish wellness. The presence of opportunistic bacteria in internal organs such as kidneys can decrease growth rate, stress resistance, and immune response. Presence of *Moraxella* sp. in farmed sea bream (*Sparus aurata*) kidney is able to induce proteome alterations correlated to metabolic alterations in fish (Addis et al., 2010b). Similarly, in families of farmed channel fish showing high and low susceptibility to infection with *Edwardsiella ictaluri*, kidney protein profiles associated with disease resistance were investigated (Booth and Bilodeau-Bourgeois, 2009).

Another topic that has been addressed using proteomics techniques is related to fertility and quality of gametes and embryos in cultured fish. In female trout, the proteome of coelomic fluid was analyzed during egg quality decrease associated with oocyte post-ovulatory ageing (Rime et al., 2004). Likewise, the proteome changes during embryogenesis underlying normal and abnormal development was analyzed in rainbow trout (*Oncorhynchus mykiss*) and masu salmon (*Oncorhynchus masou*) (Kanaya et al., 2000). In the farmed flatfish Senegalese sole, F1 males reared in captivity often show

lower sperm production and fertilization capacity than wild-caught males. Differential proteomics (DIGE) was used to study the changes in the testis proteome of wild-caught males at spermiation with that of F1 males showing different stages of germ cell development after hormone treatment *in vivo* (Forné et al., 2009). Proteomic profiles were investigated to determine proteins correlated to sperm cell motility in sperm of gilthead sea bream (*Sparua aurata*) and striped sea bream (*Lithognathus mormyrus*) (Zilli et al., 2008), and to study the effects of cryopreservation in sperm of sea bass (*Dicentrarchus labrax*) (Zilli et al., 2005).

1.6.3 Adaptation and taxonomy issues

A proteomic approach can be useful to distinguish species and population on the basis of the assessment of genetic polymorphism. Monodimensional gel electrophoresis have been used to study allozymes, variant forms of an enzyme that are coded by different alleles at the same locus, that are an important source of characters for reconstructing phylogenies among conspecific populations and closely-related species (Wiens, 2000). Similarly, two-dimensional electrophoresis analysis of proteins is a source of monogenic and codominant markers and the resulting positional polymorphism can be used as a source of genetic biomarkers for population genetics analysis and genetic variability studies (De Vienne et al., 1996). Proteomics strategies were applied in studies on marine organisms to differentiate species and populations, particularly on mussels of the *Mytilus* genus that has become a model organism for this kind of studies (López, 2005). A proteomics approach based on two-dimensional electrophoresis has been used to study the genetic variability in the *Mytilus galloprovincialis*, highlighting an elevate grade of individual heterozygosity (Mosquera et al., 2003). Similar proteomics works allowed to

identify some peptides as possible biomarkers to differentiate among the three mussel species *M. edulis*, *M. galloprovincialis* and *M. trossolus*, which are of interest in biotechnology and food industry (López et al., 2002a and b). Proteomics provides additional and basic information to understand gene expression and regulation, thus revealing to be a complementary tool to genomics. In fact, transcripts analysis provides only a limited view of gene expression, since it does not take into account regulatory steps at level of RNA translation (Agaton et al., 2004). The phenotypic traits of a particular genotype and protein function depend not only on the possible post-translational modifications, but also on the protein expression. Therefore, proteomics implies a higher level of analysis in the understanding of gene function. Interestingly, the proteome can reflect the modifications in genome expression induced by environmental changes, so proteomics can be of help in better understanding the detailed biochemical and physiological processes that occur during ecological adaptation and speciation. A proteomic study detected alterations in protein expression between intertidal and cultured populations of *M. galloprovincialis* (López et al., 2001). Similarly, protein expression patterns were compared in mussels from a hybrid zone between the two species *M. edulis* and *M. galloprovincialis* and significant differences were observed both among mussels within species and between species, revealing that hybrid mussels have more variable protein expression patterns than mussels of each species (Diz and Skibinski, 2007). Also in the case of two ecotypes of the marine snail *Littorina saxatilis*, protein expression changes occur during speciation, revealing that it is a case of sympatric incomplete speciation, originating as a by-product of adaptation to distinct habitats (Martínez-Fernández et al., 2008). A proteomics approach has been applied also to

differentiate among fish populations living in relatively homogeneous habitats that lack an apparent barrier to gene flow, thus obstaculating the detection of genetic differentiation, as it is the case of populations of the European hake (*Merluccius merluccius*); the identified protein isoforms can adequately discriminate among populations (Gonzalez et al., 2010). A few proteomics studies estimate genetic variance and heritability. One of them reported the heritability underlying egg protein expression in the marine mussel *M. edulis* (Diz et al., 2009). Maternal effects and mitochondrial DNA inheritance may have profound effects on the rate of evolution of offspring traits; the study reported that egg proteome varies significantly between individual females and different mitochondrial genomes are transmitted independently through female and male lineages.

1.6.4 Seafood Safety: Autenthication

Proteomics studies focused on species identification have been carried on also under the perspective of fish authentication in seafood. Indeed this theme is of great importance in seafood, since incorrect food labelling and deliberate or inadvertent adulteration can occur with species of different commercial value. The first proteomics studies on fish authentication dated to ’90s years and were focused on the analysis of the myofibrillar composition in the muscle of different fish species, such as salmonids (*Salmo salar* and *Salvelinus alpinus*) (Martínez et al., 1993 and 1994), herring (*Clupea harengus harengus*) (Martinez et al., 1990b), cod (*Gadus morhua*) (Martínez et al., 1990) and different species of the family Gadidae (Martinez et al., 1990c). By employing separate electrophoresis techniques like isoelectrofocusing (IEF) of native or denaturated proteins and sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE), several

polymorphisms in the subunit composition of the myosin light chains were identified, that could be used for the identification of species and muscle tissue in marine organisms (Martínez, 2004). Afterwards, the use of different electrophoretic techniques like isoelectrofocusing (IEF) and sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), alone or in combination into two-dimensional electrophoresis (2-DE or 2D-PAGE), has been successfully applied in the identification of fish species in seafood and a significant number of publications are now available. In Belgium, a databank of native IEF protein patterns of the sarcoplasmic fraction of muscle was created to identify and authenticate commercial flatfish (*Pleuronectiformes*) species in seafood (Bossier and Cooreman, 2000). Freshwater fish species which are sold under the generic label of ‘‘perch’’ could be distinguished by their IEF and 2D-PAGE protein profiles (Berrini et al., 2006). The application of 2D-PAGE also allowed the identification of closely related gadoid species by species-specific parvalbumin patterns that could not be distinguished by only conventional IEF or SDS-PAGE techniques (Piñeiro et al., 1998). Interestingly, species-specific proteins detected by 2D-PAGE were further investigated by other complementary techniques such as mass spectrometry (MS). This approach was employed for the differentiation of high commercial value hake species from other less appreciated fish species that are commercialized in Europe under the generic name of “hake” in processed seafood products. Analysis in 2D-PAGE detected the presence of a 17 kDa polypeptide specific for European hake (*Merluccius merluccius*) and Cape hake (*Merluccius capensis*), that was then subjected to electron spray mass spectrometry (ESI-MS) and revealed high homology with rat nucleoside diphosphate kinase A (NDKA), whereas coupling of 2D-PAGE with matrix-assisted laser desorption ionization time-of-

flight mass spectrometry (MALDI-TOF MS) allowed the identification of a specie-specific peptide in Southern hake (*Merluccius australis*) (Piñeiro et al., 2001). Similarly, analysis in 2D-PAGE coupled with MALDI-TOF MS of muscle parvalbumins fraction on closely-related species belonging to the Merluccidae and Macruronidae families, revealed a low intra-specific degree of polymorphism among the isoform patterns that were noticeably species-specific, thus allowing the classification between the two genus possible (Carrera et al., 2006). In addition, the proteins of interest were successively subjected to triptic digestion and the resulting peptides analyzed by MALDI-TOF MS, LC-MS/MS, and nanoESI-MS/MS. Their characterization by *de novo* sequencing revealed that the peptides belonged to different isoforms of the protein nucleoside diphosphate kinase B (NDK B); due to their high degree of homology these specie-specific peptides can be used as biomarkers for fish authentication purposes (Carrera, 2007). In one very recent work, native isoelectric focusing of water-soluble sarcoplasmic proteins was applied for the identification of several shrimp species of food interest belonging to the order Decapoda, revealing species-specific protein band profiles and low intra-specific polymorphism (Ortea et al., 2010). Also some collaborative studies on the use of electrophoretic techniques in the identification of different marine species have been carried out within several laboratories (Rehbein et al., 1995; Rehbein et al., 1999; Mackie et al., 2000).

1.6.5 Seafood Quality

In seafood, not only ensuring the correct identification of the fish species is important, but also assessing the conditions of food processing and the freshness of fish product. Proteomics proved to be valuable for detecting proteolysis and degradation in seafood and a lot of 2-DE studies on muscle food dealt with post-mortem changes in proteome.

Some authors studied protein degradation after ice storage of whole fish and fish fillets, identifying whether or not specific peptides and proteins could be used as indicators of freshness in fish (Papa et al., 1996; Papa et al., 1997; Verrez-Bagnis et al., 1999; Verrez-Bagnis et al., 2001). Jessen and colleagues also employed 2D-PAGE to study protein changes occurring in frozen fish. In fish muscle, proteins from both myofibrillar (α-actinin, actin, MLC1, MLC2, and N-terminal 70 kDa MHC fragment) and sarcoplasmic (glycogen phosphorylase, creatine kinase, and TPI) fractions are closely correlated with fish firmness. Frozen storage of fish is expected to oxidatively modify proteins, particularly inducing the formation of carbonyl groups and reducing their solubility (Kjærsgård et al., 2006). Moreover, muscle proteins can also be highly affected by proteolytic activity, thus *post-mortem* softening of fish tissue depends on protein degradation and results in low yield and decreased product quality (Godiksen et al., 2009). 2D-PAGE has also been used to examine the effects of other relevant parameters of the production method, such as the effects determined by the use of additives on the so-called “drip-loss”. This phenomenon is represented by the loss from the muscle of water containing salts, osmolytes, vitamins, minerals and other small molecules of nutritious value. Repeated freezing/thawing cycles on seafood are known to increase drip loss as well as protein denaturation and degradation. Addition of some salts, typically NaCl or polyphosphates, increases the water-holding properties of seafood but does not stop the loss of nutritionally important components (Martínez and Friis, 2004). Also the effects of hydration process on water-soluble proteins of preserved cod products, like stockfish and salted cod, were assessed (Di Luccia et al., 2005). In this study, an alternative electrophoretic technique, the sodium dodecyl sulfate-discontinuous pore gradient

electrophoresis (SDS-PPGE) was used and the results reported that in hydrated products there is presence of cross-linked protein aggregates, related to the oxidation of -SH groups during re-hydration, and a great loss of water-soluble proteins occurs during re-hydration. Also cooking induces modifications on seafood proteins and the solubility of sarcoplasmic proteins is affected by heat treatment. In fact, it causes proteins denaturation and the reduction of water-extractable protein amounts, determining changes in the IEF patterns of protein extracts, as reported in a comparative electrofocusing study between raw and cooked crabmeat (Gangar et al., 1996).

1.6.6 Ecotoxicology and Seafood Safety

Lastly, ensuring seafood safety is another challenge in which proteomics may proof to be a valuable tool. Fish and seafood may be potential carriers of pollutants and toxic compounds like heavy metals (mercury, cadmium, lead and arsenic), persistent organic pollutants (dioxins, polychlorinebiphenyls (PCBs), heterocyclic aromatic amines (HAAs), polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), dichlor-diphenyl-tricloroetane (DDT), aldrin, dieldrin, endrin, chlordane, heptachlor, hexachlorobenzene, mirex, toxaphene and furans), biologic toxins (diarrhetic shellfish poisoning or DSP toxin and ciguatera toxin) and microorganisms. In recent years, the contamination of the marine environment by chemical contaminants has risen due to the global increase of population and industrial development (Arellano et al., 1999). All these pollutants have the ability to accumulate in the biota and, being fish and shellfish the most common reservoirs of these contaminants (Francesconi, 2007), when reaching a substantially high level their toxicity may be a relevant problem for fish and humans (Islam and Tanaka, 2004). Worldwide, health authorities and consumers are deeply

concerned with the exposure to toxic contaminants in the environment and in food and several legislative actions have been taken for environmental control and conservation and for food safety assessment, like the European Union Water Framework Directive (2000/60/EC), the European Union Marine Strategy Framework Directive (2008/56/EC) and the European Union Food Law Framework Regulation (178/2002/EC). Proteomics may relevantly contribute to study and address toxicology issues and the application of ecotoxicoproteomics (Gomiero et al., 2006) is on the rise. Several articles have already reviewed the theme (Sheehan, 2007; Monsinjon and Knigge, 2007; Nesatyy and Suter, 2008; Piñeiro et al., 2010; Lemos et al., 2010). The use of proteomics in environmental toxicology has been pioneered by some groups (Adams et al., 1989; Shepard and Bradley, 2000; Shepard et al., 2000; Meiller and Bradley, 2002). These works studied seawater bivalves, particularly mussels of the genus *Mytilus*, for their peculiarity of being good indicators of water pollution. For this reason, bivalves have been often employed as natural sentinel species in studies of aquatic toxicity and pollution as well as in biomonitoring programs all around the world. A proteomic approach was employed to study on *Mytilus edulis* the effects on protein expression determined by exposition to copper, the PCB compound Aroclor 1248, and low salinity (Shepard et al., 2000). The same authors reported also the effects on lysosomal stability in gills of *M. edulis* caused by exposition to growing concentrations of copper by analyzing their protein expression (Shepard and Bradley, 2000). It is noteworthy reporting that since then the authors have already emphasized the relevance of using a proteomic approach under the two main perspectives of toxicoproteomics, namely detecting protein expression signatures and identifying possible toxicity mechanisms of action for toxicants. The mussel *M. edulis* was

employed also as a model to study the effects of oxidative stress on expressed proteome in response to menadione, a precursor of Vitamin K used as a nutritional supplement in economically developed countries and recently banned by the U.S. Food and Drug Administration (FDA) because of its potential toxicity (McDonagh et al., 2006; McDonagh and Sheehan, 2007 and 2008). Several pollutants such as menadione, organochlorines and metals can promote in biological systems a state of oxidative stress, when reactive oxygen species (ROS) may be produced. These compound can react and be absorbed by proteins, particularly reacting with the amino acids methionine and cysteine and inducing disulphide bonds cleavage. Reaction with ROS can lead to structural modifications in proteins including carbonylation, chain cleavage and glutathionylation, thus changing the protein expression pattern (PES) and complicating the expressed proteome (Davies, 2005). In many cases the biological effect of the toxicant action results in a significant decrease in the number of detected spots, since the pathways of cellular protein labelling via ubiquitination and carbonylation and of protein removal are activated by toxicant exposure (Chora et al., 2008 and 2010). Also nanoparticles can induce oxidative stress in exposed organisms with the production of reactive oxygen species (ROS), as reported by a proteomics work on mussels exposed to menadione and gold nanoparticles (Tedesco et al., 2008). The use of nanoparticles and nanomaterials has caused many concerns of their potential hazards to the aquatic environment.

In more recent works, peroxisome-enriched fraction from the digestive gland of *M. edulis* were analyzed by 2-D DIGE and MS and several proteomic signatures associated with the exposure to several marine pollutants, such as diallyl phthalate, PBDE-47, and bisphenol-A, were identified (Apraiz et al., 2006). A similar work using liquid

chromatography coupled with 2D-PAGE analyzed the changes occurring in the protein expression pattern of the digestive gland on *M. edulis* after exposure to marine pollution (Amelina et al., 2007). One study reported the modifications in the proteome expressed by blue mussel (*M. edulis*) gills induced by oil-polluted water (Manduzio et al., 20005). Similarly, a proteomic study has been conducted on gills of the zebra mussel (*D. polymorpha*) exposed to benzo(α)pyrene, revealing marked PES differences depending on the gender and exposure concentrations (Riva et al., 2011).

Some interesting ecotoxicoproteomics studies have employed for the first time the SELDI-TOF ProteinChip technology to address ecotoxicology issues. One work was focused on the proteome profiling in the blue mussel (*M. edulis*) exposed to polyaromatic hydrocarbon and heavy metals (Knigge et al., 2004;), reporting that the identified PES could be associated with the field site of origin. A second work reports the analysis with the same methodology of PES featuring chronic exposure of blue mussel to crude oil or spiked oil with alkylphenols or polycyclic aromatic hydrocarbons (Bjørnstad et al., 2006). Another one adopted the same analytical approach to discover biomarkers in crab exposed to pollutants (Gomiero et al., 2006).

Several works highlight that that many other species can be chosen besides mussel as sentinel species in proteomics studies of ecotoxicity, such as rainbow trout (*Onchorynchus mykiss*) (Albertsson et al., 2008), Atlantic salmon (*Salmo salar* L.) (Søfteland et al., 2011), Atlantic cod (*Gadus morhua*) (Berg et al, 2011), flatfish (*Limanda limanda*) (Ward et al, 2006), the Medaka fish (*Oryzias latipes*) (Mezhoud et al, 2007; Malècot et al. 2009) and the zebra fish (*Danio rerio*) (Kültz et al., 2007; Kling et al., 2008; Kling and Förlin, 2009), rare minnow (Wei et al., 2008), crabs and shrimps (Gomiero et al.,

2006; Alves de Almeida et al., 2008). Also the effects of other environmental stressors like salinity, temperature variations (Kimmel and Bradley, 2001), hypoxia or anoxia (Gardestrom et al., 2007; Brouwer et al. 2008; Pérez-Casanova et al., 2008; Wulff et al., 2008) have been tested under the perspective of studying the consequences over animal beings induced by climate changes. Another toxicant is microcystin-leucine-arginine toxin (MC-LR), an hepatotoxin with potent inhibitor activity on specific protein phosphatises, that is produced by cyanobacteria during algal blooms in water reservoirs. The effects on proteins deriving from the exposure to microcystin have been studied on Medaka fish (*Oryzias latipes*) liver with a proteomic approach (Mezhoud et al., 2008; Malécot et al., 2009).

1.6.7 Seafood Safety: Allergens

Besides being carriers of toxic compounds, fish and seafood can determine allergenic reactions, so that avoidance of the food is currently the only available treatment. Food allergy mainly affects developed countries, where it is estimated that up to 4% of children and adults is affected (Sicherer and Sampson, 2010). The most common allergies are caused by an increased production of antibodies from immunoglobulin E (IgE) in response to contact with the antigen of the allergen. Most of allergens are molecules of protein nature and antigens are usually proteins or glycoproteins with molecular masses in a range from ca. 10,000 to 70,000 kDa. Traditional methods to detect allergenic proteins in foods are mainly ELISA, polymerase chain reaction (PCR) and real-time PCR, the latter two methods detecting DNA markers but not the protein; other studies published so far use crystallography tools to characterize allergens. Proteomic tools, often combined with antibody identification by Western blotting, have been recently employed in allergy field

to characterize protein allergens, identify their genetic and phenotypic variability as well as well detect and quantify allergens in their native forms or in forms resulting from food processing (Sancho and Mills, 2010). Mass spectrometry allows unambiguous detection of allergens and their isoforms in processed allergen products as well as the possibility of detecting multiple allergens in a single analysis. Some authors reported the application of proteomics to the identification and characterization of allergens in seafood products (Tichá et al., 2002). The major fish allergen is parvalbumin, which is an acidic, calcium-binding 12 kDa protein resistant to heat and digestive enzymes. Fish muscles express multiple parvalbumin isoforms and white muscles rather than dark muscles contain higher amounts of parvalbumins (Griesmeier et al., 2010). The exposition to high molecular weight proteins, including parvalbumin aggregates, may cause an IgE-mediated response (Jeebhay et al., 2001). Moreover, polysensitization to various fish species is frequently reported and linked to the cross-reactivity of their parvalbumins. SDS-PAGE and immunoblotting tools were employed to study the IgE cross-reactivity of purified parvalbumins between fish species containing more white muscle, like cod (*Gadus morhua*) and whiff (*Lepidorhombus whiffiagonis*) and others containing more dark muscles, as swordfish (*Xiphias gladius*) (Griesmeier et al., 2010). Parvalbumins from different fish species have been identified as the major fish allergens. Natural and recombinant forms of β-parvalbumins from cod and carp were cloned and expressed by their encoding cDNAs and then purified, their molecular mass and the presence of contaminants and IgE reactivity were accurately assessed by MS and inmunoblots, respectively (Ma et al., 2008). Lastly, an allergy study using SDS-PAGE and Western blotting with allergen-specific antibodies and proteomic analysis (2D-PAGE and MALDI-

TOF-MS/MS) was carried out on the genetically-modified and non-GM individuals of amago salmon (*Oncorhynchus masou ishikawae*) to assess that new transgenic fish carrying the insertion of the growth-hormone-1 (GH-1) do not contain new allergens or higher concentrations of known allergens than the same non-GM fish (Nakamura et al., 2009).

Chapter 2

The Mytilus galloprovincialis

2.1 Evolution, Biology and Ecology.

The *Mytilus galloprovincialis* (Lamarck, 1819) is known as Mediterranean mussel or bay mussel and is a marine bivalve mollusc belonging to the genus *Mytilus* (Linnaeus, 1758), of which also the blue mussel *Mytilus edulis* (Linnaeus, 1758) and the foolish mussel *Mytilus trossolus* (Gould, 1850) are the most representative species (McDonald et al., 1991; ITIS database). There is substantial interest the genus *Mytilus* since many of its member species are eaten by humans in large amounts: like the other components of the family, the *Mytilus galloprovincialis* mussels are widely exploited as food and used in mariculture. The species is native to the Mediterranean coast and the Black and Adriatic Seas: in Italy it is commonly called “muscolo” or “peocio” in the northern regions, while in the south of Italy it is better known as “cozza”. The *M. galloprovincialis* can be found in marine habitats throughout the Mediterranean area, spreading out throughout the Atlantic coast, from North Africa to the Iberian peninsula (Cáceres-Martínez and Figueras, 1998) and to France, Britain, and Ireland (Skibinski et al., 1978). While the other species of *Mytilus* are restricted to bays and estuaries (Carlton, 1992), the Mediterranean mussel occurs in open coasts, where it can be found from exposed rocky outer coasts to sandy bottoms (Ceccherelli and Rossi, 1984).

Despite its name clearly recalls the geographical zone of origin, the Mediterranean mussel has succeeded in establishing at widely distributed points throughout temperate

regions around the globe. Populations of *M. galloprovincialis* have been found in the coasts of South Africa (Grant and Cherry, 1985; Hokey and van Erkom Schurink, 1992), California and east and west North America (McDonald and Kohen, 1988; Carlton, 1992; Anderson et al., 2002), Mexico (Ramirez and Cáceres-Martínez, 1999), Hawaii (Godwin, 2003), north-eastern Asia (Wilkins et al., 1983; Lee and Morton, 1985; McDonald et al., 1990) and South-east Australia (McDonald et al., 1991). In these regions the introduction of *Mytilus galloprovincialis* has occurred in correspondence of large shipping ports. It is agreed that the species has been accidentally introduced in these regions and ship hull fouling and transport of ballast water have been implicated in its spread (Geller et al., 1994; Carlton, 1999; Apte et al., 2000; Godwin, 2003). However, this hypothesis is still discussed (Carlton, 2003). In the zones where it was introduced, the impact of *M. galloprovincialis* on native communities and native mussels has been relevant (Sebastián Ruiz et al., 2002; Robinson and Griffiths, 2002; Branch and Stephanni, 2004), altering competition and predation interactions in the recipient community (Ruiz et al., 1997; Steffani and Branch, 2005), sometimes inducing hybridization with native mussel species (Skibinski et al., 1978; Vario et al., 1988; Hilbish et al., 2002; Coghland and Goslin, 2007; Beaumont et al., 2008; Dias et al., 2008 a and b) and sometimes the decline of native mussel species (Geller, 1999; Hanekom and Nel, 2002); the effects of the invasion by *M. galloprovincialis* has been suggested by a number of studies and observations (Carlton, 1999; Geller et al., 1994; Calvo-Ugarteburu and McQuaid, 1998; McQuaid and Phillips, 2000; Hanekom and Nel, 2002; Steffani and Branch, 2003 a, b, c; Braby and Somero, 2006; Zardi et al., 2007; Branch et al., 2010; Lockwood and Somero, 2011) and since a few years the *M. galloprovincialis* has been recognized as being amongst 100 of the world's worst

invasive species (Lowe et al., 2000; ISSG database). Nowadays, *Mytilus galloprovincialis* is currently the dominant intertidal species along the west coast and occupies over 2000 km of South African coastline (Robinson et al., 2005). In this region some experimental intertidal mussel fishery projects based on *M. galloprovincialis* have been initiated in the effort to exploit an alien marine species to generate economic benefits for local communities (Robinson et al., 2007).

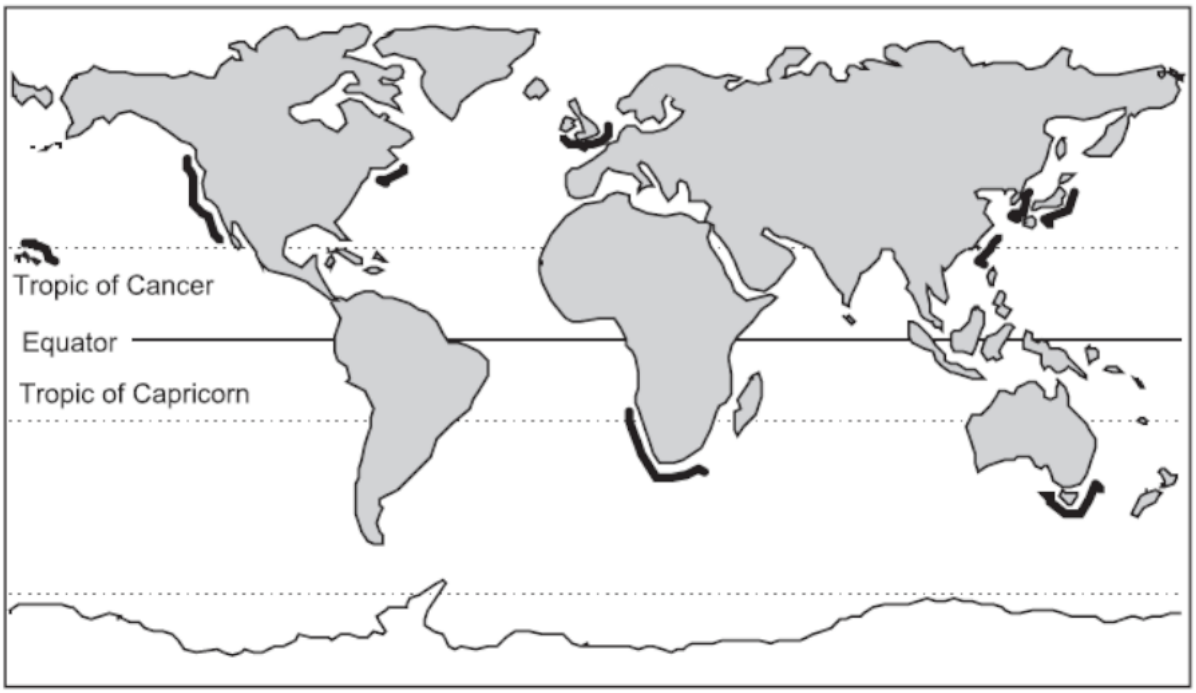


Figure 2.1 Geographical world distribution of Mytilus galloprovincialis as invasive species. From: Branch and Steffani, 2004

Thanks to the availability of excellent fossil records that facilitated the evolutionary studies, the evolution of bivalves molluscs could be dated back to the Cambrian era (500 mya) (Morton, 1992 and 1996; Harper et al., 2000; Ponder and Lindberg, 2008). The phylogeny of Bivalvia has been inferred by means of nuclear and mitochondrial genes (Giribet and Wheeler, 2002; Plazzi and Passamonti, 2010). It is generally believed that bivalves evolutionary derived from an hypothetical ancestral mollusc that originated during the Devonian and Cambrian eras (300-6500 mya). This is represented as a small,

shelled animal with a large foot creeping over the substrate of pre-Cambrian sea bottoms, scraping algae off the rocks with its mouth parts. At its posterior the animal had a pair of ciliated filamentous gills, which functioned only as respiratory organs (Morton, 1996). The genus *Neopilina*, the only representative of the primitive class Monoplacophora, includes limpet-like fossils resembling this hypothetical ancestor. Over evolutionary time, bivalves have become flattened side to side and had developed two shell valves to cover and protect the whole body. The mouth had raised off the shell with long fleshy extensions to allow primitive bivalves to feed on surface deposits by means of palps, making it possible for the animal mouth to be lifted off the substrate. The gills were primarily respiratory organs: in postulated primitive bivalves as *Nucula* and *Glycymeris* the water is capable of entering the mantle from anterior and posterior directions (isomyarian form). It is generally agreed that these animals used to burrow in soft sediments, so their foot had lost the original flat creeping sole, greatly reducing in size and becoming wedge-shaped and extending out between the valves in order for the animal to move. In some species of modern bivalves the foot has been entirely lost. Only during evolutionary time the role of catching food had shifted from the palps-shaped mouth to the two gills. The exploitation of filter feeding represented an important development in the evolution of modern bivalves, since it allowed bivalves to colonise a wide variety of habitats that had been inaccessible before, like the lower layers of bottom substrates (Morton, 1992). In order to achieve the capability of penetrating the substrate while keeping one body end in free communication with water, the site of water intake progressively moved to the posterior of the animal (heteromyarian form) (Figure 2.2). Plankton in the water current was increasingly adopted as a source of food, the gills replacing the palps processes as the

feeding organs and the mantle extending into siphons, through which water was inhaled and exhaled. Water flows in through the inhalant siphon to the gills, where filtering of suspended food particles takes place, and exit through the exhalant siphon. The chief modification of the gills for water filtering was the significant increase of the gill surface area to become an efficient ciliary feeding system. It is believed that the triangular-shaped gill filaments of primitive bivalves progressively enlarged, lengthened and folded to change into the W-shaped filaments of the modern bivalves (Barnes, 1980). Finally, from the heteromyarian form originated the monomyarian form and by consequence the adoption of the horizontal posture characterized by the lost of the adductor muscle and the reorganization of the whole body around the posterior muscle. The evolution of the heteromyarian form led to the development of a triangular shape and the capacity of living in clusters of individuals, as it happened in mussels of the family Mitilidae (Morton, 1992).

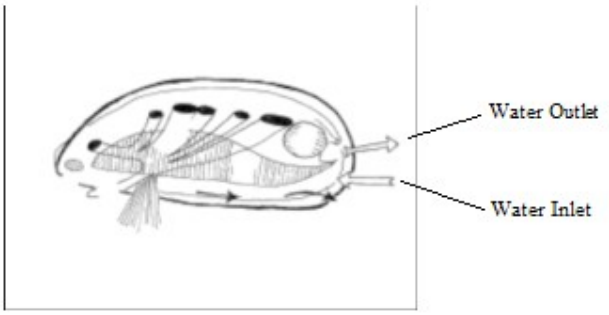


Figure 2.2 Representation of the heteromyarian form in mussels of the genus Mytilus. Adaptation from: Acquacoltura Responsabile (Cataudella, Bronzi, 2001).

The genus *Mytilus* belongs to the family Mytilidae, which dates back to the Jurassic and perhaps Devonian times (Soot-Ryen, 1969). In its original classification, Linnaeus included twenty-two species in the genus *Mytilus*; subsequently many of them have been assigned to different genera and the genus consists of only three closely related species: *Mytilus trossulus* (Gould, 1850), *Mytilus edulis* (Linnaeus, 1758) and *Mytilus*

galloprovincialis (Lamarck, 1819). *M. edulis* and *M. galloprovincialis* originated from an ancestral population of *M. trossolus* during a large-scale trans-Arctic faunal migration approximately 3,5 million of years ago (Vermeij, 1991); *M. edulis* gradually evolved from population of *M. trossolus* that became isolated during glaciation (Seed, 1992). Subsequently, *M. edulis* spread out across the North Atlantic and the Mediterranean Sea during the inter-glaciation periods. About 2 million years ago, the populations of *M. edulis* in the Mediterranean became isolated during another glaciations period (Seed, 1992), which gave rise to the gradual evolution of *M. galloprovincialis* (Barsotti and Meluzzi, 1968). Recently, the *M. californianus* (Conrad, 1837) has been recognized as being a species belonging to the same genus (Turgeon et al., 1998).

The ecology, biology and physiology of mussels has been long studied and different authors have systematically reported on these topics (Bayne, 1976; Gosling, 1992; Dame, 1996; Gosling, 2003). Mussels are characterized by two triangular shaped shell valves that are very similar in size and are hinged together at the anterior end by means of a ligament called umbo (Figure 1A). *Mytilus galloprovincialis* has a dark blue or brown to almost black shell. However, the color of mussel shell is controlled by several genes (Innes and Haley, 1977; Newkirk, 1980) and also depends on the age and location of the animal (Mitton, 1977). Bivalve shells are characterized by the presence of concentric rings that are annual in origin; these feature is traditionally used to reliably estimate the age of some bivalve species, like scallops and clams. However, shell length in mussels is very variable and rings can provide an accurate age estimate only for mussels coming from few geographical locations (Lutz, 1976); the alternative method is based on the microscope evaluation of the longitudinal section of shell valves: some growth bands can be detected

in the inner nacreous layer that form at a rate of one per year during spring (Lutz, 1976). Both shell length and shape are very variable in *Mytilus* species (Seed, 1976; Seed and Suchanek, 1992). Densely packed mussels have a more triangular shaped shell than those living in less crowded conditions (Seed and Suchanek, 1992). The typical growth length of *Mytilus* spp. is 50-80 mm, although under optimal conditions such as in the sublittoral zone, *M. galloprovincialis* attain a shell length of 100 – 130 mm, whereas in marginal conditions like high intertidal zone of exposed shores, they may measure as little as 20-30 mm even after 15-20 years (Seed, 1976). Metals and radionucleotides accumulate in the periostracal layer of the shell (Widdows and Donkin, 1992; Livingstone and Pipe, 1992), while deformities in the shell shall are induced by antibiofouliung agents like tributyltin (Laughlin and French, 1988).

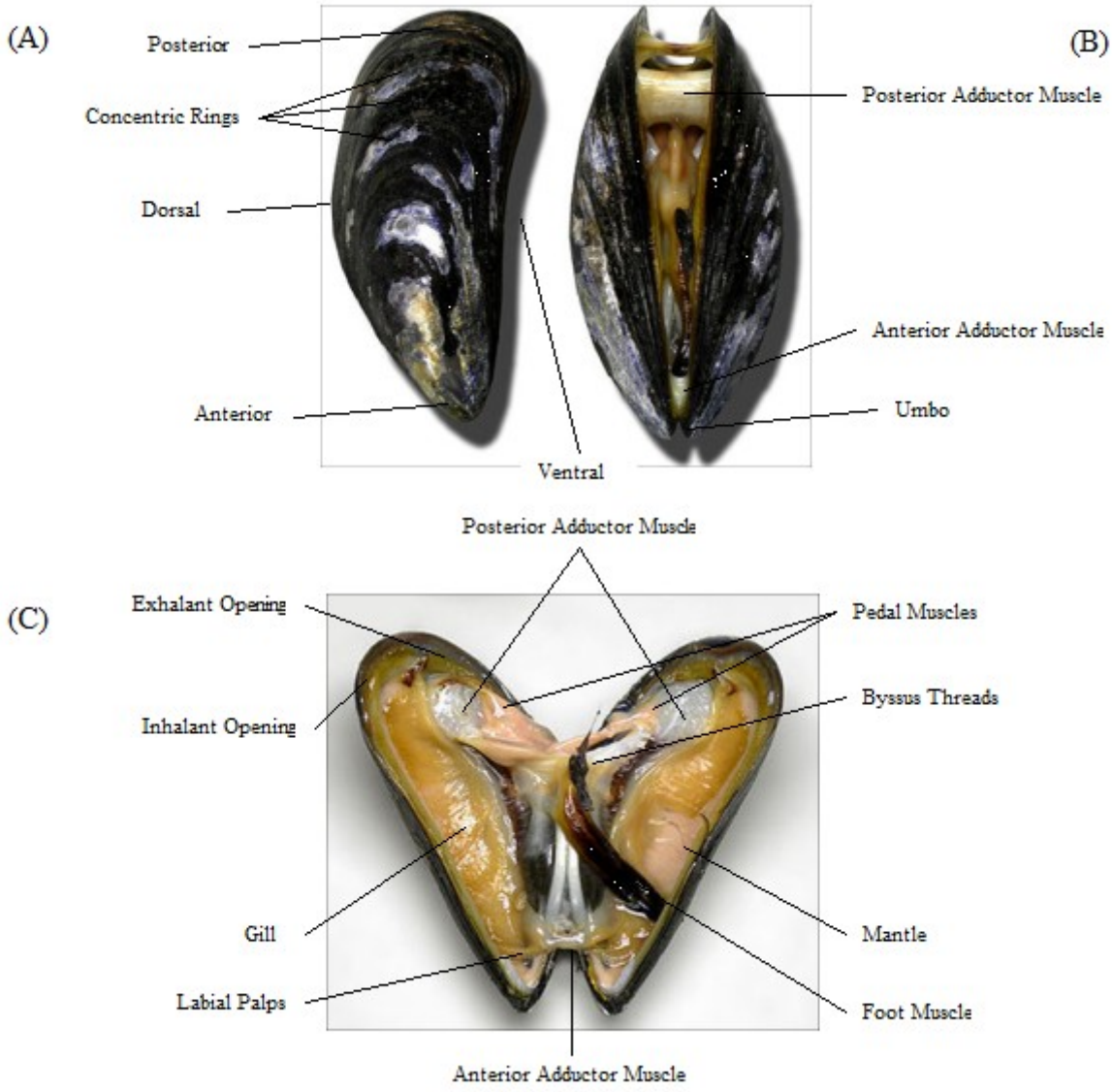


Figure 2.3 Esternal (A), Lateral (B) and Internal (C) features of the mussel Mytilus galloprovincialis.

In bivalves the mantle is composed of two lobes of tissue that completely enclose the animal within the shell (Figure 2.3C); haemolymph vessels, nerves and muscles are contained inside its connective tissue. On the inner surface, it is rich in cilia, that play an important role in directing food particles towards the gills and rejecting heavier material towards the openings. Generally, the mantle is thin and transparent in bivalves, with the exception of mussels, whose mantle contains most of the gonads. The gametes produced

by gonads accumulate in the mantle, are transported to gonoducts by means of ciliated channels and then are released into the mantle cavity. During the accumulation process the mantle gets thick and highly coloured, becoming thin and transparent only after mussels have released the gametes. In mussels the mantle is also the site for the storage of nutrients and glycogen; generally, nutrients are accumulated in the mantle during the summer to be employed in gametogenesis during autumn and winter (de Zwann and Mathieu, 1992). In mussels, together to gills, kidney and digestive gland the mantle is the site of bioaccumulation for many metals and organic contaminants (Widdows and Donkin, 1992; Livingstone and Pipe, 1992). In its peripheral part, the mantle is organized into three folds, each one destined to a different function. The outer mantle fold secretes two of the three layers of the shell, the periostracum and the prismatic layer; the middle mantle fold is deputed to sensory functions, hosting short tentacles containing tactile and chemoreceptor cells; lastly, the inner mantle fold is muscular and controls water flow in the mantle cavity (Figure 3). It is worth to note that the assumption of the sensory role by the middle fold of the mantle is involved in the bivalve evolutionary process that resulted in the loss of the head and associated sense organs. The mantle is attached to the shell by means of the muscle fibers of the inner fold and is separated from the shell by a very thin space that contains the pallial fluid, where calcareous and organic materials accumulate during the process of shell formation; the inner nacreous layer of the shell is produced by the inner part of mantle (Figure 2.3). Conversely to most of bivalves, mussel of the genus *Mytilus* produce pearls only when they have been infected by the larvae of small parasitic flatworms. The mantle in mussels is the host site for many non pathogenic viruses, potentially pathogenic protozoans, commensal cnidarians and parasitic flatworms like the

Proctoeces maculatus (Loos, 1901), that reduces glycogens reserves, disturbing gametogenesis with possible sterilization and death of the infected mussels (Bower, 1992). This parasite was identified as the probable cause of an extensive mortality in cultured mussels populations of *M. galloprovincialis* in Laguna Veneta, Italy (Munford et al., 1981). In mussels the mantle margins are organized in such a way that the exhalant opening is small, smooth and conical, and the inhalant aperture is wide, fringed and rich in sensory papillae; the mantle margin is only partially merged and siphons are missing, so that water can get inside through the wide semicircular opening of the mantle. The roominess of the mantle cavity particularly favours gills development and the efficiency of water filtration process (Figure 2.3C).

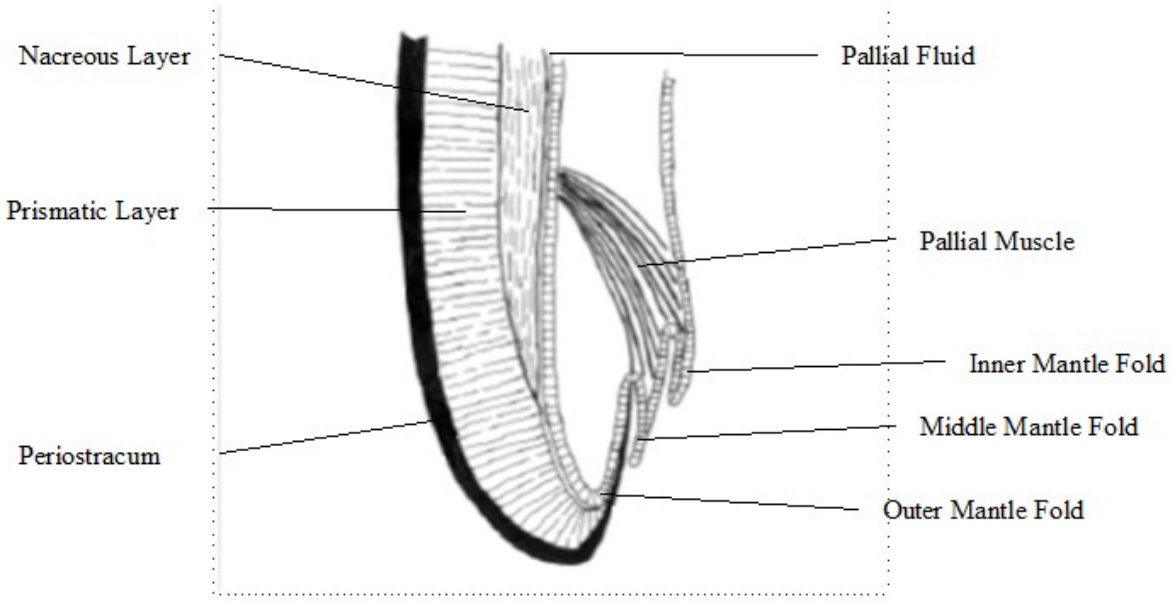


Figure 2.4 Internal structure of the shell and the muscle margins in Mytilus galloprovincialis. Adaptation from: Acquacoltura Responsabile (Cataudella, Bronzi, 2001).

Like all the other species of bivalve molluscs, the Mediterranean mussel is a filter feeder, feeding itself from what is conveyed by water masses movements and is filtrated by gills. Bivalve gills are two large, curtain-like structures hanging up from the ctenidial

axis of the dorsal margin of the mantle (Figure 2.4). Each gill is made up of numerous W-shaped filaments held together by a skeletal rod rich in collagen to strengthen each filament; the ctenidial axis contains the branchial nerves and the haemolymph vessels. Each V is called demibranch and is composed of one ascending and one descending lamella, delimiting the inner space called exhalant chamber. In mussels and other primitive lamellibranchs neighbouring filaments are attached to one another through ciliary junctions, giving a rather delicate gill type (fillibranch), while in eulamellibranchs neighbouring filaments are joined at intervals by tissue connections resulting in a more solid structure. Gills have a respiratory role, with their large surface area allowing adequate gas exchange between the water and the haemolymph, as well as a feeding role. Feeding occurs by gills filtration of water masses conveyed by the efficient internal water circulating system: the inlet water circulation brings food, sediment, chemicals and sperm, while the outlet water circulation delivers gametes and secretion products, filtering in one hour a water volume up to 30 or 60 times the volume of his body (Fianchini and Gravina, 2001). Like all the other species of bivalve molluscs, the Mediterranean mussel is a filter feeder and feeds itself with a wide range of zooplankton and phytoplankton, preferring fast-moving water that is free of sediment and thrives in regions where nutrient-rich upwelling occurs. Gills are also involved in the bioaccumulation of pesticides, soluble heavy metals and hydrocarbons (Livingstone and Pipe, 1992); high concentration of copper, zinc and lead affect the lysosomal stability in gills (Shepard and Bradley, 2000).

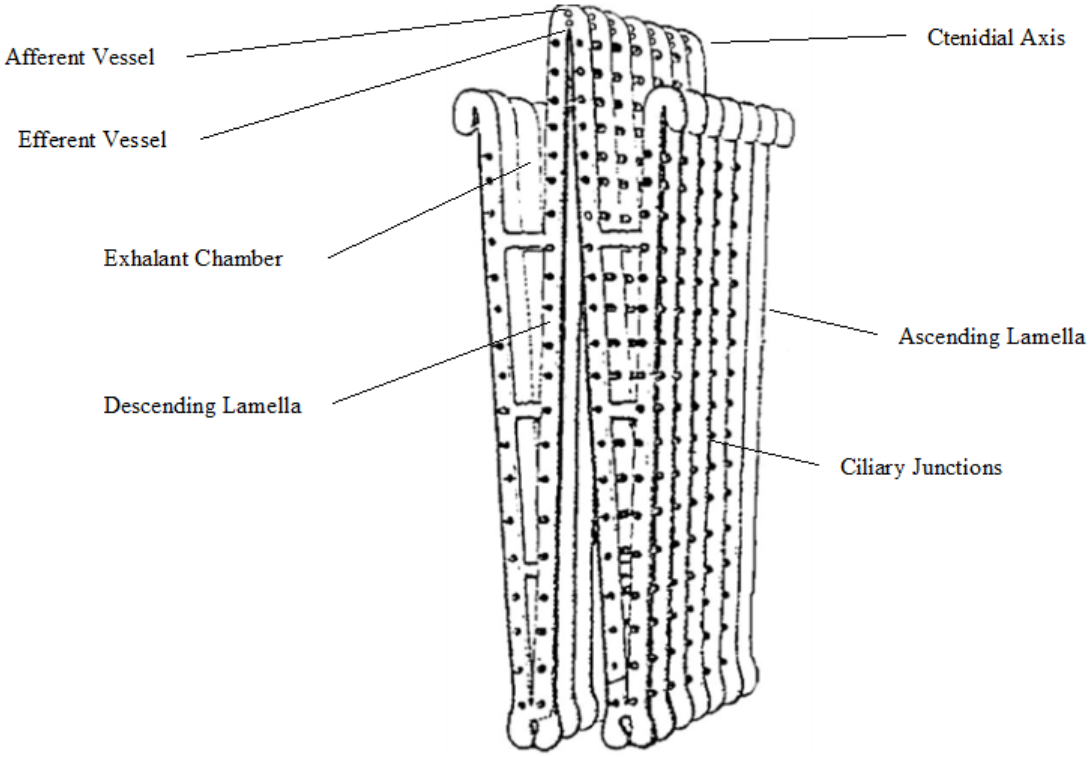


Figure 2.5 Representative scheme of gills in Mytilus galloprovincialis. Adaptation from: MEDRAP, Mediterranean Aquaculture Project (FAO, 1986).

Once conveyed and selected by cilia movements of gills and mantle, food particles are transported to the mouth where they are collected by small tentacles, called labial palps. Then, food particles are conveyed into the stomach to be digested. This organ has a structure which is peculiar to molluscs and is called crystalline style (Figure 2.6). This is a semi-transparent gelatinous rod of about 3 cm, rich in mucus and digestive enzymes which is contained inside a special sac at the posterior end of the stomach (Purchon, 1957). The stylus protrudes into the stomach cavity and is made rotating by the movement of the style sac cilia, its tip rubbing and gradually consuming itself against the stomach wall that is protected by a cuticular coating called gastric shield. A variety of digestive enzymes are released during style abrasion: amylases, cellulases, laminarinase, β-galactosidase; proteolytic activity is low in bivalves, but endopeptidases like

chymotrypsin and cathepsin have been reported (Reid, 1968). The food coming from the short esophagus amalgamates with the mucus produced by the style, forming a string wrapping around the head of the style. The low gastric pH facilitates the detachment of food particles from the string of food and mucus, that are then attacked by digestive enzymes. Inside the stomach there are ciliated tracts with ridges and grooves that act as sorting areas: smaller and digested food particles are kept in suspension by cilia ridges and swept away into the digestive duct openings; larger food particles and the small masses of waste particles (fecal pellets) forming during the digestion are conveyed are channeled into the intestine throughout a long rejectory groove on the bottom of the stomach. After several loops, the intestine ends with the anus opening, that is placed above the posterior adductor muscle near the exhaling siphon. The digestive gland is a major site for the uptake of metal and organic contaminants; a wide range of enzymes related to the bio-transformation of organic contaminants has been detected (Livingstone and Pipe, 1992); the response of this tissue to contaminant exposure has been well documented (Livingstone et al., 2000; Shepard and Bradley, 2000; Apraiz et al., 2006).

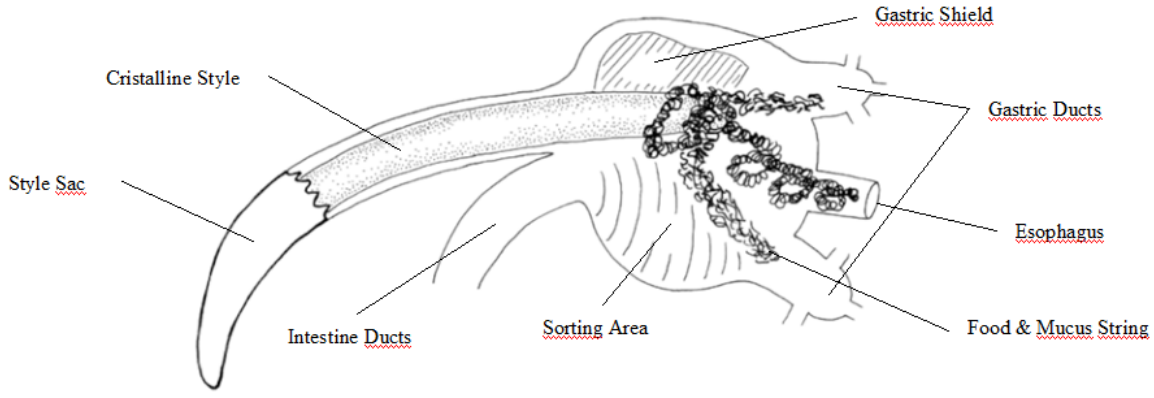


Figure 2.6 Representation of the stomach in Mytilus galloprovincialis. Adaptation from: Acquacoltura Responsabile (Cataudella, Bronzi, 2001).

Bivalves do not have a closed circulatory system: from the heart, the haemolymph flows into some main vessels and then fills all the cavities of the body except for the pericardium and the gonads, bathing the tissues. Muscle contraction forces the haemopymph to flow into certain gaps of the connective tissue, changing the form and giving temporary stiffness to some organs like labial palps, the siphons and the foot. It is clear that haemolyphm plays a number of important roles in bivalve physiology: beyond gas exchange and osmoregulation, nutrient distribution and waste collection and elimination, it also serves as fluid skeleton. The haemolymph coming from the tissues goes to the kidneys where it is purified. In the mussels of the genus *Mytilus* the circulatory system is slightly different from that of the other bivalves: some of the haemolymph from the kidney enters the gills passing throughout the ascending and the descending filaments and passes back to the kidney to be purified one more time and then from the kidney to the heart. In other bivalves haemolymph from the gills does not return to the kidney but directly flows to the heart.

Burrowing into the substrate is the habit most extensively exploited by bivalves: they burrow into sand, mud or gravel using the foot. Nevertheless, mussels of the Mytilidae family live a sessile life living permanently attached on the surface. Attachment to a hard surface is provided by the production of threads of byssus, a substance that immediately solidifies after contact with water and through which the molluscs can anchor themselves to the rock. The byssus production is possible by the retention until adult life of the byssal apparatus, a post-larval structure that evolved to temporarily attach the animal to the substrate during the vulnerable stage of larval metamorphosis. This return to the epifaunistic life of their ancestors is believed to have occurred early on in the evolution of

bivalves, about 400 mya (Yonge and Thompson, 1976). In mussels the byssus-producing gland is located at the basis of the foot; together with the foot the byssus is located in the front side (the head) of the animal, which is small and pointed. This affects also the size of the anterior adductor muscle, which is quite reduced in dimensions. By contrast, the back side of the mantle and of the shell is large, rounded and houses a large posterior adductor muscle. The punctual dwindling of the anterior end and expansion of the posterior resulted in the adoption of a pronounced triangular shape and led to significant changes in the cavity shape and in water circulation inside the mantle. The evolution of this heteromyarian form allowing free access to the water in bivalves, that tend to attach themselves to the substrate by means of byssal threads, is believed to be an adaptation of the sessile condition and of living in clusters (Morton, 1992).

The reproductive cycle of bivalves is very simple. Mussels have a gonochoristic reproduction, that is sexes are separate and males and females spawn simultaneously. As previously said, in mussels the gonads develop within the mantle: the mantle containing gametes is usually orange in females and creamy-white in males. *M. galloprovincialis* has high fecundity and spawns at the time of year with the highest water temperature (Bayne, 1976). Mussel larvae are dispersed like passive particles matching the speed and direction of surface currents generated by the wind. From the fertilized egg a larva develops, called the veliger, that is characterized for having a protein matrix plate and a large ciliated lobe called velum (Figure 2.7). The velum allows the larva to float during its migration, and through its cilia to filter from water food particles with which the larva feeds itself. Transported by the currents, bivalve larvae can cover very large distances, which would be impossible for adults, thus ensuring the spreading out of the species. At

the end of the larval stage, when bivalve larvae are about 200 µm long, larvae must find a place where to settle and start metamorphosis. The foot first appears in the juvenile stage so that larvae can start to swim and crawl, and is very large in shape and rich in haemolymph (pediveliger). In juvenile mussels the ventral side of the foot is covered in cilia and there are as many as nine different kind of glands, each of which plays its own specific role in crawling and attachment (Lane and Knott, 1975). For their settlement, mussel veligers need the presence of a filamentous substrate of diatoms and colonies of hydroids. The pediveliger sinks to the bottom by withdrawing the velum and extending the foot in order to start crawling. If the substrate is unsuitable, the foot is withdrawn and the larva swims off once again (Lutz and Kennish, 1992). When the pediveliger finds a suitable substrate, it gradually ceases moving, protrudes the foot and starts secreting the byssus threads to settle and start the metamorphosis. Byssus threads can be repeatedly broken and reformed before final settlement takes place. In adult mussels the foot has not entirely lost its locomotory capacity.

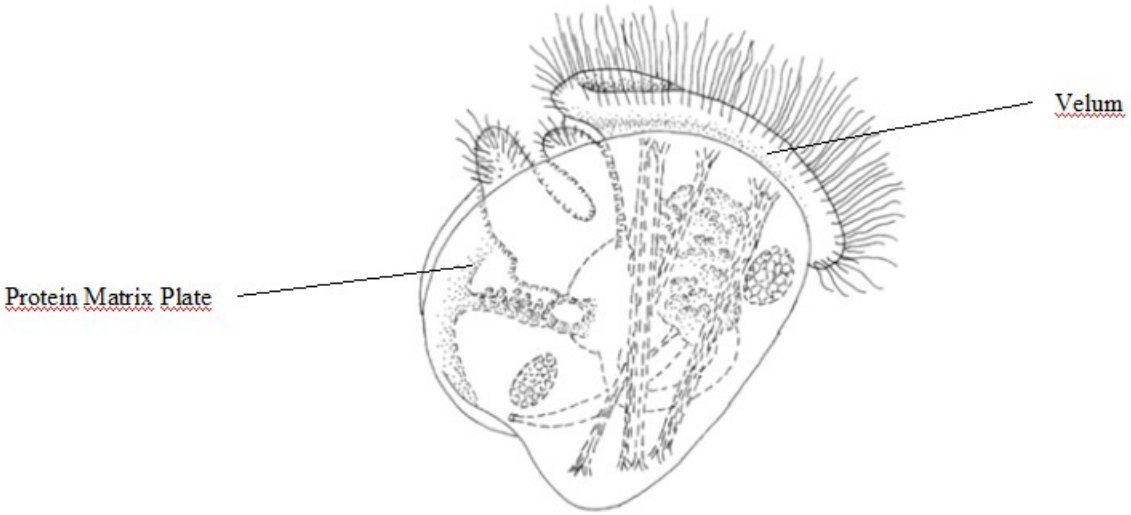


Figure 2.7 Mussel veliger. Adaptation from: Acquacoltura Responsabile (Cataudella, Bronzi, 2001).

Chapter 3
The culture of M. galloprovincialis *in Italy and Sardinia*

3.1 The State of Italian mussel culture

Shellfish culture is the principal activity of Italian aquaculture industry: despite the slight decrease registered in 2009 (-4,2%), shellfish aquaculture industry accounts for about the 68% of the national aquaculture production, surpassing also finfish aquaculture (about 32%) (ISMEA, 2010). The national shellfish aquaculture production is based almost exclusively on mussels and clams, with limited quantities of oysters. Undoubtedly, Mediterranean mussels (*Mytilus galloprovincialis*) is the most important product of the national fishery and aquaculture activity, representing almost (49,96%) half the national shellfish production (ISMEA, 2010). Italy is the main producer of Mediterranean mussels and clams inside the European Community, locating inside its territory the 60% of *Mytilus galloprovincialis* production and the 96,7% of *Ruditapes* species production, respectively. The most recent data of the national mussel production report that, after the decrease by 8% registered in 2008 that influenced the total aquaculture production (almost -6%), mussel culture production remained stable during 2009, contributing to reduce the decrease in the global shellfish farming production (-4,2%) caused by the drop down in the Manila clams production (-16%) (ISMEA, 2009 and 2010).

Italian mussel farming essentially relies on three rearing systems, namely the fixed system, the single ventia long-line and the ‘‘Trieste’’ long-line or multi ventia system. The system of mussel culture in Italy is very complex, since it is the result of two different

forces acting on it: from the one hand, the existing legacies inherited by the old farming system of extensive nature, limited to very restricted regions of the national territory, characterized by an artisanal management of the farming activity and the persistence of old traditions and practices of farming, that profoundly influenced also the social structure of the local communities; from the other hand, the introduction of newer and more modern techniques of intensive farming like the long-line, that is gradually helping to overcome the local and artisan traits of mussel farming activity by conquering new spaces off-shore and creating farming opportunities no longer constrained by environmental or sanitation concerns.

On the basis of the employment data available on the institutional channels, in 2005, in Italy there were 263 settlements dedicated to mussel culture with about 1400 workers employed (Prioli, 2008); these settlements are mainly situated in the Gulf of Taranto (Puglia), La Spezia (Liguria), the Veneto lagoon, the Gulf of Napoli and the Flegreo coast (Campania), the Gulf of Gaeta (Lazio), the Gulf of Trieste (Friuli-Venezia Giulia), the Gulf of Olbia (Sardegna), the Gulf of Goro (Emilia-Romagna), the Marche, the Abruzzo and the Adriatic coast of Puglia (Prioli, 2008).

Some of the main production areas have a long-standing tradition, where the farming systems are mostly located in lagoon areas and sheltered coastal areas. Here, Italian mussel culture is practiced in fixed systems established in lagoon areas or in sheltered coastal areas, and can be traced back to more ancient settlements that employed a modified bouchot technique. The bouchot technique has been adopted in the 13th century in France to farm *M. edulis* mussels on the Atlantic and English Channel coast, where since its origin it has been used without drastic changes (Figure 3.1). The bouchot

method consists of the setting of wooden poles (4-7 m long, 12-25 cm in diameter) fixed for half their length into the intertidal seabed, placed in rows perpendicular to the shoreline, where mussel seed is placed in tubular nets rewound around the bouchot poles for on-growing (Goulletquer and Herald, 1997). Conversely, spat bouchot are situated off-shore and consist of parallel row of poles with horizontal ropes for seed collection strung between the poles (Goulletquer and Herald, 1997).

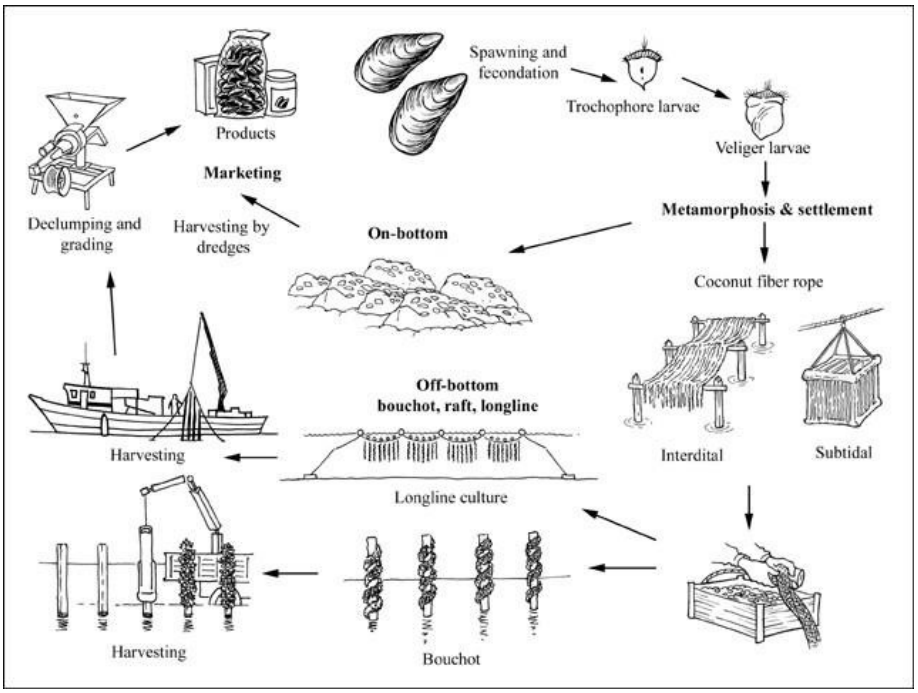


Figure 3.1 Production cycle of the Atlantic mussel Mytilus edulis with the bouchot and the long-line farming techniques. Source: FAO.

In Italy the bouchot method has been adapted with some modifications. The wooden poles, usually made of chestnut, were stuck for about 2 m into the seabed and protruded for about 1,50 m from the mean sea level; poles were linked together by marsh grass ropes or by wooden poles placed horizontally to form a structure called “ventia” (Figure 3.2A); a number from 6 to 8 mesh-stocking ropes named “pergolas” hung from each “ventia” (Figure 3.2A); each “pergola”, from 3 to 7 meters long, was intertwined with mussel spat destined to on-growing (Bussani, 1983). In this modified farming technique,

poles were placed at 10-15 meters of distance in rows perpendicular to the shoreline, reflecting the classical bouchot scheme (Figure 3.2B); but also a different organization has spread over, in which the pole units were arranged according to squares instead of rows. Each square represented a modular unit of 5 meters long that was composed of four poles, each one placed at one square corner and linked with ropes to other two units across the same square side (ventia) and the same square diagonal (crociera) (Figure 3.2A). The squared module is repeated several times to form a bigger squared structure; at the external side the single poles are substituted by groups of two poles (coppiola) or three poles (triangolo) joined together; the coppiolas are placed alongside the square, while triangolos are placed in correspondence to the corners (Figure 3.2C). This structure can make up the whole farming plant, covering an area of 500 squared meters or more of extension. This alternative organization scheme was first adopted in the Gulf of Taranto and rapidly spread out in the rest of Italy (Bussani, 1983).

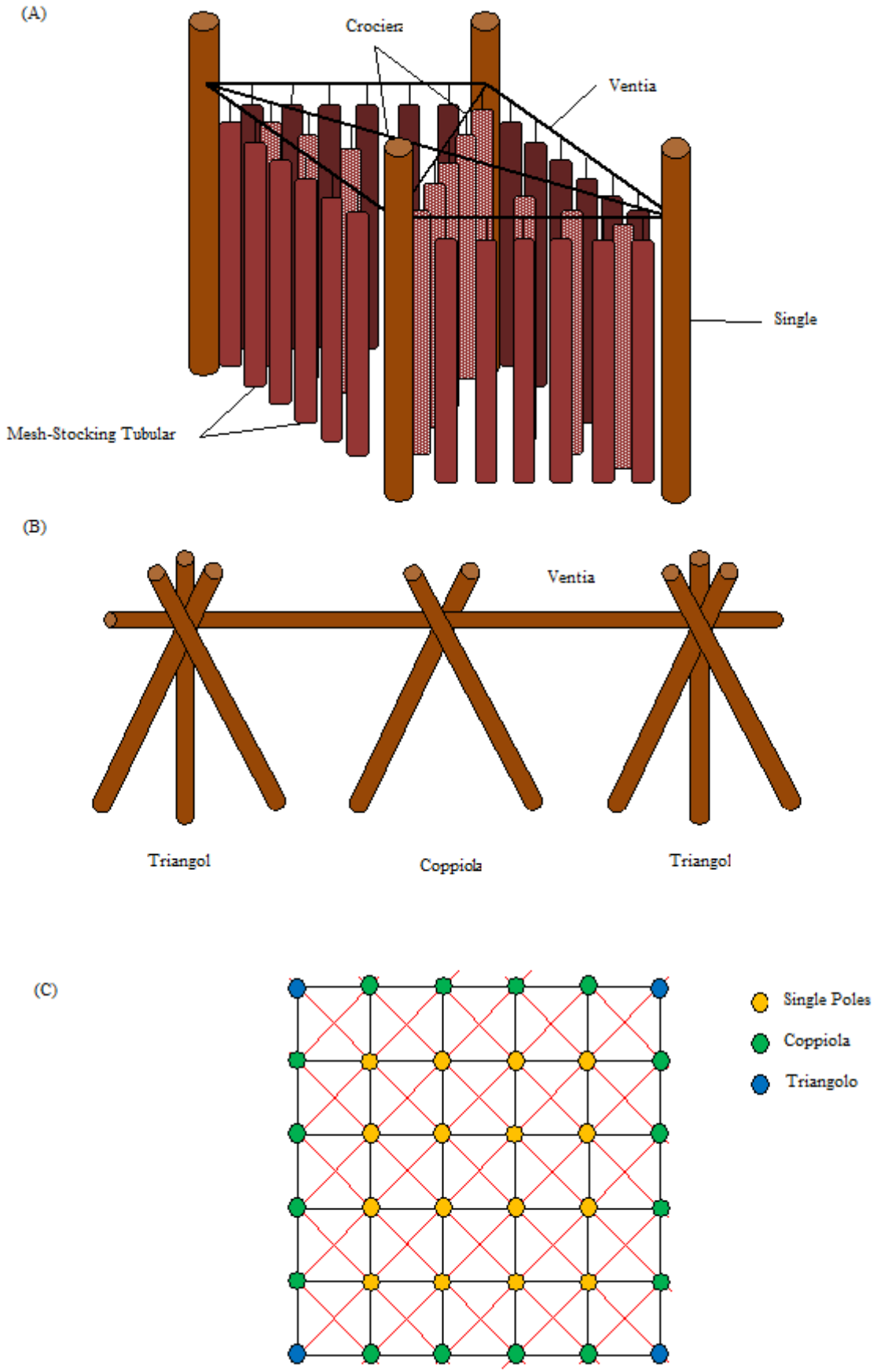


Figure 3.2 Representative scheme of the fixed systems employed in Italy for mussel farming.

Traditional farms with fixed systems were mostly located in lagoons and coastal areas of the Veneto lagoon, the Gulf of Trieste (Friuli-Venezia Giulia), the Gulf of Taranto (Puglia), the gulf of Goro (Emilia-Romagna), the Gulf of Olbia (Sardinia) and in La Spezia (Liguria) (Prioli, 2004), quite often run by families or groups of families. Productions in fixed systems could range across 12 kg/mq, but they varied a lot on the basis of different conditions depending on water currents and interchange, food resources availability and the occurrence of chemical processes of biodegradation in seawater for the production of mussel excrements (pseudofaeces). A phenomenon that highly recurred in these systems, particularly during the summer, was that the inner pergolas contained smaller and less vigorous mussels than the external ones, so that trimming the number of internal "pergolas" was necessary by moving them to a specific ventia placed around the external perimeter of the system, called "filimbindo" (Bussani, 1983).

Fixed systems facilities have been through a gradual process of modernization and some modifications have been progressively made to the original fixed systems. Chestnut wood poled have been progressively substituted with concrete poles or zinc-iron poles, since these materials are cheaper and more long-lasting than chestnut wood, while marsh grass ropes have been replaced with iron steel or nylon ropes and plastic tubular mesh-stocking nets. However, in many cases facilities modernization coincided with the adoption of other systems and the progressive replacement with offshore surface long-lines (Figure 3.1 and 3.3).

Historically, the long-line system has been developed during the years '60s in the Atlantic coast of France, where a system capable to resist storm and wave effects and particularly adapted to areas showing high tidal cycles was required (Goulletquer and

Herald, 1997). A long-line consists of a series of floats connected by horizontal lines, from which tubular nets or mesh-stocking collectors hang for the on-growing of mussels by continuously feeding in the ceaseless current (Figure 3.3). The whole structure is kept stable by two dead bodies anchors, generally two concrete blocks weighting several tonnes, placed at a distance ranging from 100 to 200 meters from the long-line, and connected by one or more cables kept in suspension by a succession of floats. Long-lines are used in Ireland and United Kingdom, while a multi-longline system using 7-9 headlines has been developed in Norway and Sweden. As in the case of bouchot system, the production of long-line systems depends exclusively on the settlement of natural seed in the system. Seed is either caught on collector ropes hung from the floating lines, or gathered from natural settlement in intertidal areas during spring. After spat has been seed inside the tubular nets, during their growth, mussels are rub-off by the original rope, separated by size and then attached to new ropes. This operation, called “thinning” is necessary to stimulate rapid and uniform growth or if the mussels grow too rapidly before harvesting and in order to ensure that all mussels reach a similar size at harvest time. Thinning and reseeding onto grow-out ropes or into stockings are carried out until the mussels reach the size of 5 cm, corresponding to the minimum marketable size allowed in Italy (D.P.R. 02/10/98, n°1639, art.89).

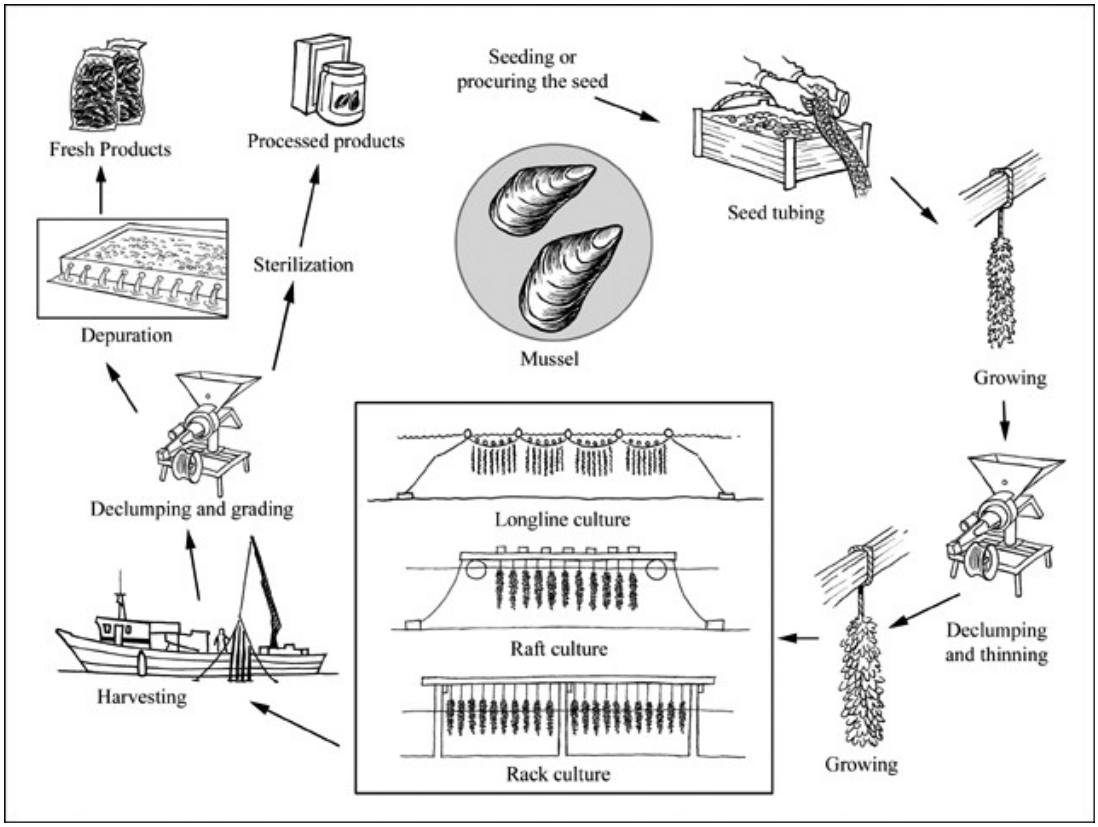


Figure 3.3 Production cycle of the Mediterranean mussel Mytilus galloprovincialis with the fixed systems (rack culture) and the long-line farming techniques. Source: FAO.

The long-line technique allows highly mechanized culture and high-yields productions of about 18-20 tonnes/ha per year (Goulletquer and Herald, 1997). Long-line systems offer great resistance against seawater events, included those of relevant magnitude, so the advent of this new off-shore farming technique allowed farmers to conquer new spaces beyond lagoon areas and sheltered coastal areas, where mussels have been farmed in traditional fixed systems with the well-known limitations. Consequently, new mussel farming facilities have started their activity also in those regions of the Italian peninsula where establishing shellfish rearing systems was impossible for the natural condition of the coastal environment, like the Marche, the Abruzzo and the Adriatic coast of Puglia. In a relatively short time, about twenty years, the single ventia long-line system have come into use in almost all regions of the Italian peninsula, where it replaced fixed systems, and today it constitutes the strong point of Italian shellfish aquaculture. The

introduction of the long-line system has brought a change also in the identity of the business ownership. In the ancient past, mussel farming facilities had been run by families or groups of families; this tradition has been reflected in the forms of running ownerships, which usually are sole proprietorships and co-operatives, but also more complex forms like partnerships and limited liability partnerships have been established after the introduction of the long-line system.

But also another farming technique, called "Triestino" or "Trieste" multi—ventia long-line is in use in the Italian waters. This system is a modification of the single ventia long-line and has originated during the 1980s along the Trieste coastline, from where it takes its name; in this case, two or three parallel lines of ropes or "ventie" whose length varies between 100 and 200 meters, are joined together and kept separate by floats (Figure 3.4); the "Trieste" system is used in partially or totally sheltered areas. Multi ventia systems are very common in Friuli-Venezia Giulia, where nearly all rearing systems have been substituted, and in Puglia, Liguria and Sardinia, as well. In Italy there are about 2.700.000 linear meters (lm) of long-line available for mussel farming, with each company managing an average of about 10.000 lm; of these, about the 75% of the linear meters available for mussel farming are accounted by single ventia plants (Prioli, 2004). The regions with the largest number of linear meters are Emilia-Romagna, Puglia, the Veneto, Friuli-Venezia Giulia and Sardinia, where the largest companies are also based (Prioli, 2004). Among them, Puglia, Veneto, Emilia-Romagna, Friuli-Venezia Giulia and Sardegna account for the 80% of mussels national production.

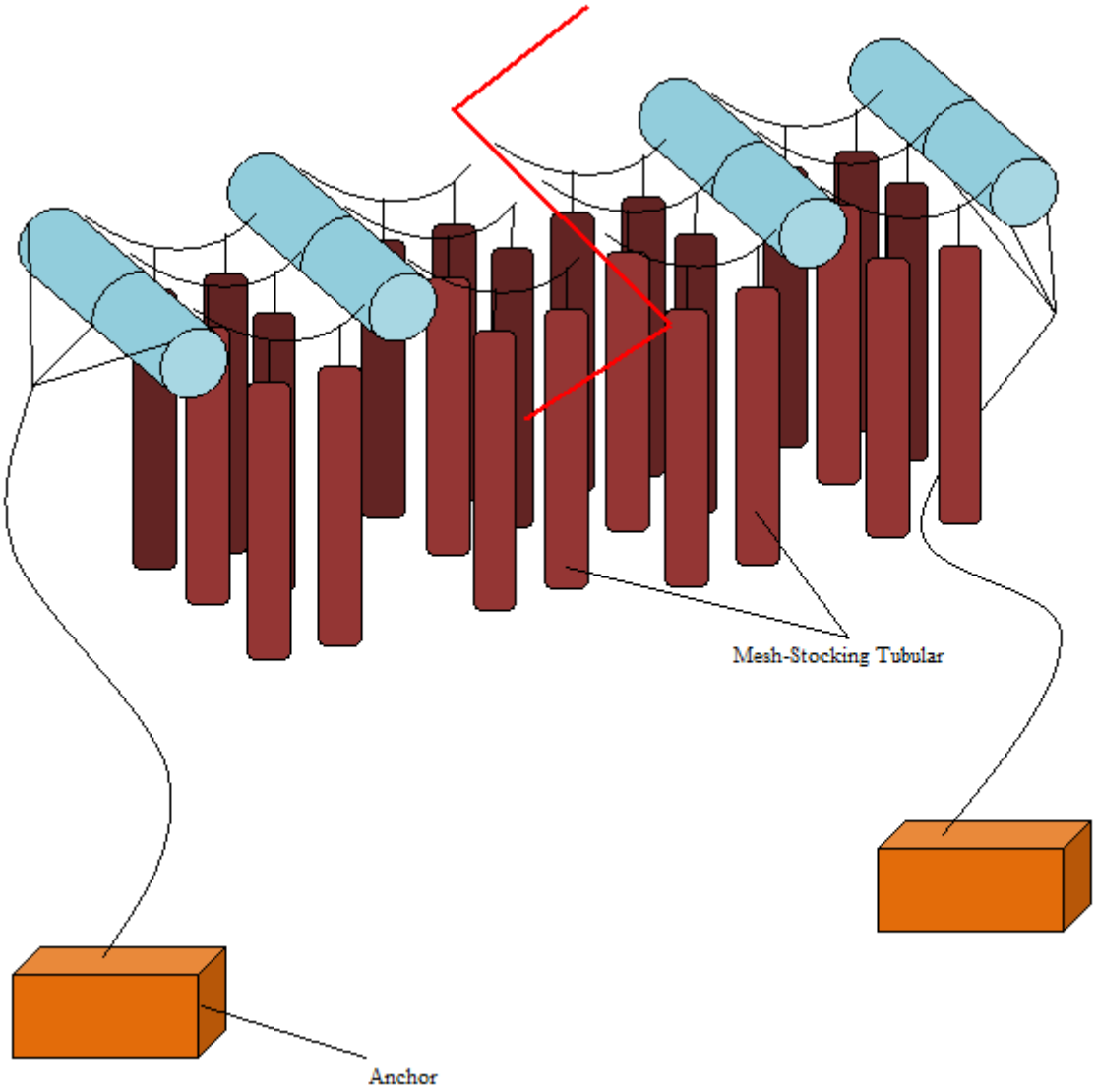


Figure 3.4 Representative scheme of the "Trieste" multiventia Long-line.

Generally speaking, hatchery production of mussel seed, even if it is a feasible activity, yet is not so widespread because of the high costs in setting up and running hatcheries if compared to the high availability of seed in nature. In Italy, mussel culture relies on the use of natural spat that may be caught from the wild or is produced in cultured stocks in the mussel farming plants or, alternatively, comes from Italian hatcheries, which are a very limited number and are situated in Puglia, the Veneto and Emilia-Romagna (Prioli, 2004).

The safety conditions that must be fulfilled during the phases of harvesting, handling, storage, transport and distribution of live bivalve molluscs are laid down by the Italian Decree Law No. 530 of 30th of December 1993, which implements the Council Directive of the European Communities of 15 July 1991 regulating the health conditions for the production and marketing of live bivalve molluscs (91/492/EEC).

On the national territory Mediterranean mussels are sold at an average production price of about 1 €/Kg (ISMEA, 2009 and 2010). However, during the 2009 a decrease in the average annual price was observed (€ 0,95 /kg), that originated also from seafood safety problems: the appearance of DSP toxins (Diarrheic Shellfish Poisoning) manifested in Trieste and numerous cases of poisoning were recorded in Northern Italy. The phenomenon occurred during summer, when the Italian product reaches the commercial size and the seafood demand is higher, and by consequence mussels sales were blocked in the North of Italy, negatively affecting the production prices (ISMEA, 2010).

It is noteworthy considering that, on the whole, in Italy fisheries and aquaculture activities occupy a secondary position in the national agricultural and food activity, accounting for about 4% of the national production and almost 5% of the added value of the whole primary sector (ISMEA, 2010). During the last years the Italian fisheries registered a relevant crisis and the year 2008 was a particularly difficult year for the Italian fish production. Italy fisheries operating in the Mediterranean sea significantly reduced their activity, both for the policy of progressive reduction of the fishing capacity adopted by the European Union and the increase of fuel prices as a consequence of the World economic crisis. The slight increase recorded in 2009 in the national fisheries in the Mediterranean area (+8,1%) was not sufficient to recover the sharp drop registered in

2008 (-19%). Only aquaculture industry have been registering a continuous increase during the last years: despite the decreases registered in 2008 (-3,9%) and in 2009 (-2,3%), the Italian aquaculture production, inclusive of finfish and shellfish production, accounts for almost half the national fish production with a mean annual increase of 2% and a total production of 232.170 tonnes (ISMEA, 2010). Thanks to aquaculture production, Italy ranks fifth in the list of the the EU-27 producing countries (ISMEA, 2010).

The World economic crisis not only caused the decrease in fisheries revenues, but also it impacted on domestic demand. A slight reduction in the domestic demand of seafood has been registered during the last years, that somehow interested also the domestic purchases of mussels: despite the slight but regular increases of mussel demand during the last years from 2007 to 2008 and from 2008 to 2009 (+2,1% and +3,1%, respectively), a general stagnation in mussel consumptions can be detected, that has been carrying on for some years: consumptions are only a bit higher than those in 2004 (ISMEA, 2010).

Italy ranks fourth in the list of top ten world importers countries of fish and fishery products (FAO, 2010). The European Union is the primary area of international trade for Italy. Italy ranks tenth in the list of EU-27 exporters and third in that of EU-27 importers (ISMEA, 2010). Together with anchovies, sardines and trouts, mussel represent half the share of Italian export (ISMEA, 2010). Italian fresh mussels are exported mainly to France (46%), Spain (36,2%), and Germany (5,2%). During the last years, mussels exports have registered a remarkable decrease: from 2007 to 2008 and from 2008 to 2009 the registered decrease was -21,8% and -24,2%, respectively, confirming the negative trend in Italian exports (ISMEA; 2010). For what concerns Italian imports, fresh mussels represent the most imported product, covering about 16% of fresh seafood imports. However,

despite the remarkable increase in the years 2007-2008 and 2008-2009 (+17,4% and +14,7%, respectively) mussel imports have been gradually decreasing since 2004 (-1,9%), resulting the only imported seafood product with a negative registered trend (ISMEA, 2010). Italy imports fresh mussels mainly from Spain (71,1% of imports) and Greece (24,3% of imports). Spain is the principal import-export partner of Italy, accounting for 18,6% of imports and 29,1% of export. However, in the countries where it exports, Italy occupies a secondary position as an exporter. Italian products often do not benefit of specific identity or features that differentiate them from those of other direct competitors, they can only compete for the price. As an example, in 2008 exported mussels were sold at a medium price of 0,97 €/Kg (ISMEA, 2009). The lack of differentiation of the Italian supply is making Italy increasingly vulnerable to competitors coming from other European countries and third countries, whose products are being sold at very low prices. For instance, Italy was the first importer of fresh mussels in France but in the last 5 years its position has worsened because of the higher sale prices of the Italian product; by consequence, Italy has been overtaken by Spain, which is now the exporter EU country leader in France (36% of market share) (ISMEA, 2010).

3.2 The Role of Sardinia in the National Fisheries and Aquaculture

Mussels have always been the most cultured species in Sardinia. The history of Sardinian shellfish culture is younger than the Italian tradition. According to a recent survey among Sardinian fishermen, shellfish culture have been practiced in the island since 1981, while finfish aquaculture started two years earlier, in 1979 (Viale, 2009). Historically, the first plant of mussel farming in Sardinia was established in the Gulf of Olbia in the early 1920s; this shellfish culture plant is the most ancient in Sardinia and one

of the most recent in Italy. Here, the first mussel farmers came from Taranto or La Spezia soon after the end of the first World War (Bussani, 1983; Tognotti, 2002). They first made an exploratory survey of the waters of the Gulf of Olbia, to assess whether the environmental conditions of the inner area of the Gulf were suitable enough for mussels breeding. The inner part of the Gulf of Olbia is neither too broad nor too deep-bottomed, is sheltered from the winds except for the greek-east wind, which however is not very frequent in the area, and is enriched by the influx of fresh water from the local river Rio Padrongianus. Moreover, the vast harbour inlet, the amplitude of the semidiurnal tides and the currents determined by them establish the ideal conditions for the development of mussel farming in this stretch of coast. Once that the sites where to settle the rearing plants were chosen, the spat began to come from the hatcheries in La Spezia, while ropes and chestnut poles came from Taranto and Torre del Greco, and the mussel culture activity began to start (Tognotti, 2002). The mussels farming system that was originally employed in the Gulf of Olbia is that of based on a modification of the bouchot method that was inherited from La Spezia farmers and is called “Italian system” (Sistema italiano) (Bussani, 1983) (Figure 3.5). Fixed installations spread out across the whole bay of the Gulf of Olbia, made up of wooden or zinc-iron poles, about 10 m long, placed parallel to the shoreline and spaced about 4 – 5 meters, with half their length embedded in the seabed. Today, after almost 90 years, the Gulf of Olbia accounts for one of the most important districts of *Mytilus galloprovincialis* culture in the Meditarranean sea (Tognotti, 2002). Other mussel culture plants have been established across Sardinia: today there are 15 plants of mussel culture, situated in the gulf of Cagliari and in the lagoons of Tortolì and Cabras (Oristano) (Viale, 2009).

Figure 3.5 An historical picture of the fixed system for mussel farming in Olbia.

Sardinian lagoons, commonly referred to as "ponds", are shallow transitional coastal system where mussel rearing is practiced extensively together with the rearing of other finfish and shellfish species. With an estimated yield of more than 2.000 tons per year, Sardinian ponds are still one of the most interesting productive realities of the Mediterranean area (Cannas, 2001). The rearing activity is practiced in 30 lagoons for a total extension of about 8.250 hectares (Cannas, 2001). The salinity of Sardinian ponds is usually high for the limited intake of freshwater and the relevant inputs of sea water; this characteristic makes Sardinian ponds a suitable environment for mussel rearing: molluscs account for 66% of the average annual production of Sardinian ponds, while the remaining share is represented by different finfish species and eels. The aquaculture and fisheries activity in the lagoons is managed by cooperatives of fishers and fish farmers, that have usually exploited the natural resources in a traditional way. This kind of conservative management of the lagoon environment has permitted the preservation of

the natural environment and its biodiversity. Over the past 30 years, important operations of environmental reclamation have been realized on 26 ponds over the 30 lagoons of Sardinia, in order to protect them from the natural process of environmental degradation. These operations have consisted in the dredging of the bottoms and the rationalization of freshwater and seawater inputs in order to facilitate the natural process of conversion into marine habitats. As a consequence, production data report a decrease in fish produced quantities and an increase in the variety of species reared in Sardinian lagoons, with great advantage for mussel culture, that registered an increase in produced amounts (Cannas, 2001).

Other rearing system employed in Sardinia as an alternative to the "Italian system" are the longline mono-ventia system and the "Trieste" multi-ventia long-line system. Together with Emilia-Romagna, Puglia, the Veneto and Friuli-Venezia Giulia, Sardinia is one of the Italian regions with the largest number of linear meters of long-line available for shellfish farming and where the largest companies are also based (Table 5.3) (Prioli, 2004). In Sardinia, mussels are reared at an average density ranging from 10 to 80 Kg of mussel per mesh tube (Viale, 2009).

Table 3.1 Regional distribution of linear meters (lm) used for mussel farming. Adapted from Prioli, 2004.

Region	Number of Companies	Total Long-line Linear Meters	Average Long-line Linear Meters per Company
Abruzzo	1	18.000	18.000
Campania	12	41.288	3.441
Emilia-Romagna	19	631.150	33.218
Friuli-Venezia Giulia	24	186.440	7.768
Lazio	4	21.295	5.324
Liguria	68	49.042	721
Marche	6	55.500	9.250
Molise	2	46.000	23.000
Puglia	31	550.270	17.751
Sardinia	16	143.660	8.979
Sicily	1	600	600
Veneto	20	303.240	15.162
TOTAL	204	2.046,485	10.032

In recent years the number of aquaculture plants in Sardinia has been greatly reduced. In 2002 there were 50 operating aquaculture plants, of which 22 were allocated to mussel culture. Since then, this number has undergone a strong reduction; today in Sardinia there are 35 operating aquaculture plants, and 15 are allocated to shellfish and mussel culture, while the remaining 20 aquaculture plants are allocated to the rearing of the two eurhyaline species sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*). Nevertheless, Sardinian aquaculture production has been increasing recently. Mussels rearing has increased its share during the period from 2002 (75,31%) to 2008 (82,60%), even if this share is comprehensive of the part of imported mussels that are traded in Sardinia during summer, the period of highest demand (Viale, 2009). During a ten years

period from 1992 to 2002, Sardinia has reached to double its mussels production and in 2008 the quantity of reared mussels accounted for 10.662,00 tonnes, equivalent to € 19.723.715,00 of income (Viale, 2009). To this amount, about another 2 tonnes of mussels coming from the fisheries must be added (IREPA, 2009). The remaining share of 2008 aquaculture production (17,4%) is interested by finfish aquaculture production, equivalent to about 15,604 millions of euro (Viale, 2009). The eurhyaline species sea bass (*D. labrax*) and sea bream (*S. aurata*) account for the most part (15,73%) of the remaining share (Table 3.2).

Table 3.2 Sardinian aquaculture production (tonnes). From: Viale, 2009.

Aquaculture species	**1992**	**2002**	**2008**
Mussels (*M. galloprovincialis*)	4.000,00	7.900,00	10.662,00
Oysters (*C. gigans*)	3,00	1,30	6,00
Sea bass (*D. labrax*)	50,00	926,00	646,00
Sea bream (*S. aurata*)	50,00	1.293,00	1.385,00
White bream (*D. puntazzo*)	1,00	67,00	16,70
Mullet (*Mugil spp.)*	1,20	12,40	10,60
Shi drum *(U. cirrosa*)	==	==	19,00
Eel (*A. anguilla*)	90,00	200,00	104,00
Trout (*Oncorhynchus* and *Salmo spp.*)	80,00	90,00	58,00
Shrimp (*Marsupenaeus japonicus*)	1,20	12,40	==

The mussel spat destined to Sardinian mussel farming partly comes from Sardinian ponds, where it is collected from the wild or that is directly present on the existing farming systems, partly comes from Italian hatcheries. During the years, mussel seed have been brought from La Spezia, Gaeta and Chioggia (Bussani, 1983) and in more recent years from Puglia, Veneto and Emilia-Romagna (Prioli, 2004). It is estimated that in 2008

Sardinian mussel culture plants bought a total of 8.462,00 tonnes of mussel seed, at an average length of 2 – 3 cm and an average price of € 0,50 – 0,70 per Kg (Viale, 2009).

The production cost for mussel rearing is about € 0,98/Kg, but mussels are sold at an average cost of about € 1,70/Kg (Viale, 2009). According to the survey carried out by the Regional Sardinian Agency Laore during 2009 among mussel farmers, this cost value is mainly influenced by three factors: on the one hand the management cost of the staff employed in the farming activity, on the other hand the price of purchase of the spat, which is remarkably high. For the mussels destined to be sold in the national market, also the cost for the transport off the island, equal to € 0,20/ kg of mussels, must be added.

Chapter 4

Research Article: A proteomic approach to the study of Mytilus galloprovincialis *in Sardinia*

4.1 Abstract

Proteomics is increasingly applied to marine species to study different biological aspects. Particularly, characterization of the proteome expressed in different tissues of mussels is important to understand many different biological, physiological and ecological aspects that may be of advantage in shellfish rearing, encompassing growth cycle, food safety, traceability, seafood authentication and quality, as well as understanding the effects on food productions brought by environmental pollution and climate change. Mussels rearing is the leading activity of Sardinian aquaculture and we believe this to be the first proteomics work in the study and characterization of mussels reared in Sardinia. The present work attempted to accomplish a systematic characterization of the foot muscle proteome of M. galloprovincialis, to gather data about its variability in physiological conditions occurring in different environments of mussel farming and to identify protein expression patterns that may be significantly correlated to growth and environmental factors. The preliminary results of this work show that there is correlation between the expressed protein pattern and the environmental conditions of the farming site, thus paving the way towards more extensive research on protein variation among different population of marine species and the discovery of possible biomarkers for environmental pollution and climate change.

4.2 Introduction

The proteome is traditionally defined as "the set of protein expressed by the genome of a cell, a tissue or an entire organism" (Wilkins et al., 1996). Thanks to the many advances made in the field of electrophoresis and MS analysis, proteomics has become a high-throughput discipline can provide a quantitative description of protein expression and its changes under the influence of biological perturbations as well as the occurrence of post-translational modifications and the distribution of proteins within the cell. In the last decade proteomics have been increasingly used in fish biology research. Proteomics strategies have been employed in seafood to better understand the biochemical mechanisms underlying some technological properties of seafood that may be relevant in food processing (Verrez-Bagnis et al., 2001; Martínez and Friis, 2004); in aquaculture studies to assess the biochemical response to specific dietary manipulations (Martin et al., 2001; Martin et al., 2003 Brunt et al., 2007) or to identify metabolic biomarkers of chronic stress induced by high stocking density (Alves et al., 2010). Proteomics has been employed also for the identification of species in seafood authentication (Piñeiro et al., 2001; Carrera et al., 2006; Berrini et al., 2006) and in taxonomic studies. Proteomics has been employed also in studies regarding the mussels belonging to the genus Mytilus, both to distinguish among species and populations (López et al., 2002a ; López et al., 2002b) and to study genetic variability and populations genetics (Mosquera et al., 2003), as well as to understand the mechanisms of the maternal effects in the heritability of gene expression (Diz et al., 2008). The biochemistry at the basis of speciation undergoes through the process of environmental adaptation. The proteome, more than the genome, is suitable to reflect adaptive changes produced in species and proteomics may

complement the analysis of populations biology. This explains the application of proteomics in ecology and evolutionary studies and the emerging of a new field in proteomics discipline, Population Proteomics (Biron et al., 2006). At least three evolutionary mechanisms occur at gene expression and gene product level during the adaptation process to distinct habitats: changes in the amino acid sequence of the protein, changes at the protein expression level, and changes in the protein environment (Somero, 2004). One of the very first studies of population proteomics has studied the expression differences between populations of M. galloprovincialis living in different ecological conditions (López et al., 2001). A proteomic approach was successfully applied in an ecology study on Littorina saxatilis, a non-model organism to understand the phenotypic variability at the basis of the evolutionary process of speciation (Martínez-Fernández et al., 2008). Similarly, another proteomics work had studied the protein expression patterns (PES) in mussels from a hybrid zone between the two species M. galloprovincialis and M. edulis (Diz and Skibinski, 2007). A very recent study has employed a population proteomics approach to investigate the protein expression changes induced by different environmental conditions, identifying putative protein biomarkers to discriminate among populations of marine species with a strong migratory attitude like the European hake Merluccius merluccius, for which the ecological adaptation have hindered the detection of genetic differentiation (González et al., 2010).

Proteomics has been applied with success on mussels and other aquatic species also on the search of biomarkers for pollutants and environmental stress. In the past decade the application of proteomics to the field of ecotoxicology has begun to develop and have been already object of review (Monsinjon and Knigge, 2007; Nesatyy and Suter, 2008).

Recently published works have been focused on the search of biomarkers for pollutants (Albertsson et al., 2008; Søfteland et al. 2011; Berg et al., 2011). Many studies have been conducted on mollusks, as they are considered as major natural sentinels for aquatic toxicity (Rodriguez-Ortega et al., 2003). Particularly, mussels belonging to the genus Mytilus have been object of several proteomics studies to identify proteomic signatures of exposure to marine and water pollutants (Knigge et al., 2004; Manduzio et al., 2005; Apraiz et al., 2006; Amelina et al., 2007) or to study the effects of oxidative stress on protein structure and degradation pathways (McDonagh and Sheehan, 2008). Also, concern about the effects of global climate change on seafood products is increasing (Piñeiro et al., 2010) and the number of studies on the topic is increasing; for instance, the proteomic response to acute thermal stress have been assessed in M. galloprovincialis (Tomanek and Zuzow, 2010).

Italy is the main producer of Mediterranean mussels and clams, locating inside its territory the 60% of Mytilus galloprovincialis production. Together with Puglia, Veneto, Emilia Romagna and Friuli, Sardinia accounts for the 80% of national production. Mussels have always been the most cultured mollusk species in Sardinia, where it has been reared since 1981. The first plant for mussels rearing was established in the Gulf of Olbia, the biggest productive site in Sardinia, in 1919, when some mussel rearers coming from Taranto established their own activity (Tognotti, 2002). The recent production data report that, accounting for 66% of total aquaculture production of the island, mussels culture is the leading product of Sardinian aquaculture (Viale, 2009). In 2009, the total mussel production of the island reached 8.462,00 tons. Another 2.200 tons of mussels imported had be added to the production value since the local production did not meet the

demand; on the whole, mussel production accounted for 83% of aquaculture species reared in Sardinia (Viale, 2009).

The present study is aimed at providing a characterization of the proteome of the mussels reared in Sardinia. In this work, by means of two-dimensional electrophoresis the protein expression patterns were characterized in the foot muscle of mussels that have been reared in three different lagoons of Sardinia (Figure 1): the lagoon of Calich, the lagoon of Porto Pozzo and the lagoon of Tortolì, each one characterized by different environmental and ecological systems. Insight on the proteome variability that may be associated to mussel size and to different environmental conditions like water parameters and quantity of heavy metals in the animals have been gathered and presented, showing preliminary results.

4.3 Materials and Methods

4.3.1 Mussel Sampling

Mussels sampling was performed within a large-scale study lasting from April 2010 to September 2010. The survey had planted the rearing of mussels in three different lagoon sites of Sardinia (Figure 1): the lagoon of Calich, the lagoon of Porto Pozzo and the lagoon of Tortolì. Each lagoon site is characterized for different environmental features. Porto Pozzo is a oligotrophic lagoon situated in the North-East of the island, with wide seawater inlets and low freshwater intakes, resulting in a annual mean salinity of about 38‰. The lagoon of Tortolì, placed in the middle-south part of the east coast, is a mesotrophic ecosystem that is seasonally supplied with relevant freshwaters intakes (Rio Girasole); the two communication channel with the sea are regimented and the annual mean salinity varies from 25 ‰ to 35 ‰; the lagoon hosts one of the most important mussels rearing

plants of Sardinia. On the North-West coast of Sardinia the lagoon of Calich is an example of coastal lagoon with very low levels of salinity, for the elevated fresh waters intakes by means of many affluents, particularly during and after the rainy season; this makes the lagoon an eutrophic ecosystem that often suffers from eutrophization and anoxia episodes.

Juveniles of Mytilus galloprovincialis of about 35-40 mm of size were supplied from a commercial mussel farm and cultured in long-line systems, kept in place by nylon nets for growth. Throughout the whole mollusks culture time, the growth parameters of mussels were monitored monthly: at every monitoring session, 60 mussel individuals were selected from each culture plant and transported alive to the laboratory, where they were measured for maximum length and width and opened; the weight of the soft part and the shell were recorded. When mussels reached the minimum commercialized length of 50 mm according to Italian regulation (D.P.R. 02/10/98, n. 1639), the total of 360 individuals collected at sampling dates of August and September were destined to proteomics analysis and metal concentration analysis. For the purpose, the entire mollusk was stored entire at -80°C until protein extraction for further proteome analysis and metal determination.

Figure 4.1 geographical locations of mussels rearing sites in the map of Sardinia, Italy.

4.3.2 Experimental design

The study compared mussels reared in three different sites of Sardinia. Provided that the number of mussels sampled (a total of 360 individuals) was elevated compared to the number of 2-DE maps that were going to be made, making a subgroup of mussels that was representative to the original population was compulsory to address the issue of biological variability between the three populations. Accordingly, individuals belonging to a locality were grouped into three size classes (small, medium and big) on the basis of the analysis of mollusks weight data. Inside each size class, three individuals were randomly selected, thus having 9 individuals representative for the population of a lagoon. The same sampling scheme was repeated for the other two lagoons and the two sampling dates. The resulting experimental scheme is in accordance with a double Latin square design and reduces to 54 the number of individuals to be submitted to chemical and proteomic analysis (see Table 4.1).

4.1 Experimental design adopted for 2-DE. Legend. Locality: PP: Porto Pozzo; T: Tortolì; C: Calich. Size Class: S: Small; M: Medium; B: Big. Sampling Time: A: August; S: September; 1, 2, 3: different individuals.

TSA 1	**TSS 1**	**CSA 1**	**CSS 1**	**PPSA 1**	**PPSS 1**
TSA 2	**TSS 2**	**CSA 2**	**CSS 2**	**PPSA 2**	**PPSS 2**
TSA 3	**TSS 3**	**CSA 3**	**CSS 3**	**PPSA 3**	**PPSS 3**
TMA 1	**TMS 1**	**CMA 1**	**CMS 1**	**PPMA 1**	**PPMS 1**
TMA 2	**TMS 2**	**CMA 2**	**CMS 2**	**PPMA 2**	**PPMS 2**
TMA 3	**TMS 3**	**CMA 3**	**CMS 3**	**PPMA 3**	**PPMS 3**
TBA 1	**TBS 1**	**CBA 1**	**CBS 1**	**PPBA 1**	**PPBS 1**
TBA 2	**TBS 2**	**CBA 2**	**CBS 2**	**PPBA 2**	**PPBS 2**
TBA 3	**TBS 3**	**CBA 3**	**CBS 3**	**PPBA 3**	**PPBS 3**

4.3.3 Protein extraction

A portion of about 40 µg of foot muscle tissue was collected using a clean scalpel from 54 selected individuals. The tissue was minced with a clean scalpel, put in a in 2 ml Eppendorf safe-lock tubes (Eppendorf, Hamburg, Germany) with 1 mL of lysis buffer with composition 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT and protease inhibitors mix (Complete Mini, Roche Applied Science, Mannheim, Germany) in 0,1 x concentration (one tablet for 10 mL buffer lysis). The homogenated mixture was incubated in ice for 30 additional minutes, then subjected to three cycles of Ultraturrax Homogeneizer (IKA-Werke, Staufen, Germany) at 13.500 rpm for 30 seconds followed by 1 minute of ice incubation each. All extracts were clarified by centrifugation for 20 min at 14.000 rpm at 4 °C (Eppendorf, Hamburg, Germany), then divided in 100 µL aliquots that were stored at −80 °C until needed.

The quantification results and the quality of the protein extracts was performed by the modified Bradford method (Ramagli and Rodriguez, 1985), measuring the samples extracts in duplicates and using bovine serum albumin (BSA) as protein standard. The

results of the quantification were checked by running 25 µg of each protein extract on a denaturing sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) gel.

4.3.4 Determination of environmental parameters and mussel metal concentrations

During the whole period of mussel growing, several environmental parameters were recorded on-site for each farming site twice a month, with the only exception of September when only one monitoring session was performed for unscheduled causes. The values of temperature and pH, salinity and oxygen dissolved in water were monitored using a field – analysis water analyser, the multiparameter Ocean Seven 316 plus CTD probe (Idronaut, Milan, Italy).

After the sampling for the proteomics analysis, the mussels coming from each lagoon, deprived of the foot muscle, were pooled according to size, washed to take away sand an impurities that may interfere with the analysis, and minced with a clean scalpel to obtain a homogeneized mixture. About 1 g ± 0,01 g were weighted, mixed with 5 ml of nitric acid and 5 ml of bidistilled (MilliQ) water in teflon vessels and microwaves digested in a conventional oven model Mars 5 (CEM Corporation, Matthews, USA) according to the conditions of temperature, pression and time described in Table 4.2. The digested solutions were cooled to room temperature, then transferred in 50 ml tubes and made up to 50 g ± 0,1 g with bidistilled water. An aliquot of the resulting solution was diluted to a 1:5 ratio and analyzed for zinc, copper, manganese, arsenic, cadmium, mercury and lead by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) according to the international regulation (EPA Method 6020a, Regulation (EC) No. 1881/2006). The ICP-mass spectrometer was an Agilent 7500ce with Octopole Reaction System (Agilent Technologies, Palo Alto, USA), equipped with a ASX-520 autosampler (Cetac Technologies,

Omaha, USA). The instrument has a mass range 5-240 m/z (AMU), a 1 m/z (AMU) resolution and a 5% half-height-peak width. Before samples analyzing, the instrument had been tuned for mass calibration, sensitivity and presence of interfering substances using a solution consisting of 1 mg/l each of Li, Y, Ce, Tl and Co in 2% HNO3 and 0,2% HCl. The accuracy and precision of the method were controlled by comparison with a certified standard: mussel tissue reference material CRM 278 R from the Community Bureau of Reference (CBR) (Commission of the European Communities, Brussels, Belgium).

Table 4.2 Experimental conditions of Microwave-assisted digestion for heavymetals ICP-MS determination.

Stage	**1**	**2**	**3**	**4**	**5**
Run Time (min)	10	10	10	10	6
Power (%)	20	30	40	60	60
Pressure (psi)	40	40	85	130	130
Temperature (°C)	100	100	100	150	150
Fan Speed (%)	100	100	100	100	100

4.3.5 Protein mono-dimensional gel electrophoresis

Denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12,5% denaturing polyacrylamide gels using the Laemmli tris-glycinate electrode buffer system (Laemmli, 1970) on a Protean Tetra Cell (Bio-Rad, Hercules, USA) following the manufacturer instructions. Briefly, protein extracts were mixed to the desired concentration with Laemmli loading sample buffer of composition 30% glycerol, 2% SDS, 62,5 mM Tris-HCl pH 6.8, 100 mM DTT, 1% BBF, boiled during 5 minutes, cooled

to room temperature and loaded onto the gel. The stacking gel was composed of 4% T acrylamide-bisacrylamide (29:1), 12,5 mM Tris HCl (pH 6.8), and 0,1% SDS, while the resolving gel was composed of 12,5% T acrylamide-bisacrylamide (29:1), 37,5 mM Tris HCl (pH 8.8), and 0,1% SDS. Running buffer was made of 0,025 M Tris, 0,192 M glycine, and 0,1% SDS, final pH 8.3. Gels were run at constant voltage at 90 V for 15 minutes, followed by 150 V until BBF reached gel bottom (about 60-70 minutes). Gels were stained with SimplyBlue Safe stain Coomassie staining (Invitrogen, Carlsbad, USA).

4.3.6 Protein two-dimensional gel electrophoresis

For 2D-PAGE, protein extract were diluted in rehydration buffer with composition 7 M urea, 2 M thiourea, 2% CHAPS, 1% DTT, 0,5% IPG Buffer pH 3-11 NL, 0,002% BBF. Resuspended proteins, approximately 200 µg, were absorbed overnight into 7 cm Immobiline Drystrip pH 3–11 NL (GE Healthcare, Buckinghamshire, UK) and focused at 50 µA per strip according to the manufacturer's protocol, for a total of 6.500 Vh (Table 4.3) in an IPGphor isoelectrofocusing unit (GE Healthcare). After focusing, strips were equilibrated in 7 M urea, 75 mM Tris HCl, pH 8.8, 2% SDS, 30% glycerol, supplemented with 1% DTT for 15 min, and then with 2,5% iodoacetamide for 15 min. The second dimension SDS PAGE was performed on 12,5% denaturing polyacrylamide gels (12,5% acrylamide-bisacrylamide (29:1), 37,5 mM Tris HCl (pH 8.8), and 0,1% SDS) using the Laemmli tris-glycinate (0,025 M Tris, 0,192 M glycine, and 0,1% SDS, final pH 8.3) electrode buffer system (Laemmli, 1970) on a Protean Tetra Cell (Bio-Rad) (Figure X) following the manufacturer instructions (90V for 15 minutes, then 150V until the BBF reached the gel bottom, approximatively 60-70 minutes). Gels were stained with EZBlue Coomassie staining (Sigma Aldrich, St Louis, USA).

Table 4.3 Experimental conditions of isoelectrofocusing for 2D-PAGE.

Voltage Mode	Voltage (V)	Time (h:min)	kVh
1 Step and Hold	50	0:30	0,015
2 Step and Hold	300	0:30	0,09
3 Gradient	1000	0:30	0,3
4 Gradient	5000	1:20	4,0
5 Step and Hold	5000	0:25	2,0
6 Step and Hold	50	Until stop	NA
TOTAL		3:15	6,515

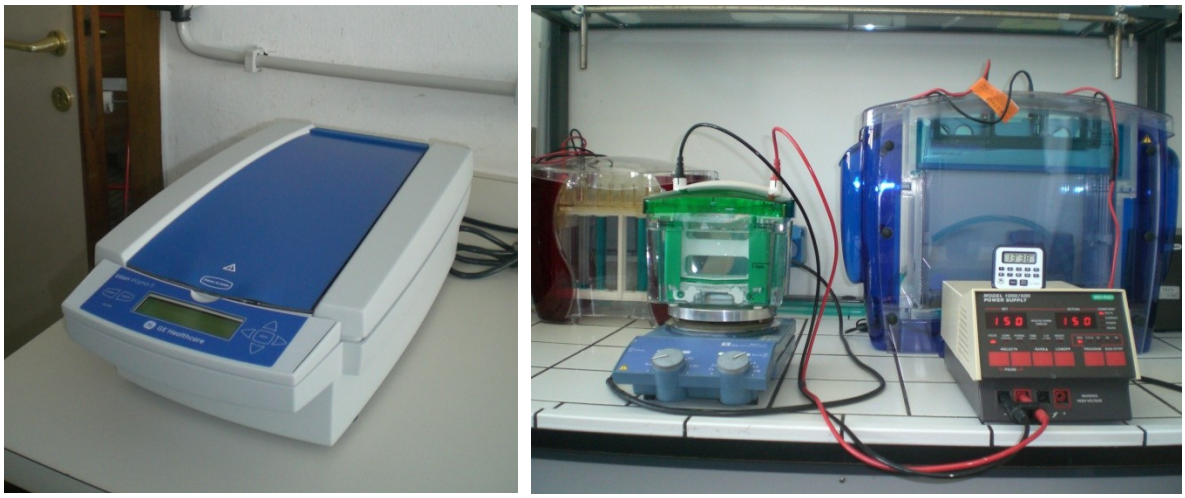


Figure 4.2 Instruments used for 2-DE. Isoelectrofocusing system unit (left), vertical SDS-PAGE system unit (right).

4.3.7 Assessment of 2-DE technical and biological variability

To estimate the technical variability of two-dimensional electrophoresis, a Reproducibility and a Repeatability Experiment were carried out. On one day, two individuals of M. galloprovincialis were analysed by 2-DE. The same protein extracts were then analyzed by 2-DE on a second day and on a third day (Reproducibility Experiment). The two samples were analyzed by 2-DE in duplicate (Repeatability Experiment).

Moreover, in order to evenly split the part of the technical error related to gel running conditions over the whole number of samples that were going to be submitted to 2-dimensional electrophoresis, the samples were distributed across the various 2-DE experimental sessions according to a Balanced Latin Square design (Table 4.4).

Table 4.4 Scheme of sample distribution across the laboratory sessions for the 2-DE. Different colors correspond to different 2-DE sessions (Yellow: 1st 2-DE session; blue: 2nd 2-DE session; green: 3rd 2-DE session; violet: 4th 2-DE session; red: 5th 2-DE session).

TSA 1	TSS 1	CSA 1	CSS 1	PPSA 1	PPSS 1
TSA 2	TSS 2	CSA 2	CSS 2	PPSA 2	PPSS 2
TSA 3	TSS 3	CSA 3	CSS 3	PPSA 3	PPSS 3
TMA 1	TMS 1	CMA 1	CMS 1	PPMA 1	PPMS 1
TMA 2	TMS 2	CMA 2	CMS 2	PPMA 2	PPMS 2
TMA 3	TMS 3	CMA 3	CMS 3	PPMA 3	PPMS 3
TBA 1	TBS 1	CBA 1	CBS 1	PPBA 1	PPBS 1
TBA 2	TBS 2	CBA 2	CBS 2	PPBA 2	PPBS 2
TBA 3	TBS 3	CBA 3	CBS 3	PPBA 3	PPBS 3

4.3.8 Image analysis

All gels were digitalized after run using a GS-800 imaging densitometer (Bio-Rad), with a resolution of 63,5 x 63,5 µm resolution (Figure 4.3). Mono-dimensional SDS-PAGE gels were then analyzed with the Quantity One software, version 4.6.9 (Bio-Rad).

Figure 4.3 The densitometer Bio-Rad GS-800 employed for gel image acquisition.

Protein patterns in digitalized 2D-PAGE gel images were analyzed using the PDQuest Advanced software, version 8.0.1 (Bio-Rad). The digitalized gel images were subjected to the software analysis workflow, consisting of spot detection, spot matching and spot analysis. The spot detection and matching steps performed by the software were manually checked in order to eliminate errors such as artifactual spots, splitted spots and missed spots. All the experiments of gel image analysis were visually double – checked for better efficiency of control. As for spot quantification, the raw values of protein spot volumes were normalized by dividing each volume value by the total for that gel and the resulting values were logarithmically transformed for better analysis robustness. All the spot analysis steps consisting in spot volume quantification, volume ratio normalization and logarithm transformation were performed with the same PDQuest software. In order to identify the possible up or down-regulated candidate proteins, a 2-fold threshold volume value and a statistical significance of a two-tailed Student's t-test with a 95% confidence level ($P < 0,05$) were considered as analysis constraints to compare protein

expression levels. The significant results, indicated as interesting spots, were selected and submitted to statistical analysis.

4.3.9 Statistical Data Analysis

Mussels weight data were submitted to Exploratory Data Analysis (EDA) to assess the normality of their distribution and identify possible outliers in order to define size classes. Statistical analysis was performed to study and interpret the expression data of the identified protein spots. Comparisons between groups were made using Student's t-test, one-way and multifactorial ANOVA. Then, the relations among the expression levels of the proteins of interest, the quantities of heavy metals in mussels and environmental parameters were predicted using an unsupervised principal component analysis (PCA). The softwares Excel 2007 (Microsoft Corp., Redmond, USA), Statistica version 6.1 (StatSoft Inc., Tulsa, USA) were used for conducting data manipulation and statistical analysis, while the software The Unscambler X (CAMO Softwares, Osòlo, Norway) was employed for Principal Component Analysis (PCA).

4.4 Results and Discussion

4.4.1 Design strategies

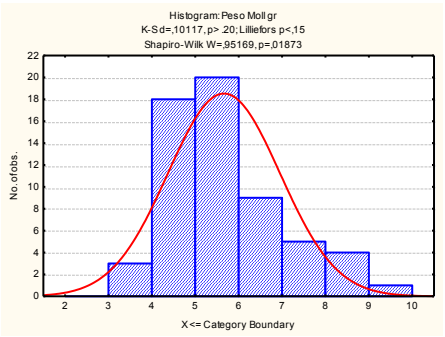
The choice of analyzing the foot muscle was addressed by two main reasons. On the one hand, even if it has been proved that PES can be used as biomarkers even without the need of protein identification (Monsinjon and Knigge, 2007), nevertheless a reliable identification of novel proteins is rather difficult in some cases, because of the lacking of protein primary structure and genome information for some organisms. The foot muscle is the tissue most studied in proteomics works on mussel (López et al., 2001; López et al., 2002a; López et al., 2002b; Mosquera et al., 2003; López, 2005), thus is the one for which

more records in proteomics and genomics databases exist. On the other hand, disposing of a tissue with good resolution patterns was necessary. The mantle could have been chosen as an alternative, but this tissue contains most of the mussel gonads and therefore can undergo gametogenic cycles that may alter the protein expression patterns. Therefore, it would be difficult to determine whether the changes detected in the protein expression patterns were to be ascribed to environmental stimuli or to hormonal production induced by the gametogenic cycle. Other interesting tissues like the gills or the digestive gland could have been selected, but other variables must be evaluated in these cases. Recent studies have analyzed by 2-DE and MS the proteome expressed by the peroxisome-enriched fraction of the digestive gland to identify PES in response to the exposure to several marine pollutants of the coastal environment (dyallil phthalate, PBDE-47, bisphenol A) (Apraiz et al., 2006). Similarly, another proteomics study was performed on zebra mussels (*D. polymorpha*) gills to evaluated the effects on proteome of the exposure to benzo(α)pyrene (Riva et al., 2010). In both studies the experiments have been performed on mollusks after that they had been sampled from the wild and kept under controlled laboratory exposure for a period of time (1-3 weeks), during which time mussels could “clean” their digestive gland and gills from whatever might have been present. In natural contexts, both tissues can be interested by exogenous protein contaminants, particularly from microorganisms, that could alter the 2-dimensional pattern and give false results.

4.4.2 Data Analysis of Sampled Mussels for selection of the individuals for 2-DE analysis

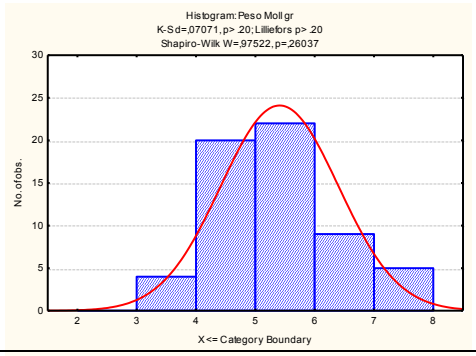
In order to choose a small group of mussels that was representative of the original population, the mussels individuals sampled for each site and sampling date were

grouped into three size classes (Small, Medium and Big) according to their value of mollusk weight. For the purpose, the mollusk weight data were statistically analyzed to assess the normality of their distribution within each sampling group (sampling site x sampling date), eventually exclude outliers and identify the boundaries of the size classes. The distribution histograms and the parameters values for descriptive statistics are reported (Figure 4.4). The mussel weight values of everyone of the six samples groups sampling site x sampling date adequately approximates the normal distribution. When displaying the data of the six populations in a box plot (Figure 3), it can be shown that the two populations of Calich and Porto Pozzo increased their mean weight from August (Porto Pozzo: 5,74 ± 1,04 g; Calich: 9,97 ± 2,64 g) to September (Porto Pozzo: 7,54 ± 1,35 g; Calich: 12,27 ± 2,94 g). On the contrary, the population of Tortolì slightly reduced its size during the same period (August: 5,66 ± 1,29 g; September: 5,41 ± 0,99 g). Analyzing the distribution of weight values, we noticed that for some localities the weight distribution of August overlapped with that of September; for instance, this was the case of the Calich lagoon population. To avoid the possibility that selecting over or under-grown individuals could affect the results, the distribution range and intervals were shrunk by excluding the individuals with the extreme weight values. The size classification with the respective class interval values and the names of the 54 selected individuals is reported in Figure 4.5.



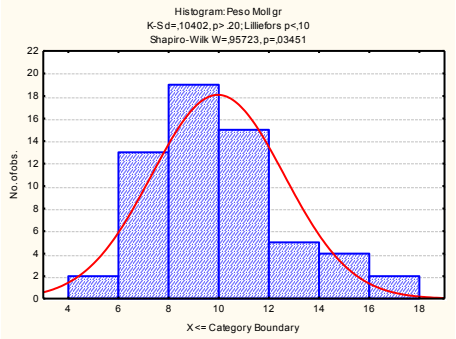
Tortolì
02.08.2010

N=60
Xm=5,66 g
Dev Std=1,29 g
Min=3,38 g
Max=9,02 g
Skewness=0,74
Curtosis=0,03



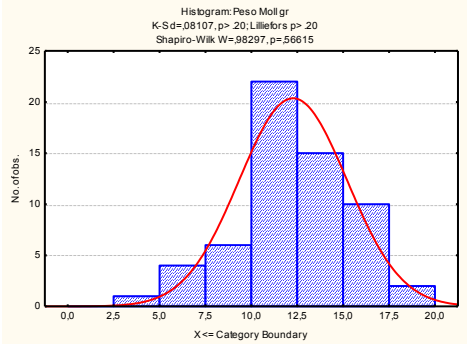
Tortolì
06.09.2010

N=60
Xm=5,41 g
Dev Std=0,99 g
Min=3,39 g
Max=7,87 g
Skewness=0,51
Curtosis=0,10



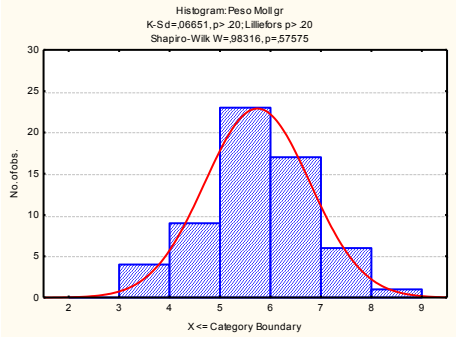
Calich
04.08.2010

N=60
Xm=9,97 g
Dev Std=2,64 g
Min=5,34 g
Max=16,40 g
Skewness=0,62
Curtosis=0,09



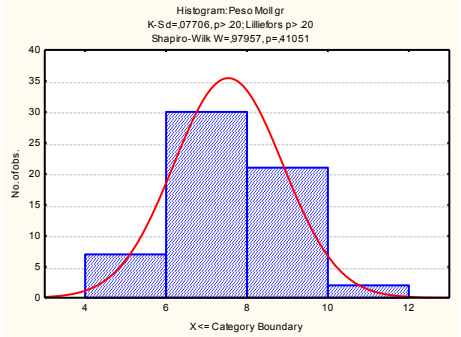
Calich
07.09.2010

N=60
Xm=12,27 g
Dev Std=2,94 g
Min=4,85 g
Max=18,42 g
Skewness=-0,29
Curtosis=-0,02



Porto Pozzo
03.08.2010

N=60
Xm=5,74 g
Dev Std=1,04 g
Min=3,40 g
Max=8,24 g
Skewness=-0,09
Curtosis=0,22



Porto Pozzo
08.09.2010

N=60
Xm=7,54 g
Dev Std=1,35 g
Min=5,16 g
Max=11,39 g
Skewness=0,39
Curtosis=-0,18

Figure 4.4 Exploratory data Analysis of mollusk weights for the sampled mussels

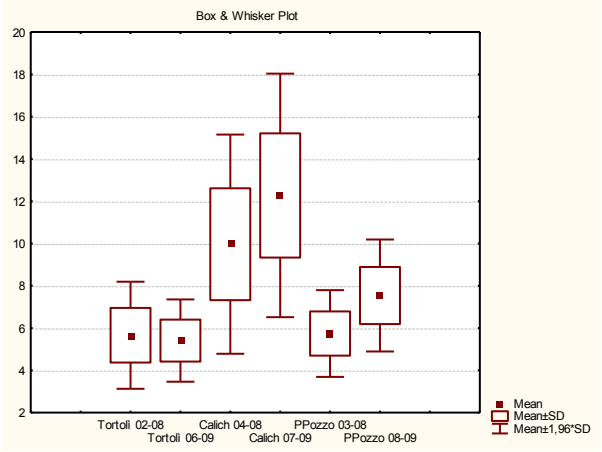


Figure 4.5 Box and Whiskers Plot of mollusk weight for all the sampling sites and sampling dates.

Table 4.5 Size classification table with the class intervals the class identification and the names of the selected individuals for each sampling place and date.

Sampling Date	**August**											
Locality	**Tortolì**				**Porto Pozzo**				**Calich**			
Size Class	**Class Weight Values**	**Class Weight Id.**	**Sample Name**	**Sample Weight (g)**	**Class Weight Values**	**Class Weight Id.**	**Sample Name**	**Sample Weight (g)**	**Class Weight Values**	**Class Weight Id.**	**Sample Name**	**Sample Weight (g)**
Small	Min=3,86 g Max=5,39 g	TSA1	602	4,12	Min=3,43 g Max=4,80 g	PPSA1	502	3,54	Min=6,37 g Max=8,30 g	CSA1	605	6,53
		TSA2	204	4,57		PPSA2	403	4,56		CSA2	604	7,25
		TSA3	306	4,84		PPSA3	104	4,64		CSA3	502	7,70
Medium	Min=5,40g Max=6,92 g	TMA1	308	5,65	Min=4,81 g Max=6,17 g	PPMA1	105	5,46	Min=8,31 g Max=10,23 g	CMA1	210	9,60
		TMA2	410	5,97		PPMA2	506	5,91		CMA2	606	9,99
		TMA3	102	6,63		PPMA3	306	6,05		CMA3	106	10,76
Big	Min=6,93 g Max=8,45 g	TBA1	104	7,37	Min=6,18 g Max=7,54 g	PPBA1	409	7,25	Min=10,24 g Max=12,16 g	CBA1	110	11,76
		TBA2	208	8,14		PPBA2	509	7,34		CBA2	203	11,91
		TBA3	408	8,44		PPBA3	106	7,53		CBA3	202	12,16
Sampling Date	**September**											
Locality	**Tortolì**				**Porto Pozzo**				**Calich**			
Size Class	**Class Weight Values**	**Class Weight Id.**	**Sample Name**	**Sample Weight (g)**	**Class Weight Values**	**Class Weight Id.**	**Sample Name**	**Sample Weight (g)**	**Class Weight Values**	**Class Weight Id.**	**Sample Name**	**Sample Weight (g)**
Small	Min=3,78 g Max=4,91 g	TSS1	408	3,84	Min=5,25 g Max=6,90 g	PPSS1	602	5,53	Min=6,56 g Max=9,91 g	CSS1	502	6,60
		TSS2	107	4,08		PPSS2	210	6,02		CSS2	206	7,65
		TSS3	607	4,46		PPSS3	607	6,40		CSS3	507	7,74
Medium	Min=4,92 g Max=6,04 g	TMS1	510	4,79	Min=6,91 g Max=8,55 g	PPMS1	205	7,34	Min=9,92 g Max=13,26 g	CMS1	605	10,42
		TMS2	503	5,19		PPMS2	503	7,80		CMS2	606	11,41
		TMS3	102	5,69		PPMS3	101	8,36		CMS3	307	12,35
Big	Min=6,05 g Max=7,16 g	TBS1	109	6,26	Min=8,56 g Max=10,2 g	PPBS1	408	9,25	Min=13,27 g Max=16,61 g	CBS1	306	13,58
		TBS2	310	6,38		PPBS2	303	9,50		CBS2	508	15,14
		TBS3	201	6,54		PPBS3	110	9,55		CBS3	406	16,21

4.4.3 Determinations of environmental parameters and metal concentrations in mussels tissues

The environmental data values of water temperature, pH, salinity and dissolved oxygen that were recorded during mussel sampling are reported in Table 4.6.

Table 4.6 Environmental monitoring data for the Sardinian lagoons that were object of study.

Locality	Calich		Tortolì		Porto Pozzo	
Sampling Date	**August**	**September**	**August**	**September**	**August**	**September**
Mean temperature (°C)	24,82	24,34	27,04	25,64	25,23	24,79
Mean Salinity (‰)	31,01	33,21	37,75	37,78	39,51	39,39
Mean Dissolved Oxygen (%)	114,7	98,58	80,51	79,83	90,91	83,92
Mean Dissolved Oxygen (mg/L)	7,94	6,80	5,17	5,24	5,96	5,54
Mean pH	8,36	8,17	8,04	8,08	8,05	8,01

There are no differences between the values of the environmental parameters recorded in August and September (p=0,528), while Calich lagoon differs from Tortolì and Porto Pozzo for the lower values of mean salinity and dissolved oxygen and the higher pH value (P=0,002); this data is in accordance with what expected on the basis of the ecological features of Calich lagoon.

The concentration values for the heavy metals assessed by ICP-MS in the selected mussels are reported in Table 4.7. All metal concentrations are below the concentration limits defined by the European regulation for the levels of contaminants in food products (EU Regulation n. 1881/2006 of 19th of December 2006). However, metal concentration values differently characterize the mussels coming from the three farming sites, with

Porto Pozzo having highest concentrations of arsenic and mercury (P=0,00) and substantially relevant concentrations of cadmium, while Tortolì is characterized for the highest content of cadmium among the three lagoons (P=0,00).

Table 4.7 Metal concentration (ppb) values determined by ICP-MS with the corresponding analytical reference values.

Sample Name	Chromium	Manganese	Copper	Zinc	Arsenic	Cadmium	Mercury	Lead
C0408 Big	48,24	2844	780,4	23260	1167	14,56	4,725	60,97
C0408 Medium	76,49	4246	1186	23150	1236	12,57	4,684	91,36
C0408 Small	72,52	3886,5	841,5	31140	1253	2,789	2,598	60,59
C0709 Big	607,2	2504,5	1156	34920	1397	3,378	5,085	68,88
C0709 Medium	71,59	3667	1309	33410	1654	7,298	3,948	99,54
C0709 Small	84,24	3266,5	935,8	38360	1280	18,21	3,42	102,1
PP0308 Big	88,75	2642	1461	40450	3543	59,57	9,662	72,81
PP0308 Medium	164	5245,5	985,4	36220	3715	75,66	11,06	287,1
PP0308 Small	477,9	2968	1064	40380	2514	85,88	9,056	83,09
PP0809 Big	83,66	2231	1311	20980	7664	68,41	14,34	134,6
PP0809 Medium	110,3	2421,5	1060	30330	6039	58,6	10,55	64,57
PP0809 Small	122,9	2515,5	1040	24100	6227	43,52	11,54	64,84
T0208 Big	56,1	3147	1033	31380	2717	129,6	3,187	63,58
T0208 Medium	66,91	1913,5	720,2	33290	2179	183,5	2,94	50,37
T0208 Small	89,53	4897,5	650,1	17390	1937	102,6	2,686	72,17
T0609 Big	69	2139,5	741,6	25320	1869	119,9	2,134	63,2
T0609 Medium	49,99	2613,5	556,6	30940	2090	190,4	2,169	82,4
T0609 Small	66,07	3845	1079	29820	2059	207,5	2,803	101,6
PP0809 Medium + STD 50 ng	165,9	2332	1023	27440	5623	121,6	60,11	127,9
BRC 214 mussel tissue 12/09	643,6	10660	9627	83070	6120	350,1	164,8	1931
BRC 214 mussel tissue 12/09 Reference values	780	7690	9450	83000	6070	348	196	2000

4.4.4 Protein Extraction and Quantitative Analysis

Foot muscle protein extracts were obtained using the extraction buffer and procedure previously described. The quality and quantity of each sample extraction was assessed using the modified Bradford method previously cited and the results were checked on a

SDS-PAGE gel. The concentration of the protein extracts are reported in Table 4.8. The concentration values of the extracts range from a minimum value of 8,68 ±1,45 µg/µL to a maximum value of 17,67 ± 3,17 µg/µL and the standard deviations of the mean values for each locality and sampling date show low variability.

Table 4.8 Concentration values of the protein extracts.

Sampling Date					**August**					
Locality					**Tortolì**					
Sample Name	**602**	**204**	**306**	**308**	**410**	**102**	**104**	**208**	**408**	**Mean ± Ds**
	15,65 ± 1,98	13,84 ± 1,45	16,37 ± 1,68	14,77 ± 1,55	13,01 ± 0,27	16,62 ± 3,15	13,12 ± 2,89	17,67 ± 3,17	12,92 ± 3,17	**14,89 ± 1,02**
Locality					**Calich**					
Sample Name	**605**	**604**	**502**	**210**	**606**	**106**	**110**	**203**	**202**	**Mean ± Ds**
	10,84 ± 1,18	10,19 ± 1,76	13,64 ± 0,34	10,20 ± 2,00	8,68 ± 1,45	11,18 ± 2,18	13,11 ± 1,93	11,52 ± 1,56	12,03 ± 1,20	**11,27 ± 0,56**
Locality					**Porto Pozzo**					
Sample Name	**502**	**403**	**104**	**105**	**506**	**306**	**409**	**509**	**106**	**Mean ± Ds**
	9,03 ± 1,32	11,53 ± 1,05	10,59 ± 1,71	9,14 ± 0,58	9,74 ± 0,64	10,44 ± 0,77	9,70 ± 0,44	11,56 ± 0,78	12,96 ± 1,46	**10,52 ± 0,44**

Table 4.9 Concentration values of the protein extracts.

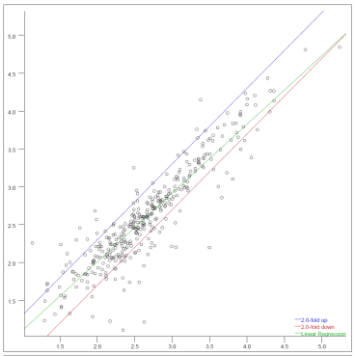
Sampling Date					**September**					
Locality					**Tortolì**					
Sample Name	**408**	**107**	**607**	**510**	**503**	**102**	**109**	**310**	**201**	**Mean ± Ds**
	15,41 ± 1,76	13,96 ± 1,29	13,56 ± 1,21	13,93 ± 0,42	12,60 ± 1,29	17,40 ± 3,98	14,69 ± 0,24	17,10 ± 3,06	15,89 ± 3,02	**14,95 ± 1,28**
Locality					**Calich**					
Sample Name	**502**	**206**	**507**	**605**	**606**	**307**	**306**	**508**	**406**	**Mean ± Ds**
	13,00 ± 1,31	10,82 ± 1,77	12,51 ± 1,61	12,40 ± 1,67	16,31 ± 2,04	13,64 ± 4,94	13,34 ± 1,87	12,89 ± 2,25	13,74 ± 1,98	**13,18 ± 1,08**
Locality					**Porto Pozzo**					
Sample Name	**602**	**210**	**607**	**205**	**503**	**101**	**408**	**303**	**110**	**Mean ± Ds**
	12,25 ± 0,94	14,24 ± 2,05	12,05 ± 1,14	11,79 ± 1,40	13,03 ± 2,11	13,03 ± 1,99	11,89 ± 1,62	13,73 ± 1,52	12,60 ± 1,18	**12,73 ± 0,43**

4.4.5 Assessment of technical and biological variability

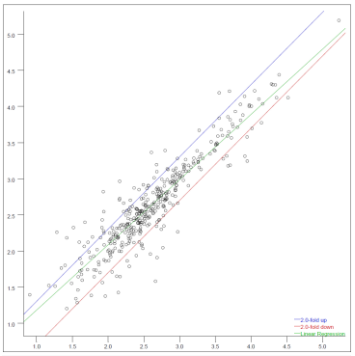
Replication is central to experimental design and allows more robust data analysis. Experimental replicates were split into two groups: technical replicates and biological replicates. Technical replicates, also called repeated measures, address the technical error, or noise, in the experiment. In electrophoresis 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 duplicate 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 and the reproducibility of the experimental technique (Figure 4 and 5). In these graphs, each protein spot has been plotted according to its intensity in the first gel (x-axis) versus its intensity in the second gel (y-axis). Protein intensity values were reported as normalized values. For what concerns the Repeatability Experiment (Figure 4.6), two samples (T0609-504 and PP0809-106) were selected and were run in duplicate during three analytical sessions. The intensity values of each protein spot resulting in the first and the duplicate gel were plotted in the x-axis and in the y-axis, respectively. The cluster of points along the diagonal in all the scatter plots clearly indicates a good degree of repeatability. The values of the correlation coefficient r2 shows a good accordance between replicates; its mean value (r2 = 0,897) between the two replicates A and B of the same sample (T0609-504 and PP0809-106) indicates that the technical variability contributes for the 10,3% of the total variability. For what concerns the Reproducibility Experiment (Figure 4.7), two samples (T0609-504 and PP0809-106) were selected and were run in duplicate during three analytical sessions on

three different days. The results obtained indicate a mean value of r2= 0,922, corresponding to 7,8% of total variability for the Reproducibility Experiment.

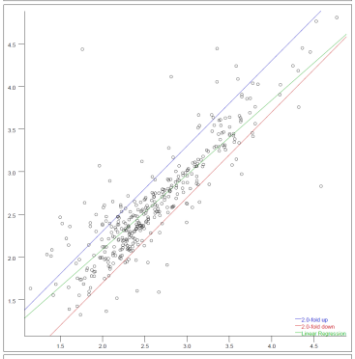
The 54 mussels individuals selected for 2-DE analysis and metals determination were considered as biological replicates in the classical statistical sense, that is different samples measured across different conditions. For each size class, locality and sampling date, three individuals were randomly selected to obtain a sub-population of mussels that was representative of the originary population. This strategy was adopted on the basis of the theoretical assumption that the random sampling of biological replicates across a population is normally distributed and thus gives an adherent representation of that population. Accordingly, if different populations are subjected to different treatments or conditions, the random sampling permits to make inferences for each population about the effects of the treatments or altering conditions in relation to the biological noise of the system. Lastly, since previously proteomics studies conducted on mussels of the genus Mytilus to differentiate among the species have evidenced the occurrence of genetic variability among these mussels species (López, 2005), the strategy adopted for the comparative analysis of protein expression among the groups took into consideration exclusively quantitative differences in the expressed proteins that are common to all the individuals studied, without taking into considerations proteins isoforms.



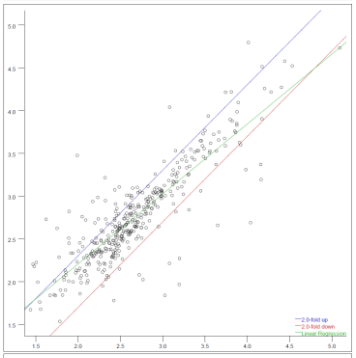
X-axis: T0609-504A-Rip 1
Y-axis: T0609-504B-Rip 1
Corr. Coeff. = 0,908909
Slope = 0,907874
Intercept = 0,193023
Spot count = 356
Norm Units = PPM



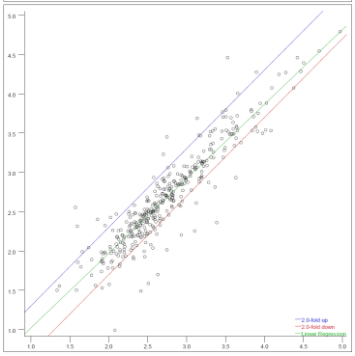
X-axis: PP0809-106A-Rip 1
Y-axis: PP0809-106B-Rip 1
Corr. Coeff. = 0,919054
Slope = 0,903126
Intercept = 0,277065
Spot count = 376
Norm Units = PPM



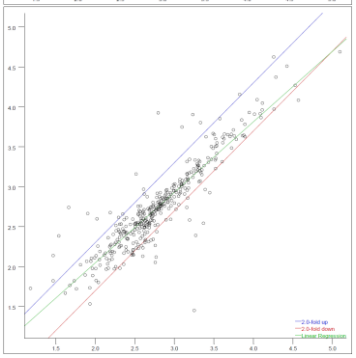
X-axis: T0609-504A-Rip 2
Y-axis: T0609-504B-Rip 2
Corr. Coeff. = 0,862546
Slope = 0,874512
Intercept = 0,341042
Spot count = 367
Norm Units = PPM



X-axis: PP0809-106A-Rip 2
Y-axis: PP0809-106B-Rip 2
Corr. Coeff. = 0,867357
Slope = 0,818083
Intercept = 0,570083
Spot count = 377
Norm Units = PPM

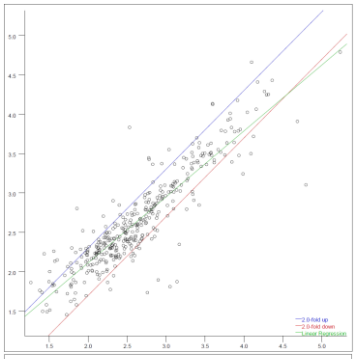


X-axis: T0609-504A-Rip 3
Y-axis: T0609-504B-Rip 3
Corr. Coeff. = 0,919452
Slope = 0,948115
Intercept = 0,073616
Spot count = 362
Norm Units = PPM

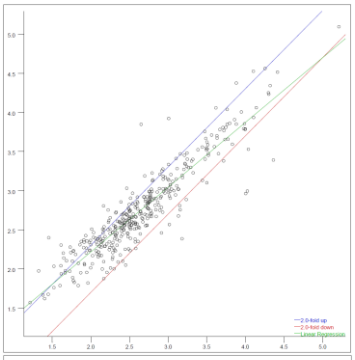


X-axis: PP0809-106A-Rip 3
Y-axis: PP0809-106B-Rip 3
Corr. Coeff. = 0,906188
Slope = 0,882934
Intercept = 0,281100
Spot count = 370
Norm Units = PPM

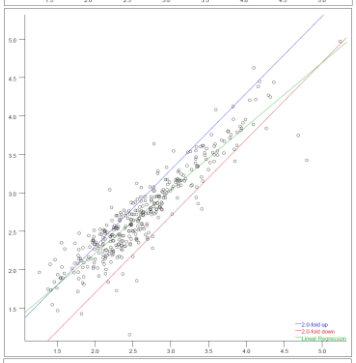
Figure 4.6 Repeatability scatter plots.



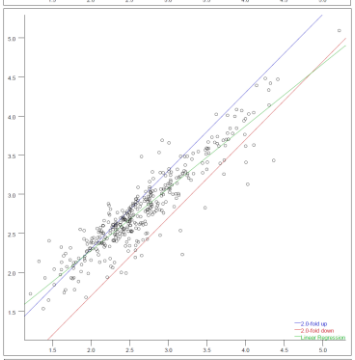
X-axis: T0609-504-Rip 1
Y-axis: T0609-504-Rip 2
Corr. Coeff. = 0,898388
Slope = 0,837131
Intercept = 0,439140
Spot count = 373
Norm Units = PPM



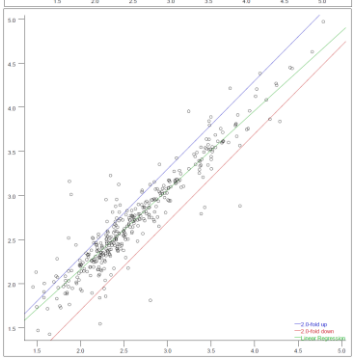
X-axis: PP0809-106-Rip 1
Y-axis: PP0809-106-Rip 2
Corr. Coeff. = 0,920074
Slope = 0,827478
Intercept = 0,560246
Spot count = 383
Norm Units = PPM



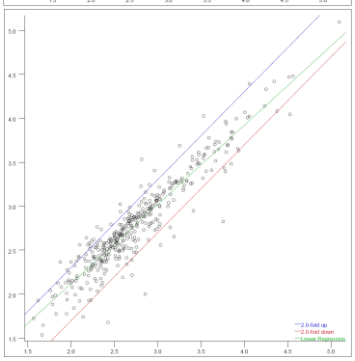
X-axis: T0609-504-Rip 1
Y-axis: T0609-504-Rip 3
Corr. Coeff. = 0,919637
Slope = 0,827176
Intercept = 0,558404
Spot count = 373
Norm Units = PPM



X-axis: PP0809-106-Rip 1
Y-axis: PP0809-106-Rip 3
Corr. Coeff. = 0,918065
Slope = 0,796430
Intercept = 0,685611
Spot count = 379
Norm Units = PPM



X-axis: T0609-504-Rip 2
Y-axis: T0609-504-Rip 3
Corr. Coeff. = 0,936730
Slope = 0,899494
Intercept = 0,362440
Spot count = 374
Norm Units = PPM



X-axis: PP0809-106-Rip 2
Y-axis: PP0809-106-Rip 3
Corr. Coeff. = 0,938878
Slope = 0,900967
Intercept = 0,316640
Spot count = 382
Norm Units = PPM

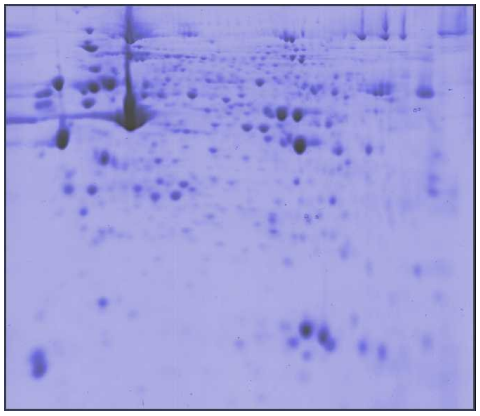
Figure 4.7 Reproducibility scatter plots.

4.4.6 Analysis 2-DE protein maps

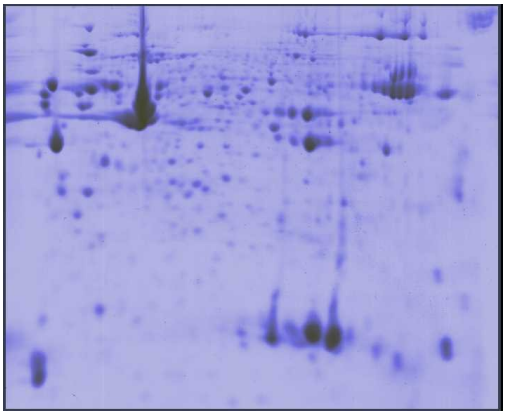
The protein extracts were analyzed by 2D-PAGE within isoelectric point pI and molecular mass intervals of 3 – 11 pH units and 10 – 250 kDa, respectively. Some 2-DE maps of foot muscle are reported in Figure 4.8, that are related to one individual for one size class, each locality and each sampling date. Spots were quite homogeneously distributed across the entire pI range, although some significant protein clusters of high molecular weight proteins appear. Some proteins have been identified by MS analysis from 2D-PAGE maps of foot muscle by some authors (López et al., 2002; Mosquera et al., 2003; Diz et al., 2009) and are available in the proteins databases of ExPASY server. A summarizing picture of the mainly expressed proteins is reported in Figure 4.9. In general, the proteins expressed in mussel foot muscle that have been studied and identified can be classified into the following functional groups: Cell metabolism and energy, Cell signaling, Oxidative stress and Protein structure, as illustrated in the Table 4.10. Skeletal proteins like actin, tropomyosin, myosins and tubulins are the most relevantly expressed proteins as well as proteins involved in energy and metabolic processes, like creatine kinase, ATP synthase, malate dehydrogenase and NADH-ubiquinone oxidoreductase; but also oxidative stress enzymes can be detected in 2-DE maps, like superoxide dismutase and protein disulfide isomerase. It is interesting to note that some chaperone proteins like hsp 25, hsp 70 and hsp 90 are detected on 2-DE map of foot muscle tissue.

The conditions of gel electrophoresis and analysis allowed to discriminate a mean of 749,5 ± 73 protein spots on each gel, among which a mean of 16% has been matched. Among the analyzed gels, a total of 77 spots has been detected as differentially expressed as reported in Figure 4.10; the relative numbers are reported in the table immediately

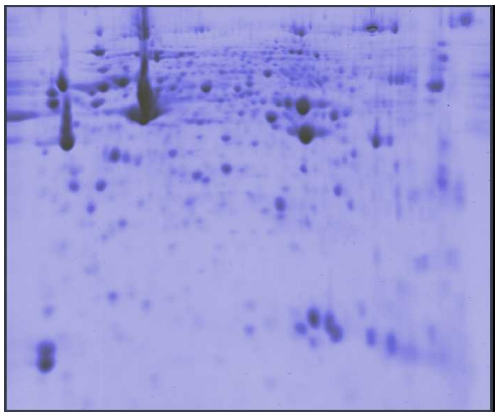
below. The 77 selected spots have been considered as spots of interest for statistical data analysis.



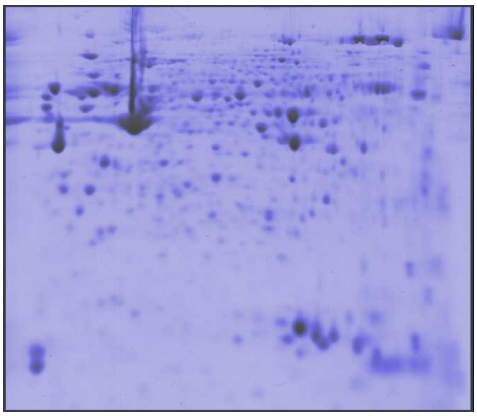
T0208 – 602 (TSA1)



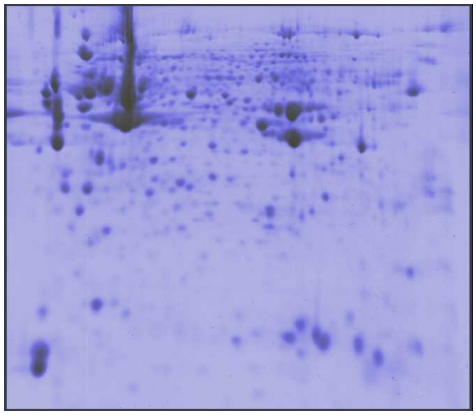
T0609 – 408 (TSS1)



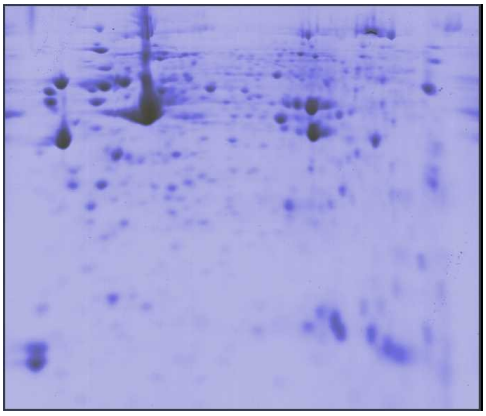
C0408 – 202 (CBA3)



C0709 – 406 (CBS3)



PP0308 – 506 (PPMA2)



PP0809 – 503 (PPMS3)

Figure 4.8 2-DE gel image of mussel foot muscle. For each locality, the 2-DE foot muscle map are shown for two individuals at the two different sampling dates.

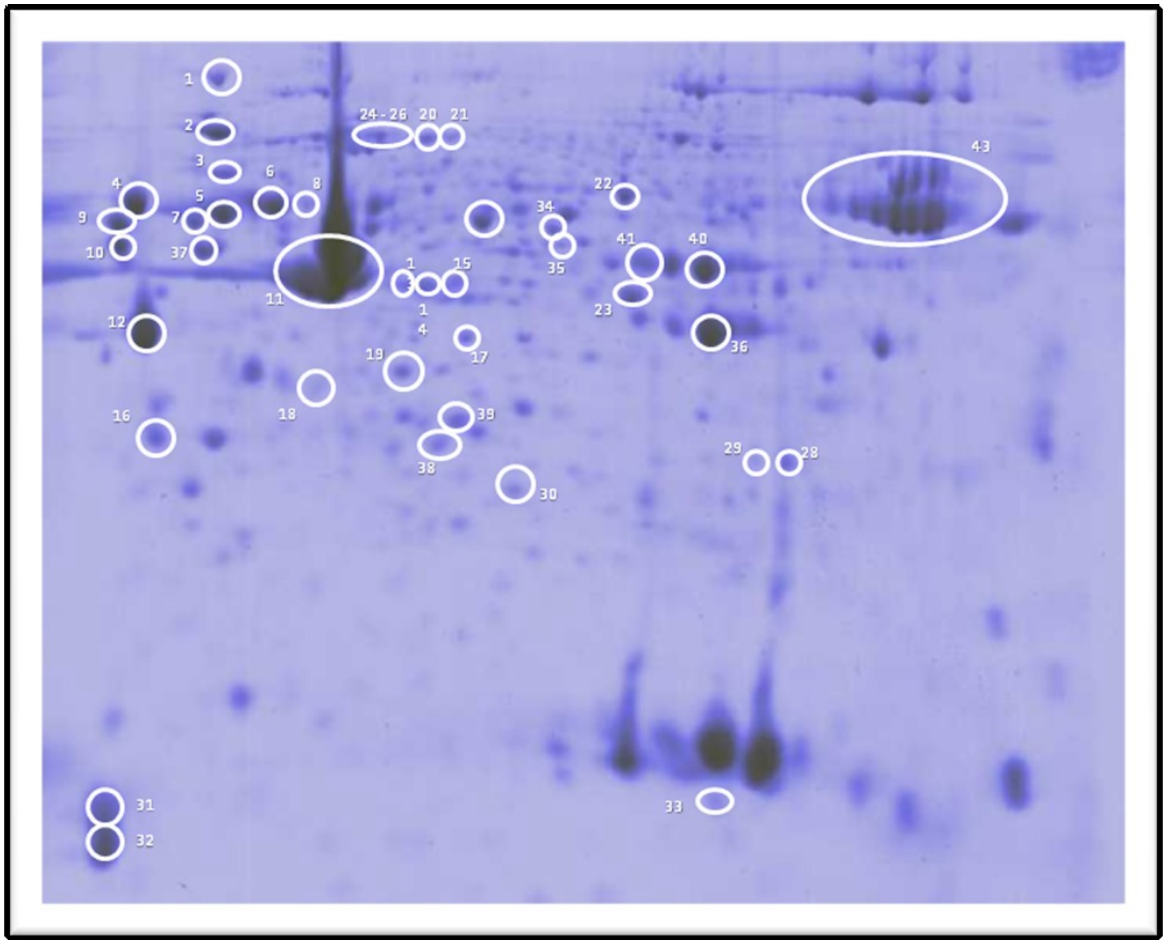
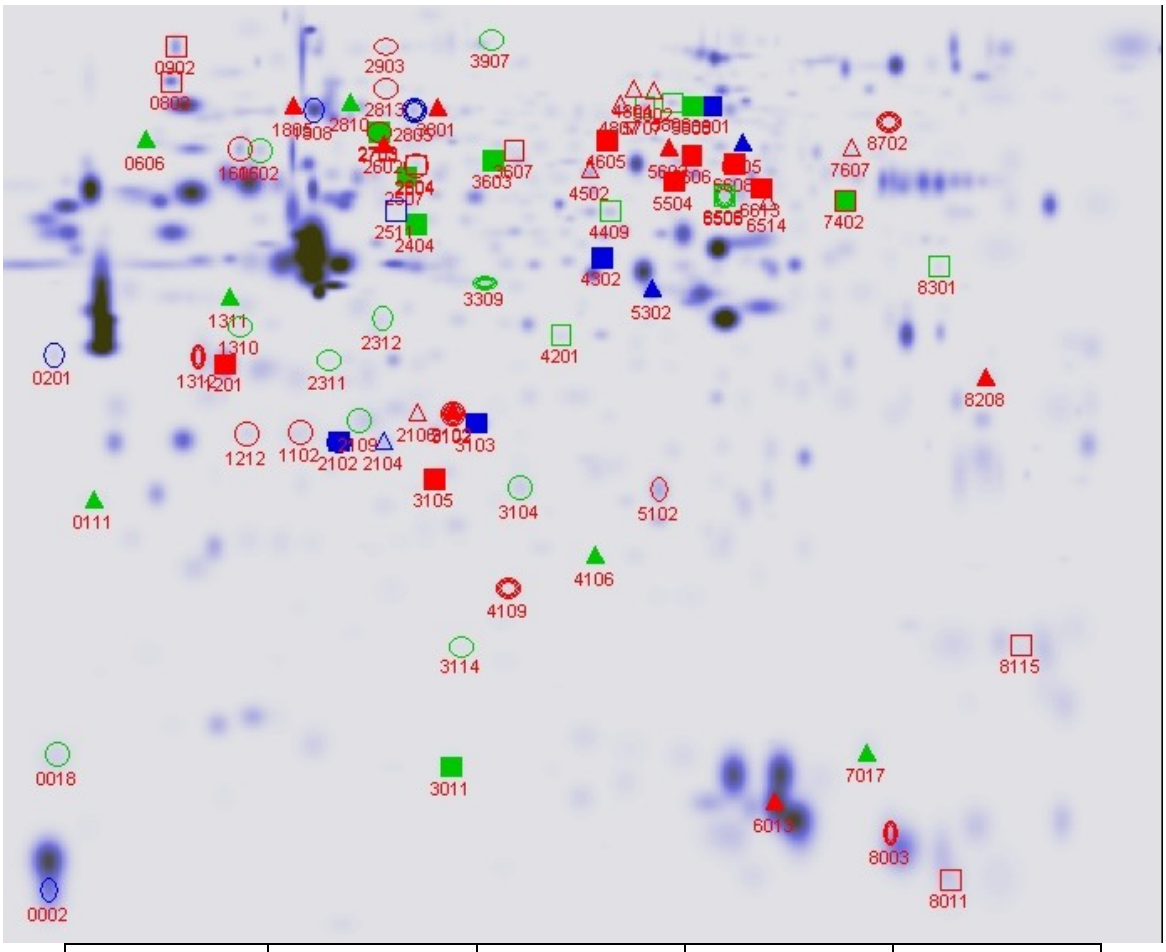


Figure 4.9 2-DE map of the foot muscle in M. galloprovincialis with the identification of some of the mainly expressed proteins.

Table 4.10 List of some of the mainly expressed proteins identified in foot muscle tissue of Mytilus galloprovincialis.

Spot Number	Protein Name	Functional Category	SWISSPROT accession number	Theoretical pI using ExPASY tools	Theoretical Mr (KDa) using ExPASY tools	Reference
1	Heat shock protein 90	Molecular chaperones	C0Z203	4.84	83.12	Tomanek & Zuzov, 2010
2	Kelch-related protein 1		O60662	4.96	82.22	Gelfi et al., 2003
3	Heat shock protein cognate 70	Molecular chaperones	Q3LF64	5.35	35.00	Tomanek & Zuzov, 2010
4	ATP synthase β-chain	Energy metabolism	P06576	5.26	56.56	Manduzio et al., 2005
5	β Tubulin	Cytoskeletal	P05217	4.79	49.83	Tomanek & Zuzov, 2010
6	α Tubulin	Cytoskeletal				Tomanek & Zuzov, 2010
7	Calreticulin			4.80	52.60	Lopez et al., 2002
8	Heat shock protein 70	Molecular chaperones	Q3LF66	5.34	69.48	Lopez et al., 2002
9	Transporter ATP-binding protein-ho			4.50	52.10	Lopez et al., 2002
10	DNA-directed RNA polymerase			4.30	49.10	Lopez et al., 2002
11	Actins	Cytoskeletal		5.60	41.60	Lopez et al., 2002
12	Tropomyosin α-chain, skeletal muscle type	Cytoskeletal	P91958	4.62	32.77	Lopez et al., 2002
13	Actin	Cytoskeletal	Q9Y0D6	5.46	41.75	Mosquera et al., 2003
14	Actin	Cytoskeletal	Q9Y0D6	5.46	41.75	Mosquera et al., 2003
15	Actin	Cytoskeletal	Q9Y0D6	5.46	41.75	Mosquera et al., 2003
16	Cyclin D1			5.48	30.70	Manduzio et al., 2005
17	G-protein β subunit	Signaling				Tomanek & Zuzov, 2010
18	Pyrophosphatase	Energy metabolism				Tomanek & Zuzov, 2010
19	G-protein β subunit	Signaling				Tomanek & Zuzov, 2010
20	Heat shock protein 70	Molecular chaperones	Q3LF66	5.34	69.48	Tomanek & Zuzov, 2010
21	Heat shock protein 70	Molecular chaperones	Q3LF66	5.34	69.48	Manduzio et al., 2005; Tomanek & Zuzov, 2010
22	Glutamyl tRNA synthetase			5.79	50.70	Manduzio et al., 2005
23	cytosolic Malate dehydrogenase	Energy metabolism	Q3S892	6.02	36.42	Tomanek & Zuzov, 2010
24	Major vault protein	Unspecified	A0SXG1	5.69	31.71	Tomanek & Zuzov, 2010
25	Major vault protein	Unspecified	A0SXG1	5.69	31.71	Tomanek & Zuzov, 2010
26	Major vault protein	Unspecified	A0SXG1	5.69	31.71	Tomanek & Zuzov, 2010
28	Cu - Zn superoxide dismutase	Oxidative stress				Tomanek & Zuzov, 2010
29	Cu - Zn superoxide dismutase	Oxidative stress				Tomanek & Zuzov, 2010
30	ATP synthase α-chain	Energy metabolism	C3VQ17	6.68	25.79	Lopez et al., 2002
31	Myosin regulatory light chain 2	Cytoskeletal		4.20	16.00	Gelfi et al., 2003
32	Myosin light chain 2	Cytoskeletal		4.20	16.00	Lopez et al., 2002
33	NADH ubiquinone oxidoreductase chain 6	Energy metabolism	A9X4T0	4.86	16.91	Lopez et al., 2002
34	Protein disulfide isomerase	Oxidative stress		6.40	49.10	Lopez et al., 2002
35	Matrilin			6.70	42.40	Lopez et al., 2002
36	Actin	Cytoskeletal	Q9Y0D6	5.46	41.75	Tomanek & Zuzov, 2010
37	β Tubulin	Cytoskeletal	P05217	4.79	49.83	Lopez et al., 2002
38	Heat shock protein 27	Molecular chaperones	P04792	5.98	22.78	Tomanek & Zuzov, 2010
39	α -crystalline-Heat stock protein 23	Molecular chaperones				Tomanek & Zuzov, 2010
40	Creatine Kinase	Energy metabolism	P06732	6.77	43.10	Gelfi et al., 2003
41	Creatine Kinase	Energy metabolism	P06732	6.77	43.10	Gelfi et al., 2003
42	Arginine kinase	Energy metabolism				Tomanek & Zuzov, 2010
43	Byssal thread matrix proteins					Sagert & Waite, 2009



SSP0002	SSP2106	SSP3103	SSP3201	SSP8301
SSP0018	SSP2109	SSP3104	SSP4605	SSP6608
SSP0111	SSP2311	SSP3105	SSP4804	SSP6613
SSP0201	SSP2312	SSP3114	SSP4807	SSP6801
SSP0606	SSP2404	SSP3309	SSP5102	SSP7017
SSP0902	SSP2507	SSP3603	SSP5302	SSP7402
SSP1102	SSP2511	SSP3607	SSP5504	SSP7607
SSP1201	SSP2602	SSP3801	SSP5603	SSP8003
SSP1212	SSP2703	SSP3907	SSP5606	SSP8011
SSP1310	SSP2805	SSP4106	SSP5707	SSP0803
SSP1602	SSP2810	SSP4109	SSP5802	SSP8115
SSP1605	SSP2813	SSP4201	SSP5806	SSP8208
SSP1805	SSP2903	SSP4302	SSP5808	SSP8301
SSP1808	SSP3011	SSP4409	SSP6013	SSP8702
SSP2102	SSP3102	SSP4502	SSP6506	SSP6605
SSP2104	SSP1311	SSP2604	SSP6514	SSP6608

Figure 4.10 Map and list of the identified proteins of interest.

4.4.7 Statistical Data Analysis of the Identified Protein Expression Patterns and Environmental Principal Component Analysis between 2-DE data and environmental data

4.4.7.1 Multifactorial Analysis of Variance

The data about the identified proteins were analyzed by a multifactorial analysis of variance (ANOVA) in the attempt to identify interaction effects between multiple independent variables. Protein data were screened according to three factors: size class, locality and sampling time.

Analyzing the pool of data on the whole, no statistical differences of protein expressions were found among individuals of different size class, neither locality nor sampling date. However, analyzing each single spot, some general trends could be identified in the expression levels of some specific proteins. A clear increase in protein expression levels was registered within the individual mussels from Porto Pozzo sampled both in August and September with respect to the individual samples from the other two localities (Tortolì and Calich).Particularly, for the spots named as SSP1605, SSP2601, SSP2404 only an increasing trend of expression can be observed. The increase in the expression levels was statistically relevant for the spots number SSP2, SSP2703 and SSP8208. Similarly, also mussels from Calich lagoon showed an increasing trend in the levels of protein expression with respect to Tortolì and Porto Pozzo lagoons, particularly for the proteins number SSP2813, SSP4109, SSP4201, SSP3104, SSP2507, SSP4302 and SSP6609. As for mussels farmed in Tortolì, they showed very low variations in protein expression levels, generally without marked trends. It is noteworthy the trend showed by the protein number SSP18, with a statistically significant high expression level in the

mussels of Tortolì sampled during September with respect to the individual of Tortolì and Calich of August, and Calich and Porto Pozzo of September.

4.4.7.2 Principal Component Analysis

Considering that the studied system is characterized by an elevated level of biological complexity, a multivariate analysis criteria has been chosen to study the relations occurring among the expression pattern of the proteins of interest, the recorded environmental data values and the concentrations of heavy metals assessed in the studied individual mussels, in the attempt of somehow simplifying the biological system complexity and to identify possible correlations and trends. The Principal Component Analysis (PCA) is a useful method of categorization, since it separates the dominating features in a data set and tries to reduce complexity by replacing these features with a limited number of components. PCA has been quite recently introduced for the statistical treatment of proteomics data and already used in a few proteomics works (Apraiz et al., 2006; Gonzalez et al., 2010), proving to be a reliable tool for proteomics data analysis. However, an attempt of data analysis with PCA showed that a statistical explanatory model could be difficultly identified at first, as it is reported by the score plot below (Figure 4.11).

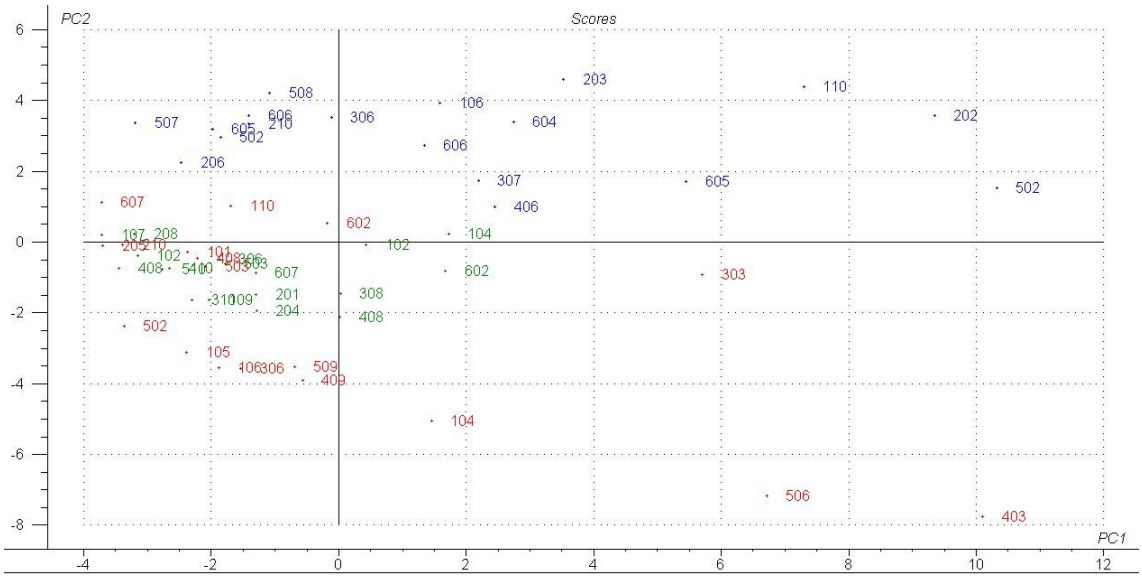


Figure 4.11 Organization of Proteomics data according to Principal Component Analysis. Blue: Calich; Red: Porto Pozzo; Green: Tortolì.

The phenomenon was determined by the fact that a number of individual samples strongly influenced the statistical model by deforming it in such a way that it was not possible to make any predictions from the model resulting from the PCA analysis. These outlier samples could be easily identified as they are placed on the right-up and right-down side of the score plot and precisely correspond to the samples reported in Table 4.11.

Table 4.11 Identifyers of the samples considered as outliers by the PCA analysis.

Tortolì		**Calich**		**Porto Pozzo**	
T0208	602	C0408	502	PP0308	104
T0609	102	C0408	110	PP0308	403
T0609	201	C0408	202	PP0308	506
T0609	310	C0408	605	PP0809	303
T0609	503				

By removing these outliers, the PCA showed a clearer separation along the first two components of the loadings plots (Figure 4.12). Thus, in a plot based on only two principal components, some clear clustering of samples could be observed. Samples separated

according to locality in the first principal component, in particular the clustering according the three farming localities can be observed while along the second principal component samples were separated on the basis of the sampling date; this separation can be appreciated only for the two localities Porto Pozzo and Calich. The same clustering trend of the samples according to locality and sampling date is confirmed also when analyzing the effects of environmental parameters and metal concentrations together with the studied protein expression signatures (PES) (Figure 4.13). No clustering patterns have been found regarding the mussels size classes.

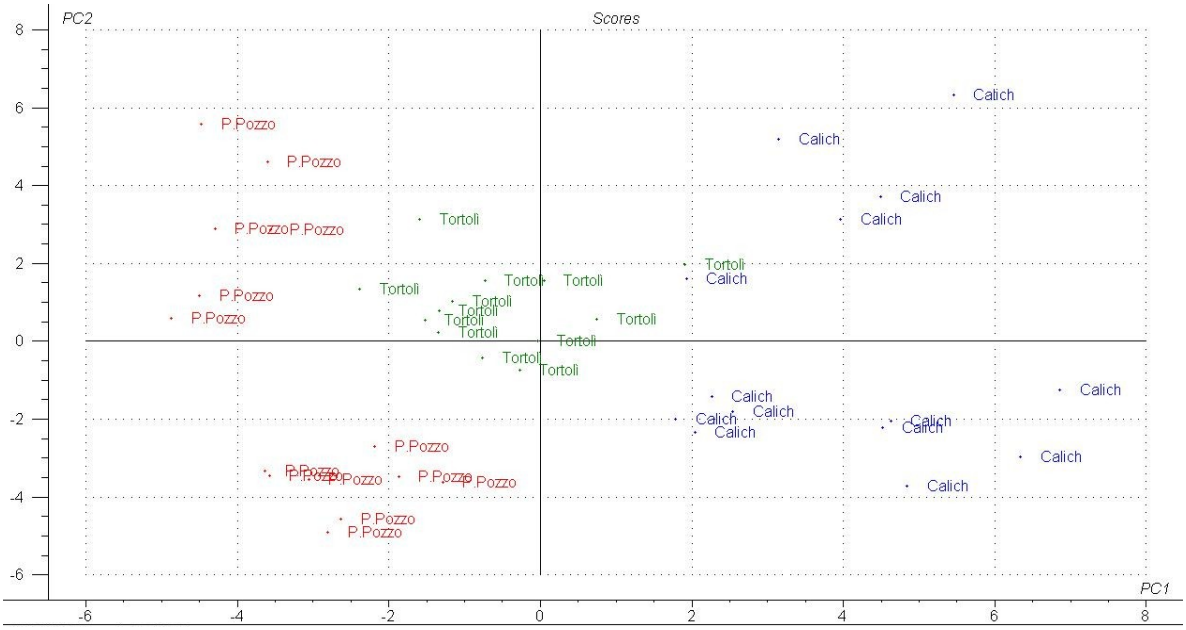


Figure 4.12 Organization of Proteomics data by Principal Component Analysis of PES alone without outliers samples.

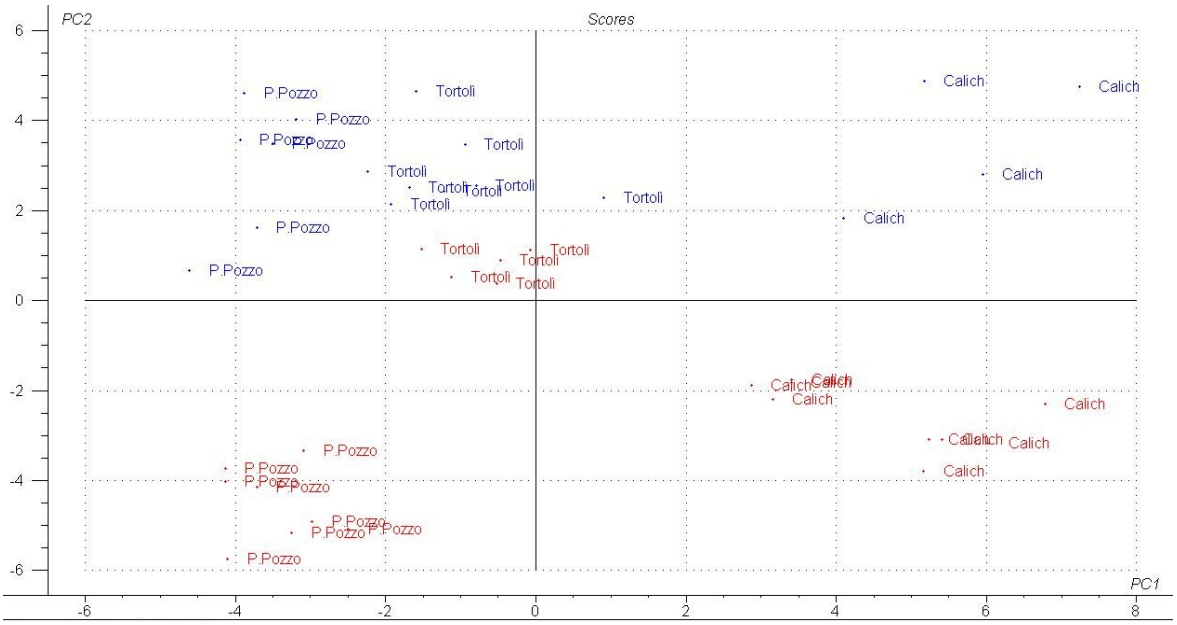


Figure 4.13 Organization of Proteomics data by Principal Component Analysis of PES including environmental and metal parameters. Blue: August; Red: September.

The performed PCA analysis could explain only a small share, about 20%, of the total variance of the studied system, while much of the remaining variance depended on other causes that yet remain undefined, but probably are related to the individual variability. At this regard, some considerations must have been made regarding the effects of outliers samples on the variability of the observed biological phenomenon. Outliers samples are linked to biological variability and contribute to address the issue of biological noise of a system. Biological variability is intrinsic to all organisms and can arise from genetic or environmental factors. In the case of protein expression, the use of individual biological replicates is essential to identify changes that significantly arise above the natural variation observed within a population; this is the most common traditional approach for studies where genetically defined populations do not exist, for example in human proteomics studies. This also could be the same case, since the mussels populations derived from a single rearing system and genetic variants should not have been present.

However, mollusks are one of the taxonomic groups in the animal kingdom where the highest levels of genetic variability have been reported (Ward et al., 1992). In the case of mussels, they present a high degree of genetic polymorphism, with genetic divergence occurring for allozymes (Skibinski et al., 1983) and mitochondrial DNA (Edwards & Skibinski, 1987). The M. galloprovincialis is a mussel species where a particularly high value of heterozygosity for enzyme loci has been found (H=0,240; Grant and Cherry, 1985) and this results have been confirmed in proteomic study (Mosquera et al., 2003). For its higher separating power, two-dimensional electrophoresis rather than monodimensional SDS-PAGE allows to discriminate a high number of protein-encoding loci. When these loci are monogenic and codominant, then the individuals express two genic products or isoforms of proteins at the same time that can be resolved and detected by 2-DE. The consequences of such a high level of heterozygosity is that the expressed protein pattern is affected and complicated by haplotypes frequencies, which correspond to qualitative changes in the expressed protein pattern due to the presence or absence of one or more isoform protein spots (López, 2005). At this point, it is easy to understand that multi-loci protein spots highly contribute to increase the share of biological noise depending from the individual genetic variability. In fact, when analyzing quantitative changes in the expression levels of a protein, a relevant quote of variability in quantitative levels is splitted into the number of polymorphic loci expressed for that protein. This phenomenon is consistent with the small share of variance explained by the PCA model.

The samples separation and clustering trends resulting from the PCA model are consistent with what is naturally observed. Analyzing the loading plot and the bi-plot PCA

graphs (Figure X), some associations among locality, environmental variables and protein expression signatures can be identified. The expression of the proteins named SSP1605, SSP2404 and SSP2601 is correlated with water salinity, which is higher in the lagoon of Porto Pozzo than in the two remaining lagoons, while it is oppositely correlated to pH and the quantity of dissolved oxygen; these environmental features are in accordance with the characteristics of the Calich lagoon, an eutrophic lagoon system with low salinity, and are associated with the expression of the proteins named SSP2507, SSP2813, SSP3104, SSP4109, SSP4201, SSP4302 and SSP6609. Also temperature values can influence the expression of some proteins and precisely those identified with the names SSP803 and SSP2805 (positive effect) and SSP8301 (negative effect). Some previous works have studied the association between environmental temperature and proteins expression signatures. Heat shock proteins (Hsps) levels are differentially induced as a consequence of thermal response. The effects of thermal stress on the induction of Hsps was examined in scallops species used to living in different thermal conditions: the northern bay scallop (Argopecten irradians), a relatively heat tolerant estuarine species, and the sea scallop (Placopecten magellanicus), living in cold and deep waters (Brun et al., 2008); the electrophoretic and immunochemical study revealed that a prolonged heat shock elicited a significant response in Hsps expression. Similarly, the heat shock protein Hsp 90-α has been identified as a possible biomarker for temperature in the carp (McLean et al., 2007), as well as the heat shock cognate 70 (Hsc 70) was detected as putative biomarker for temperature stress in populations of European hake (Gonzalez et al., 2010). These works suggest the future chance that, together with other proteins like creatine kinase, heat shock proteins may be suitable biomarkers for the assessment of the physiological and

metabolism consequences induced in living beings by adaptation to climate change (Gonzalez et al., 2010). In our case, the comparison with the results of protein identification of previous works suggests that the protein identified with name SSP2805 in the present work may correspond with a putative Hsp 70. However, it is necessary to confirm the hypothesis through MS analysis and identification in protein database.

Similar trends can be identified also for the expression of some proteins and the metal content of mussels individuals. PCA results show that, on the one hand, the high cadmium content influences the expression of the proteins named SSP2, SSP18, SSP201, SSP5802 and SSP8208 in mussels from Tortolì and Porto Pozzo lagoon. On the other hand, Porto Pozzo lagoon is characterized by a correlation between high content of arsenic and mercury and the expression patterns of the proteins identified as SSP2404, SSP1605 and SSP2703. Further studies are necessary to identify all the identified proteins through MS analysis and identification in database research.

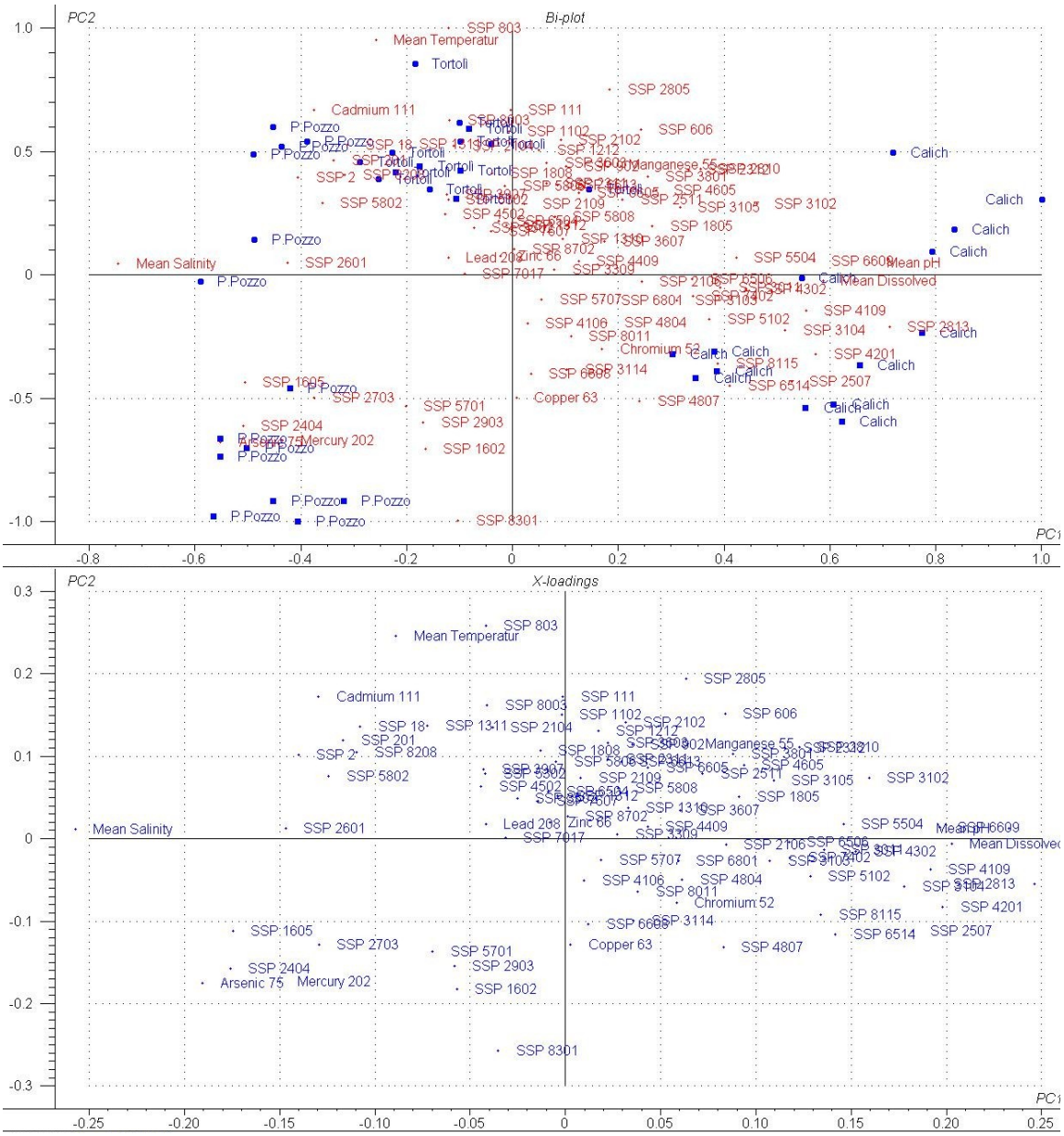


Figure 4.14 Organization of Proteomics data by Principal Component Analysis of PES and environmental and metal parameters. Upside: Bi-plot; down-side: loadings plot.

4.5 Conclusions

This work is the first attempt ever to study and characterize the proteome expressed by mussels of the specie Mytilus galloprovincialis reared in Sardinia. The 2D-PAGE protein reference map was reported, elucidating the most relevant proteins expressed in the mussel foot tissue. It was possible to observe that skeletal and energy metabolism proteins are the most abundantly expressed proteins in the studied mussels tissue. Moreover, by using two-dimensional electrophoresis, gel image analysis and statistical data treatment, changes in protein abundances among three populations of M. galloprovincialis reared in different environmental conditions were explored. Some correlations between the expression patterns of specified proteins of interest had been found, that may be considered as putative biomarkers for environmental pollution and climate change.

However, statistical data treatment highlighted that the population trends in protein expression patterns could be somehow masked by the high degree of individual variability of protein expression, that very frequently occurs among mollusks, thus confirming the high heterozygosity level previously described for this specie by other authors.

In order to diminish the high noise effect caused by the high individual variability over the trends in mussels populations, it is necessary to identify through MS analysis the various allomorphic protein variants and find the significant pairwise combinations for the relative loci associations. Alternatively, an individual pooling approach can be adopted in the making of two-dimensional electrophoresis maps. It has been demonstrated that there is a reduction in biological variance in pooled samples, confirming the important assumption of the pooling approach (Diz et al., 2009). This solution could prove useful in

the case of treating biological samples with intrinsic high variability, as in the case of the proteome expressed by mussels.

Future work will be oriented at identifying the protein of interest by means of MS analysis and database research, confirming or eventually integrating the preliminary findings of this study with the results of MS analysis and with further protein 2-DE maps, and lastly, testing the validity of the eventually identified biomarkers by increasing the number of specimens employed in the analysis.

Chapter 5

Review Article: The role of Biosensors and Proteomics in Ecotoxicology issues

5.1 Abstract

Various organisms, anthropogenic and natural substances influence the marine environments and may have diverse effect on human health. Toxicant measurements both at real time and at spatial scale are required to adequately monitor these hazards both at environmental and food chain level in order to understand their distribution, the magnitude of their effects and manage the consequences.

A great pace ahead is represented by biosensors technology that integrates the high specificity and sensitivity of biochemical recognition systems with modern electronics, resulting in the availability of fast, easy-to-use devices that can be used in environmental and food toxicology monitoring.

Proteomics have been recently applied to ecological studies in the marine environment, proving to be a valuable tool to spot biomolecular events involved in toxicant responses, to elucidate the mechanisms of stress and to identify novel biomarkers of ecotoxicity.

The contribution of "-omics" technologies in general, and of proteomics in particular into the progress of environmental and food toxicology allows the identification of even more biomarkers and, by consequence, the availability of more biosensors for environmental and food biomonitoring programs. This review tries to summarize the most recent advances in biosensors technology and its application as high-throughput

predictive screening devices in food and environmental toxicology; also, the contribution brought by proteomics in the discovery of new toxicology biomarkers for the design of new biosensors is discussed.

5.2 Introduction

The persistent anthropogenic activity of the last centuries has lead to the continuous contamination of the environment by release of high levels of many chemical compounds: heavy metals, polyaromatic hydrocarbons (PAHs), anti-biofouling and other endocrine disrupting chemicals, pesticides and so on. Once introduced in the environment, these compounds are assimilated by organisms, affecting their physiological condition. Most pollutants (pesticides, antifouling agents, PCBs, dioxins and furans) and toxic elements (e.g. Hg, Cd, Pb and As) are persistent and accumulate in aquatic ecosystems, so that aquatic organisms are exposed during the whole life cycle and over generations. Seafood is also systematically affected by the accumulation of chemical contaminants, that by this way can trace back the food chain until reaching humans, with extremely dangerous effects for human health (Islam and Tanaka, 2004). Anthropogenic activity has also contributed to exacerbate climate change. Although it is commonly accepted that climate change is a natural process taking place as a result from natural forces on various timescales and spatial scales (FAO, 2009), also human activities are contributing to increase climate change effects at a relatively local scale. The greenhouse gas CO_2 emitted by human activities have been accumulating not only in the atmosphere, but it is also permeating oceanic waters, resulting in a reduction of water oxygen levels and contributing in worsening the global warming, the continue rising in the average temperature of Earth's atmosphere and oceans. That is, oceans are becoming warmer,

less oxygenated, more saline and richer in toxic compounds. Climate change and human pollution are affecting the frequency and intensity of sea currents (FAO, 2008), as well as inducing changes in the environmental ecological balances: the increasing in the occurrence of harmful algal blooms, or "red tides" is just an example; but also the distribution of marine and freshwater species is changing (FAO, 2008). As a consequence of increased temperature and polluted environment, fish and shellfish are trying to adapt themselves to the new stressing condition by changing their physiological processes and metabolic routes: species with shorter life span will change their lifetime and spawning cycles, resulting in life stages mismatches between prey and predators in the same food chain; this phenomenon will sum to the changes in the nutrient supply induced by increasing temperatures. The outcomes can affect not only seafood availability, primarily influencing fishery and aquaculture production, but also the safety and the quality of seafood; seafood contamination by algal phycotoxins is just another example.

The seriousness of the environmental problem explains the growing number of initiatives and legislative actions for environmental control and the increasing number of scientific research programs in this field: the Stockholm Convention on persistent organic pollutants, establishing the evaluation of the effective reduction of persistent organic pollutants; the European Union Water Framework Directive (2000/60/EC), assessing a wide range of priority chemical compounds to be monitored in aquatic environments through adequate monitoring programs; the EU Marine Strategy Framework Directive (2008/56/EC), establishing the community strategy for the protection and conservation of the marine habitats and environment. The European Commission also made a call for a "more cross-boundary and multidisciplinary approach (…) to understand the impact that

humans have on the terrestrial and aquatic environments and its effects" (JMM of the Helsinki and OSPAR Commissions, 2003). Also for food, both in the White Paper on Food Safety (Commission of the European Communities, 2000) and in the food law framework Regulation 178/2002/EC, the European Union laid down the procedures in matters of food safety and the controls required to ensure that acceptable safety standards are retained, in compliance with the general opinion that food safety must be ensured in all aspects of the food production chain, in the perspective of the farm-to-fork continuum. Moreover, the Community political measures on food safety are supported by specific research actions adhering to three main perspectives: improving the knowledge of the relation between food and health; developing tools to control food-related risks, with the contribution of "-omics" biotechnologies; controlling health risks associated with environment and climate change. Accordingly, the European Framework 6 Programme (FP6) had patronized the development of some research programs aimed at employing "-omics" (transcriptomics and proteomics) technologies and approaches to discover biomarkers and develop biosensors linked to exposure to toxic compounds such as phytoestrogens, mycotoxins and organochlorine pesticides; BioCop is one of them (www.biocop.org/theproject_packages.html).

Ecotoxicology is aimed at understanding the effects induced on aquatic organisms and seafood by environmental stressors, being them chemical toxicants or changes in temperature, UV light or salinity induced by climate change. The primary aim of ecological risk assessment is to predict any adverse effect of anthropogenic pollutants on the ecosystem. To assess the hazard of toxicants, a robust methodological approach is necessary, that is focused in the employment of complementary analytical methods.

Traditionally, heavy metals and organic pollutants have been detected by analytical methods based on chromatographic techniques (LC/MS, GC/MS and ICP/MS); however, despite being extremely sensible and reproducible, these methods are time-consuming, require trained operators and do not provide information on the cumulative toxicity effects induced on a biological scale. These analytical methods cannot be employed as predictive tests, while this would be extremely useful in the assessment of toxicity and ecotoxicity of compounds, particularly for the implementation of the European Community Regulation on the registration of chemicals and their safe use (REACH) (EC 1907/2006). Biological techniques, like bioassays and biosensors, constitute a relative low-cost screening method for the quantitative detection of toxicants. Latest development in improving biosensors performance have been focused on the multi-analyte detection and on the study of their cumulative biological effects, thus making them suitable devices for a high-throughput predictive screening.

5.3 Biosensors and toxicological issues in food and environment

A biosensor is defined as an analytical device that in a solid surface integrates a sensing element of biological origin and a physical signal transducer, the first being in charge of capturing a signal related to biological, chemical or physical processes affecting the system under detection process, and the second of the physical transduction of the biochemical signal. The biological element is usually an enzyme or a high-affinity compound as monoclonal or polyclonal antibodies or their fragments. These biochemical molecules are characterized for their capacity of recognizing and linking their substrate with high specificity and sensitivity; their use in biosensing allows to contemporaneously and real-time measure different analytes at very low concentrations (micro or

nanomolar) even in complex matrices. Also molecular receptors, peptides, nucleic acids and biomimetic molecules can be employed in a biosensor, up to intact cells or tissues (Kröger S et al., 2002; Turner, 2000). As an example, a rapid bioluminescent bioassays based on recombinant cells have been developed to sensitively detect endocrine-disrupting compounds (Michelini et al., 2005).

The first biosensor was invented by Leland C. Clark during the ’60 years and was a an enzymatic electrode that employed the glucose oxidase to quantify glucose levels in blood. He eventually made the first prototype of biosensor by immobilizing the enzyme onto the oxygen electrode (Heinemann and Jensen, 2006); nowadays, this device is still used by millions of diabetics daily. A great pace ahead in the development of biosensors was determined also by the use of antibodies in detection assays (immunoassay), where the high specificity and selectivity of the reaction antigen – antibody is exploited. Great diffusion in the use of biosensors was determined also by advances in the development of modern methods of measurements, from microtiter plate-based assays to the integration of optical, electrochemical or piezoelectric signal transducers to biological components. The development made in the fields of modern electronics and in the automation of biomonitoring have resulted in the availability of fast, easy-to-use and low-cost monitoring devices with good signal stability. The forefront in biosensors technology is represented by the development of biomarkers analyzers embedded in silicon chips with digital microfluidic or electrokinetic manipulation (Jebrail and Wheeler, 2010); the resulting miniaturized and compact biosensors can be inserted in easy handling and portable instrumentation for *in situ* monitoring, both for field analysis (environmental sensors) and foodstuff.

A disadvantage of biosensors based on enzymes or antibodies is their scarce robustness, that is the capacity to keep stable analytical measurements even in presence of interfering substances or in complex matrices. Antibodies, receptors and enzymes can suffer from lack of stability, thus compromising the performance of the assay. Alternative approaches are based on the use of synthetic materials like molecular imprinted polymers (MIPs). The molecular imprinting technology introduces in a synthetic polymer recognition properties similar to those of biological receptors using appropriate templates that have same geometry and orientation of the functional groups in the molecule of interest (Wulff, 1995). MIPs have similar high affinity and specificity to their templates but higher stability in comparison with antibodies. Recent studies have been carried out on their employment in the development of biosensors with artificial receptors for algal toxins (Yu and Lai, 2010) and other marine biotoxins (Chianella et al., 2003) and pesticides (Alizadeh, 2010). However, this topic will not be discussed in this review.

Finally, the field of biosensors development is taking big advantage from the development of nanoscience. Nanoparticles (NPs) and nanomaterials usually have dimensions at nanoscale, that is 1–100 nm, with physico-chemical properties and biological effects differing from the corresponding macroparticles due to the increased numbers of surface-exposed atoms (Moore, 2006). Nanotechnology employs molecules, particles and materials for the construction of structures and devices at nanoscale. The continuous progress in nanotechnology is giving great contribution in many aspects of biosensors design and development, particularly to time of response and detection performances. Coupling nanoparticles to the biosensing element results in the availability

of different possibilities of interface immobilization, with different detection performances; in the possibility of coupling different recognition systems (antibodies, enzymes, DNA probes or cells) to simultaneously measure multiple parameters with high specificity and sensitivity and power of real-time response. The advances in nanotechnology allow the development of new transducing devices with better sensitivity, wider applicability range and lower prices. In one word, this is the capability of making high-throughputs measurements with high performances. Moreover, the continuous progression in micro/nanoelectronics and in micro/nanofluidic is helping reducing the size and the energy requirements of biosensor devices, thus contributing to lowering their prizes and improving their employment in biomonitoring programs.

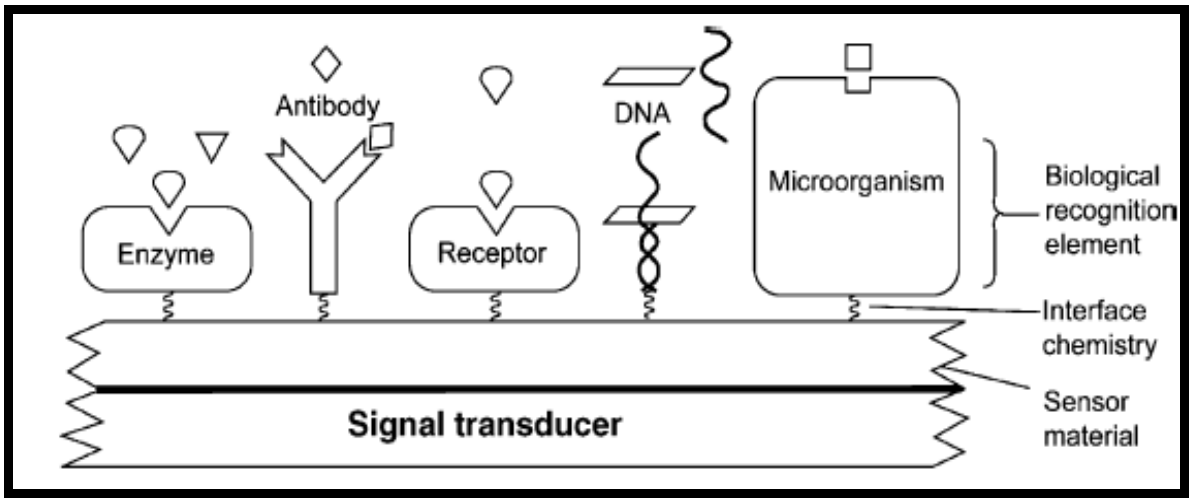


Figure 5.1 A schematich representation of a biosensor. (Courtesy: Prof. Àfrica Gonzáles-Fernández, Vigo University.)

5.4 Applications of Biosensors

5.4.1 Environmental and Water Monitoring

Human activities (fishing, disposal of waste, nutrient inputs from agricultural and rearing activities, aggregate extraction and other industrial activities) can impact and

deeply affect many aspects of marine ecosystems: primary and secondary production, animal movement and species distributions, the occurrence of disease in organisms following pollution events (ecotoxicology), biogeochemical cycles. On the general consideration that, on the whole, impacts can determine cumulative and combined effects on the environment, looking individually at each anthropogenic impact is not sufficient.

Biosensors have found broad application in monitoring marine ecosystem status, with a variety of aspects where they can be successfully employed: in monitoring nutrients, pollutants and other hazards, or species distribution. A variety of biosensors have been designed for water monitoring applications that use different types of bioreceptors (enzymes, receptors, antibodies, DNA or microorganisms) combined with electrochemical, optical or mechanical signal transduction devices (Rodriguez-Mozaz et al., 2005; Zielinski et al., 2009). Moreover, biosensors can be used in *in situ* observations or in remote sensing through a satellite or aircraft connection. The definitions and exhaustive classifications of biosensor systems for environmental monitoring and marine control have been widely reviewed (Rogers, 2006; Badihi-Mossberg et al., 2007; Jiang et al., 2008; Kröger et al., 2009; Zielinski et al., 2009; Eltzov and Marks, 2010), so it is not in the intentions of this review to discuss the topic much in detail. Only some examples about the application of biosensors in marine environmental monitoring will be discussed here; a compilation, however not exhaustive, of the most recent studies on the topic is reported in Table 5.1, while the various classes of biosensors are discussed in the following paragraphs.

5.4.1.1 Immunosensors

Many toxic compound can be detected in waters by means of immunosensors. Immunosensors are based on the highly selective antigen (Ag) — antibody (Ab) reaction. The bioreceptor immobilized on the transducer can be either an Ab or an Ag, the last one being chemically modified for the immobilization on a solid surface (hapten). Accordingly, there are different assay formats: competitive direct assay, direct inhibition assay, sandwich assay, displacement assay and indirect competitive inhibition assay. The last one is based on the competition between the immobilized antigen (hapten) and the free antigen (the analyte) for a fixed amount of antibody and is the most used assay format in immunosensors design (Shankaran et al., 2007). Immunosensors have been coupled with optical transducers, like optical immunosensor based on total internal reflection fluorescence (TIRF) (Tedeschi et al., 2003) and surface plasmon resonance (SPR) (Shankaran et al., 2007). Briefly, in SPR, when a thin metal film is excited by an incident beam of light of appropriate wavelength at a particular angle, an evanescent electromagnetic field is generated on the metal surface (Homola et al., 1999). The phenomenon is physically explained as a variation in the charge density that occurs at the interface between two media of oppositely charged dielectric constants (the metal and the exterior). The evanescent electromagnetic field can generate only under total reflection conditions, that is under fixed angle of incidence, and exponentially decreases as the distance of penetration from the interface increases. Biomolecules immobilized on the metal influence the resonance condition, so that any conformational change of the linked biomolecule, for instance determined by the binding to an antibody, causes the change in the refractional index at the interface and a shift in the resonance angle, being accurately determined. A lot of information on the analyte can be obtained from the

resonance angle shift, like the affinity for the antibody, the association (or dissociation) kinetics and the amount of bound analyte. The coupling between the specificity of antibody-antigen recognition with the sensitivity and reliability of signal detection of SPR has proven to be of great advantage in the high-performance detection of compounds in environmental monitoring (Farre et al., 2007), particularly when a multi-analyte detection of small molecular-weight analytes in complex matrices is required (Kim et al., 2007). SPR devices for studying biomolecular interaction have had great diffusion and several companies (like BIAcore) manufacture SPR instrument with a variety of options. Recent developments have been focused on the employment of immunosensors with SPR (Mitchell, 2010).

Immunosensors have also been coupled with electrochemical transducers as electrochemical impedance spectroscopy (EIS) (Prodromidis, 2010), as well as with some potentiometric transducers like semiconductor-based field-effect devices (FEDs) and ion selective electrodes (ISEs) (Bratov et al., 2010). All these types of signal transducers, as well as SPR, can be easily miniaturized and integrated in microfluidic platforms and microarrays and thus they are suitable for the creation of miniaturized analytical systems for environmental monitoring.

In the perspective of the EU Water Framework Directive (2000/60/EC) of prioritizing the monitoring of various pollutants at very low detection limits to protect water resources and control water quality (Allan et al., 2006), several projects for the development of biosensors for environmental monitoring have been funded under different framework programs during the last ten years (Rodriguez-Mozaz et al., 2005). One of the research project supported by the European Commission under the Fifth

Framework Programme, the Automated Water Analyser Computer Supported System (AWACSS) project, has developed a remote-controlled station that by means of biosensors is able to evaluate levels of various water pollutants (Tschmelak et al., 2005). Thanks to an integrated optical chip based on fluorescence-labelling as TIRF immunoassay technique, it is possible to monitor up to 32 different analytes among pesticides, natural toxins, antibiotics, carcinogens and industrial wastes in less than 20 minutes without any prior sample treatment except for water filtration to remove particulate matter. Many of the analytes can be analyzed at a level below the detection limit defined by the European Union (nanogram per liter). The data are acquired on real-time and transmitted to a central computer by a remote control (Proll et al., 2005).

5.4.1.2 Biosensors based on enzymatic inhibition assays

Also biosensors based on enzymatic inhibition assays have been proved to be of remarkable use in environmental monitoring. Organophosphorous and carbamate pesticides used in agriculture as insecticides inhibit cholinesterase activity, inducing severe toxic effects to the nervous system; also other chemical compounds, as heavy metals or detergents, as well as other nerve agents and aflatoxins, inhibit esterases. Biosensors for the detection of the neurotoxicity of these substances are based on the direct measurement of the inhibition activity of two enzymes belonging to this family, acetylcholinesterase (AchE) or butylcholinesterase. This type of biosensors have recently been object of review (Arduini et al., 2010). Recent advances in the development of neurotoxicity biosensors are oriented at improving sensor performances, either by genetic modification of the enzyme (Bucur et al., 2006), or by a better immobilization of the enzyme on the electrochemical or optical transducer. The use of nanomaterials like

gold, silver, zirconia, cadmium sulphide or iron nanoparticles, quantum dots or carbon nanotubes significantly increase the sensitivity of the biosensor; some recent reviews report the last advances in nanosensors for organophosphate pesticide detection (Liu et al., 2008) and in the nanomaterials employed (Periasamy et al., 2009). The most recent studies have also attempted to immobilize AchE on the nanomaterial via a histidine tagging (Ganesana et al., 2011), or by absorbing the (AChE)-coated Fe_3O_4/Au (GMP) magnetic nanoparticulate (GMP-AChE) on a screen printed carbon electrode (SPCE) (Gan et al., 2010). In some cases, bi-enzymatic biosensors have been developed for detecting two distinct families of pollutants, as it is the case of the conductometric biosensor using immobilised *Chlorella vulgaris* microalgae as bioreceptors. The two algal enzymes alkaline phosphatase and acetylcholinesterase are specifically inhibited by heavy metals and pesticides (organophosphates and carbamates), respectively (Chouteau et al., 2005).

5.4.1.3 Enzyme-based biosensors

Also a large number of enzyme-based biosensors have been used in environmental monitoring. The employed enzymes usually belong to hydrolase, oxidase or reductase families. According to the type of parametric variation that they locally detect, they are coupled with different transducers: hydrolase enzymes are usually coupled with potentiomentric or conductometric transducers, as they detect local changes in pH or in conductivity; oxidase and reductase enzymes with amperometric or conductometric transducers that record electronic transfers. Different enzyme-based biosensors have been employed in the assessment of various organic and inorganic pollutants. The enzyme tyrosinase catalyzes the hydrolation of various monophenols and the oxidation of diphenols into quinones; this reaction has been extensively exploited for the

development of amperometric and optical enzyme-based biosensors for the determination of phenolic compounds. Recent advances are oriented at improving sensing performances and detection limits. Various electrode materials have been used for this purpose, such as gold (Wang et al., 2010) and carbon-derived materials (Carralero et al., 2006; Lee et al., 2007; Chen and Jin, 2010; Yuan et al., 2011), glass microarrays (Jang et al., 2010), as well as different methods to entrap the enzyme on the transducer surface, by using polyacrylamide micro gel (Hervas Perez et al., 2006), carbon nanotubes and chitosane composite (Kong et al., 2009), Au nanoparticles (Carralero et al., 2006) and ZnO nanorods (Zhao et al., 2009). Also the enzyme organophosphate hydrolase (OPH) is commonly used in amperometric biosensors for organophosphorous compounds detection and different performance-improving strategies have been recently developed for this kind of biosensors (Du et al., 2010; Lee et al., 2010).

As regards nitrate and nitrite, they are two nutrients at the basis of phytoplankton growth and ocean productivity, they can conveniently be measured by biosensors that use biological sensing systems, particularly the activity of the enzyme nitrate reductase. They are the nitrate biosensors studied and engineered for most time (Cosnier et al., 1994 and 2008); one of the last developments of the device employs the enzyme nitrate reductase from yeast immobilized on a glassy carbon electrode that can directly determine nitrate in an unpurged aqueous solution with the aid of an appropriate oxygen scavenger, the methyl viologen (Quan et al., 2010). Recent advances in the development of the biosensor have been focused on obtaining better performances (Adeloju and Sohail, 2011; Can et al., 2011). Another nitrate biomonitoring systems is based on the

utilization of nitrate and nitrite by denitrifying bacteria *Agrobacterium radiobacter* (Larsen et al., 1997) and now is widely commercialized (www.unisense.com).

5.4.1.4 Estrogen Receptor-based Biosensors

Various organic compounds such as herbicides (2,4-dichlorophenoxyacetic acid), nonylphenol and its derivates, surfactants (2-hydroxybiphenyl), plasticizers (bis-2-ethylhexylphtalate), polybrominated diphenyl ethers (PBDE), polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and dioxins are endocrine disruptors compounds (EDCs), that is, chemical substances that cause hormonal imbalances by interferring with the synthesis of endogenous hormones or their receptors. Their assessment have been considered a priority in the European Water Framework Directive 2000/60/EC. There is a growing demand for biosensors for screening the presence of xenoestrogens in the environment. Biosensors for EDC detection are estrogen receptor-based, that is, they rely upon the binding of the disrupting substance to the estrogen receptor immobilized on the transducer surface. Alternatively, EDCs biodetection is based on the direct substance recognition by means of an antibody or an enzyme (AchE, tyrosinase), discussed earlier. The most used transduction systems in EDCs biosensors are electrochemical (amperometric) or optical (fluorescence, SPR). Portable systems based on SPR have also been proposed (Habauzit et al., 2007). Most recent advances in the development of EDCs biosensors have concerned the use of a field effect transistor functionalized with a carbon nanotube to significantly improve signal conductivity (Sanchez-Acevedo et al., 2009), the estrogen immobilization on a gold electrode (Im et al., 2010), or on a bilayer lipid membrane modified with Au nanoparticles (Xia et al., 2010).

5.4.1.5 Nucleic acid-based Biosensors

Many pollutants like heavy metals, benzo(*a*)pyrene, PAHs and PCBs also interact with DNA or RNA strands, causing genetic damage (genotoxicity). Nucleic acid-based biosensors have been finding increasing use for the detection of these kind of toxic compounds. In this case, the sensing element is a nucleic acid strand that interacts as a receptor with the specific chemical molecule. The molecules binding to DNA are detected either directly, by quantifying the redox potential change during the oxidation–reduction process of DNA bases, particularly guanine, or indirectly, by employing electrochemical probes. The signal transducers associated in nucleic acid biosensing are electrochemical, optical, mass magnetic and micromechanical. Many examples of DNA biosensors for the detection of DNA damage and interaction have also been reported (Palchetti and Mascini, 2008). Although the application of DNA/RNA biosensors for environmental pollution detection is quite new (Ahn et al., 2009; Ben-Yoav et al., 2009; Eltzov et al., 2009), nucleic acid biosensing and its coupling with recombinant biotechnologies (Hwang et al., 2008; Song et al., 2009; Kotova et al., 2010) and nanomaterials (Nowicka et al., 2010) is already an emerging field. First advances in the development and applications of nucleic acid-based biosensors for environmental application have already been reviewed (Palchetti and Mascini, 2008). Lastly, DNA-based biosensors have been using also for marine species detection. In this case, DNA or RNA is detected through hybridization with immobilized DNA or RNA probes immobilized on the transducer support. In DNA/RNA microchips, a collection of immobilized probes are used and, when the array of probes match with the target genetic sequence, a specific signal is produced that depends on the pattern of hybridizing probes. Biosensors based on a rRNA hybridization approach have been developed for the identification of microbial and phytoplankton species (Zhou, 2003;

Scholin et al., 2008), providing information onto many ecosystem processes and interactions. Genetic probes can be used also to detect functional groups of bacteria in various environments, as water or sediments: nitrifiers bacteria can be recognized by means of a single gene-fluorescent in situ hybridization technique (FISH) applied to whole cells to detect nitrite reductase gene (Pratscher et al., 2009). The availability of genetic information on marine species is necessary for the design of new genetic probes and microarrays, as well as new methodologies for in-situ determinations; so far, a big quantity of genetic data is accumulating as a result of ongoing sequencing projects.

Table 5.1 Application of Biosensor for Environmental Monitoring

Application Area	Analyte	Biosensing method	References
Eutrophycation (nutrients)	Nitrate and nitrite	Nitrate reductase	Cosnier et al., 1994 and 2008; Larsen et al., 1997 and 2000; Quan et al., 2010; Zhang et al., 2009; Adeloju and Sohail, 2011; Can et al., 2011.
	Phosphate	Enzyme (pyruvate oxidase)	Engbloom, 1998; Kwan et al., 2005; Zhang et al.,. 2008; Gilbert et al, 2010
Pollutants	Phenolic compounds	Amperometric enzyme-based (tyrosinase) sensor	Carralero et al., 2006; Hervas Perez et al., 2006; Lee YJ et al., 2007; Wang et al., 2008; Zhao et al., 2009; Chen and Jin, 2010; Wang et al., 2010; Kong et al., 2011; Yuan et al., 2011
	Phenolic compounds	Optical enzyme-based (tyrosinase) sensor	Abdullah et al., 2006; Jang et al., 2010; Fiorentino et al., 2010
	Herbicide (Atrazine)	SPR-Immunosensors EIS-Immunosensor TIRF-fluorescence Immunosensor	Farre et al., 2007 Hleli et al, 2006 Tschmelak et al., 2005
Persistent organic pollutants	Polycyclic aromatic hydrocarbons (PAH)	Amperometric immunosensors	Ahmad et al., 2011
	Benzo(*a*)pyrene	SPR-Immunosensor	
	Polychlorobiphenyls (PCBs)	Whole-cell sensors	Gavlazova et al., 2008;
	Polychlorobiphenyls (PCBs)	Piezoelectric immunosensors	Přibyl et al., 2006
	Pesticides (Dichlorvos)	Amperometric enzyme-based (tyrosinase) sensor	Vidal et al., 2008
	Pesticides (Chlorpyrifos)	SPR-Immunosensor	Mauriz et al., 2006
	Pesticides (Isoproturon)	TIRF-fluorescence immunosensor	Tschmelak et al., 2005;
	Pesticides (Picloram)	Amperometric-HRP labeling Immunosensor	Chen L et al., 2010
	Pesticides (Organophosphates)	Amperometric enzyme-based (organophosphste hydrolase) sensor	Du et al., 2010; Lee JH et al., 2010
	Pesticides (organophosphates)	Acetylcholinesterase sensors	Suri et al., 2002; Bucur et al., 2006; Du et al., 2008 a and b; Gong et al., 2009; Viswanathan et al., 2009; Du et al., 2010; Gan et al., 2010;
	Pesticides (carbamates)	Acetylcholinesterase sensors	Suwansa-ard et al., 2005;
	Pesticides (DDT)	SPR-Immunosensor	Mauriz et al., 2007
	Antibiofouling agents (TBT)	Recombinant bacteria sensors	Durand et al., 2003; Thouand et al., 2003; Horry et al., 2007; Gueuné et al., 2009
Endocrine disrupting compounds	Testosterone	TIRF-Immunosensor Amperometric Immunosensor	Tschmelak et al., 2006 Eguilaz et al., 2010
	17β-estradiol	Impedance receptor-based biosensor EIS Receptor-based biosensor	Xia et al., 2010 Im et al., 2010
	Bisphenol A	TIRF-Immunosensor EIS-Immunosensor FET Receptor-based biosensor	Marchesini et al., 2005; Tschmelak et al., 2005; Rahman et al., 2007 Sanchez-Acevedo et al., 2009
	Surfactants (2-hydroxybiphenyl)	SPR-Immunosensor	Kim et al., 2007;
	Herbicide (2,4D)	SPR-Immunosensors AuNP-Immunosensor	Kim et al., 2008 Chandra Boro et al., 2011;

		TIRF-fluorescence Immunosensor	Long et al., 2008
Toxins	Microcystin -LR	TIRF-fluorescence Immunosensor	Long et al., 2008
Trace Metals	Cd, Zn,	Whole-cell sensors (*Chlorella vulgaris*)	Chouteau et al., 2005; Guedri et al., 2008; Chong et al., 2008
	Pb^{2+}, Hg^{2+}, Cu^{2+}	Whole-cell sensors (*Vibrio fischeri*)	Komaitis et al., 2010
	Hg, Cu, Zn, Ni,	Whole-cell sensors (*Escherichia coli*)	Wang H., et al., 2008
	KCN, As_2O_3, Hg^{2+}	Whole-cell sensors (*Escherichia coli* DHα)	Liu C.,et al., 2009

Application Area	**Analyte**	**Biosensing method**	**References**
	Atrazine, DCMU, Formaldehyde	Amperometric algal sensor (*Chlorella vulgaris*) *Chlorella vulgaris* / *Pseudokirchneriella subcapitata*/ *Chlamydomonas reinhardtii*	Shitanda et al., 2009; Tatsuma et al., 2009
	Mitomycin C, ethidium bromide,H_2O_2, toluene, pyrene, benzo[*a*]pyrene, MMS Mitomicyn C, pentachlorophenol, H_2O_2	Whole-cell sensors (*Acinetobacter baylyi ADP1 recA::luxCDABE*) *(Escherichia coli K12 recA ::luxCDABE* and *ColD::luxCDABE*)	Song et al., 2009 Kotova et al., 2010

AchE: acetylcholinesterase, AP: alkaline phosphatase; 2,4D: 2,4-dichlorophenoxyacetic acid; DCMU: 3-(3,4-dichlorophenyl)-1,1-diethylurea, DDT: dichlorodiphenyltrichloroethane; IQ: 2-amino-3-methylimidazo[4,5-f]quinoline, MNNG: 1-methyl-1-nitroso-N-methylguanidine, MMS: Methyl methanesulfonate , 4-NQQ: 4-nitroquinoline N-oxide; TBT: Trybutiltin.

5.4.2 Detection of Marine toxins and Pollutants in Seafood.

5.4.2.1 Marine Toxins

A large number of animals from different phyla, including snails, jellyfish, sea anemones, sea urchins, sponges, bivalve, fish, etc., produce highly bioactive substances including potent toxins and venoms; these substances can be hazardous to human health. Most marine biotoxins are produced by microorganisms, such as fungi, microalgae (dinoflagellates and diatoms) and bacteria including the prokaryotic cyanobacteria (blue-green algae). Toxins from algal origin (phycotoxins) may go up through the food chain via the consumption of microalgae by fish and shellfish and accumulate in seafood and higher trophic levels without causing any toxic effect, but representing a risk for human consumers' health. Phycotoxins are produced in big quantities and released in the environment during the cycles of algal occurrence, termed harmful algal blooms (HABs) or red tides for the red-brown pigmentation of waters, also characterized by the excessive accumulation of biomass (Masó and Garcés, 2006). Phycotoxins can long persist in waters after the extracellular release (Lawton et al, 1994), thus biomass absence after bloom resiliency does not necessarily mean absence of toxins in the waters. Considering that the frequencies and intensity of HABs is increasing worldwide and that the phenomenon may be exacerbated by increasing pollution and climate change, there is growing concern for phycoytoxins persistence in the marine environment, not only for public health but also for fishing and aquaculture industry, tourism economy and, more in general for environmental protection.

Marine toxins are low-molecular weight, non-proteinaceous compounds with very diverse chemical structure and mechanism of action. The most widespread accepted

classification of marine toxins related to seafood poisoning is based on the physiological effects and syndromes caused in humans by the involved toxin (Cámpas and Marty, 2007). At cellular level, the biochemical mechanism of action of the different phycotoxins is quite similar, as they bind to specific control sites of cellular sodium channels, being receptor proteins or regulatory proteins. The resulting effect is a loss in the control in the inward flow of sodium ions, with different outcomes depending on the nature of the toxin and the interested cell or tissue. In neurons, blocking the sodium channels affects the propagation of the action potential with the release of synaptic neurotransmitters (acetylcholine) and depolarization of neuronal cells. Paralytic shellfish poisoning (PSP) toxins, amnesic shellfish poisoning (ASP) toxins, neurologic shellfish poisoning (NSP) toxins and ciguatera fish poisoning (CFP) toxins are potent neurotoxins that cause neurological symptoms ranging from mild to acute, even fatal in the case of ciguatera toxin. When sodium channels controlling sodium secretion in intestinal cells are interested, the outcome is a sodium release and a loss of liquids, responsible for a diarrheic episode, as it is the case of diarrheic shellfish poisoning (DSP) toxins and okadaic acid.

5.4.2.2 Biosensors for Detecting Marine Toxins

The *in vivo* retention of intraperitoneal mouse assay still remains the method internationally accredited and worldwide used to detect marine toxins in potentially marine toxins-contaminated seafood (Fernández et al., 2003); this bioassay is still the EU reference method for detecting PSP toxins in shellfish (Aune et al., 2007), while high performance liquid chromatography (HPLC) is the analytical reference method for the detection of other phycotoxins (Campás and Marty, 2007).

A summary of the most recently developed biosensors for detecting marine toxins is reported in Table 5.2. Some groups have attempted to realize biosensors coupling the Biacore SPR detector chip with good results for PSP toxins detection (Fonfría et al., 2007; Campbell et al., 2007). A rapid analytical optical biosensor-based immunoassay was developed and validated for the detection of okadaic acid (OA) and its structurally related toxins dinophysistoxins (DTX) from mussels (Stewart et al., 2009). The employed SPR immunosensor uses a monoclonal antibody which binds to the OA group of toxins in order of their toxicity, resulting in a pseudofunctional assay. In the last decade several biosensors for the detection of various phycotoxins have been developed, that couple different biosensing systems (rat brain sodium channels, immunoassays and enzyme inhibition) with different signal transduction systems, either electrochemical or optical (chemiluminescence and SPR) (Campás et al., 2007; Campbell et al., 2011a). Most of the recent advances have focused at improving the performances of the traditional biosensors by coupling the detecting enzyme with another one of the same biochemical pathway (Volpe et al., 2009). In most of the SPR-based immunosensors, the antibody is immobilized on a carboxymethylated dextran matrix chip (CM5) (Petz, 2009). Recently, a competitive indirect enzyme-linked electrochemical immunosensor has been developed for the detection of okadaic acid (OA) that is not expensive and suitable for field analysis and routine use (Hayat et al., 2011a). In this biosensor, the analyte OA was immobilized through biotinylation on a streptavidin-coated magnetic beads support. In another work, the same author immobilized the OA on a screen printed carbon electrode (SPCE) via a diazonium-coupling reaction, with satisfactory results for the biosensor detection performance (Hayat et al., 2011b). Also the possibility to perform a multi-toxin detection

via immobilization on a chip surface has been considered (Campbell et al., 2011 b). The existing biosensors can detect phycotoxins with appropriate sensitivity, hundreds to thousands of times below the mouse bioassay defined by AOAC (Fonfría et al., 2007; Haughey et al., 2011) and are increasingly employed in seafood toxin monitoring programs (Campbell et al., 2011a). These detection systems have proven to be highly performing, reproducible and rapid and they may represent a valid alternative to mouse bioassay; however, they still need to be validated according to an internationally recognized protocol in order to replace the mouse assay in the international regulations (Campbell et al., 2011c).

Table 5.2 Application of Biosensors in Seafood Safety

Application Area	Analyte	Biosensing method	References
Marine toxins	Phycotoxins (PSP)	SPR-Immunosensors	Fonfría et al., 2007; Campbell et al., 2007; Campbell et al., 2011b; Haughey et al., 2011; Yakes et al., 2011.
	Phycotoxins (Okadaic Acid; DTX)	SPR-Immunosensors Amperometric Immunosensor SPE-electrochemical Immunosensors Enzyme-based sensors	Llamas et al., 2007; Stewart et al., 2009; Prieto-Simón et al., 2010. Campás et al., 2008 Hayat et al., 2011a and b Hamada-Sato et al., 2004; Volpe et al., 2009;
	Phycotoxins (Domoic acid)	SPR-Immunosensor	Traynor et al., 2006; Stevens et al., 2007
Persistent pollutants	Dioxin-like Plycholorinated biohenyls (DL-PCBs) 2,3',4,4',5-pentachlorobiphenyl (PCB 118)	SPR-Immunosensor	Tsutsumi et al., 2008
	Detergents (Nonylphenol)	SPR-Immunosensor	Samsonova et al., 2004
	Antiobiotics (Fluoroquinolones - Norfloxacin)	SPR-Immunosensor	Huet et al., 2008 and 2009; Weigel et al., 2009
	Antibiotics (Chloramphenicol)	SPR-Immunosensor	Dumont et al, 2006

5.4.2.3 Biosensors for Pollutants Detection in Seafood

Optical biosensors based on surface plasmon resonance are increasingly used in food safety assessment also to detect and quantify residues of persistent contaminants like dioxin-like PCBs (Tsutsumi et al., 2008) and antibiotics chloramphenicol (Dumont et al.,

2006) and fluoroquinolones (Huet et al., 2008 and 2009; Weigel et al., 2009). Similarly, However, the number of biosensing methods that have been published so far on antibiotics, pesticide residues, polychlorinated biphenyls and other persisting small molecules is still very limited (Petz, 2009).

5.4.3 Whole-cell Biosensors

5.4.3.1 Features and Functioning of Cell Biosensors

The types of biosensors that have been presented in this review until now can detect at a time a single compound or at least a group of structurally-related compounds. The information gathered and transmitted by the biosensor does not give any indication about the biological effect determined by the detected compound or compounds. Nevertheless, this information is highly important, since compounds with different nature or chemical structure may induce the same biological effect, or alternatively, they can interact with each others with diverse biological effects; furthermore, each compound can exist in different forms and availabilities, each one differently inducing toxicity in living beings. The use of a whole-cell organism as biological monitor allows the evaluation of the total toxic effect of compounds and groups of compounds in quite real time.

Whole-cells biosensors have been engineered to test the effects of various types of contaminants, such as genotoxicants, cytotoxicants or toxicants producing oxidative damage or damage to cellular proteins and membranes. Bacteria, algae and yeasts are unicellular organisms most used in whole-cell biosensors. They act on the basis of acute-toxicity response, providing results within 1 or 2 hours and fast alarm when contamination peaks occur. However, they are not suitable to detect some important

contaminants whose toxicity is induced on the basis of a long-term exposure, like some classes of genotoxicants and endocrine disruptors compounds (EDCs). This problem have been partially solved by employing yeast or mammalian cells lines instead of bacteria strains. Indeed, eukaryote cells can provide more relevant toxicity information for humans than prokaryotes. However, these compounds can also be evaluated with high sensitivity employing biosensors based on different bioreceptors, mainly DNA probes for the detection of DNA damage induced by genotoxicants, and immunologic biosensors for EDCs.

Compared to other biosensing systems, the advantages in the use of whole-cells biosensors are many: not only cells can be easily produced in large amounts and can easily adapt to physico-chemical conditions of the medium where they find themselves, they can also be programmed to specifically respond to a particular stimulus, like the one induced by the contaminant. Cellular programming is realized through genetic engineering. Briefly, a sensing element and a reporter gene are inserted inside the cellular genome. The sensing element is a promoter gene sequence that is readily activated by the analyte and induces the expression of the reporter gene and other regulatory proteins. The reporter gene encodes a protein product in a highly reproducible and proportional quantity that can be easily detected. Various reporters have been employed: *lacZ* (β-galactosidase) and *phoA* (alkaline phosphatase) (Paitan et al., 2004), *gfp* (green fluorescent protein) (Roberto et al., 2002), and *luc* (firefly luciferase) or *lux* (luminescent luciferase) genes. In general, the functioning of biosensors systems is based on two different assay schemes: one is the light-on scheme, where exposure to the toxic compound induces the signal expression in the cellular biosensor, registering an increase

in the signal intensity; the other is the light-off scheme, where analyte toxicity negatively affects the expression of a constitutive cellular feature, leading to an intensity reduction of the detected signal. Various types of cells have been employed, from well-studied and characterized cell-lines, commercialized for their wide applicability as they harbor a wide range of products and substrates, to genetically-engineered organisms that have been constructed to specifically detect some molecules or classes of molecules, as it is the case of various engineered *E. coli* strains. Similarly, a variety of transducers have been coupled with whole-cells biosensors: electrochemical, optical, mass-sensitive and thermal, with different performance outcomes, as it has been reported by some reviews on the topic (Gu et al., 2004, Lei et al., 2006, Eltzov and Marks, 2011).

The strategy to adopt to immobilize cells to the transducer surface depends on the nature of both the biosensor and the transducer; for this reason, care must be taken in designing the interface. In general, cells can be immobilized on the transducer surface by adsorption (Gavlasova et al., 2008), cross-linking (Chouteau et al., 2004), entrapment by covalent binding of the cells through a choice of functional groups (Premkumar et al., 2001), sol-gel entrapment with calcium alginate (Polyak et al., 2001; Chouteau et al., 2004), Langmuir-Blodget deposition (Zhang et al., 2002, Hou et al., 2004) and self-assembled biomembranes (Ottova and Tien, 1997).

Whole-cell biosensors have been finding their ideal employment in the assessment of toxicity induced by multiple exposure to various compounds, like heavy metals, antibiotics and genotoxicants; the most recent applications are reported in Table 5.3.

Table 5.3 Applications of whole-cell biosensors for Environmental Monitoring and Toxicology

Application Area	Analyte	Biosensing method	Transduction	References
Organic Pollution	Biochemical Oxygen Demand (BOD)	*Escherichia coli DH5a* *Photobacterium phosphoreum* *Saccharomyces cerevisiae* *Bacillus licheniformis, Dietzia maris, Marinobacter marinus*	Potentiometric Fluorimetric Amperometric Optical fibre	Chiappini et al., 2010 Sakaguchi et al., 2007 Nakamura et al., 2010 Lin et al., 2006
Surfactants	Sodium dodecyl sulfate	Pseudomonas and Achromobacter strains	Amperometric	Taranova et al., 2002
Organophosphates	Paraoxon, parathion Fenitrothion, EPN Paraoxon, Parathion, Carbofuran Paraoxon	*Pseudomonas putida* JS444 OPH *Pseudomonas putida* JS444 OPH *Chlorella vulgaris* *Flavobacterium*	Amperometric Amperometric Conductometric Potenziometric	Lei et al., 2005 Lei et al., 2007 Chouteau et al., 2005 Gaberlein et al, 2000
Aromatic Compounds	Benzene Benzene, Toluene Toluene, Xylenes	*Pseudomonas putida L2* *Pseudomonas putida* mt-2 pGLPX::*luc* *Escherichia coli MC1061–pXylRS–LacZ, MC1061–pXylRS–AP*	Amperometric Amperometric Amperometric	Lanyon et al., 2006 Behzadian et al., 2011 Paitan et al., 2004
Organic Compounds (Solvants)	Trichloroethylene	*Pseudomonas aeruginosa J1104* *Pseudomonas putida F1*	Potenziometric Conductometric	Han et al., 2002 Hnaien et al, 2011
Persistent Organic Compounds	PAHs	*Escherichia coli - luxCDABE*	Bioluminescence	Lee et al., 2003
Polychlorobiphenyls (PCBs)	2,3,4'-trichlorobiphenyl, 2,4,4'-trichlorobiphenyl, 2,5,4'-trichlorobiphenyl, PCBs misture (Delor 103, Delor 106), also in association with PAHs	*Pseudomonas sp* P2	UV-VIS Spectrophotomeric	Gavlazova et al., 2008;
Phenolic Compounds	Phenol Phenol (protein and membrane damage) *p*-nitrophenol Hydroquinone	*Pseudomonas putida DSM50026* *Escherichia coli DnakA::lacZ, E.coli grpE::lacZ, E.coli fabA::lacZ* *Pseudomonas spp. MTCC-2619* Hamster lung fibroblasts V79	Amperometric Amperometric Amperometric Potenziometric	Timur et al., 2007 Popovtzer et al., 2006 Banik et al., 2008 Wang et al., 2010
Trace Metals	As	*Escherichia coli IRC140 asR::lux* *Rhodopeudomonas palustris 7*	Fluorimetric Colorimetric	Roberto et al., 2002 Yoshida et al., 2008
	Hg	*Escherichia coli RBE27-13 (pECFP) zntA:lacZ*	Fluorimetric	Biran et al., 2003
	Cd^{2+}, Zn^{2+}	*Chlorella vulgaris*	Conductometric Amperometric	Chouteau et al., 2005; Guedri and Durrieu, 2008; Chong et al., 2008
	Cd^{2+}, Co^{2+}, Ni^{2+} and Pb^{2+}	*Chlorella vulgaris*	Conductometric	Berezhestkyy et al., 2007
	Cu	*Saccaromices cerevisiae 19.3 C CUP1::lacZ* *Saccaromices cerevisiae SEY6210 CUP1::lacZ*	Amperometry	Tag et al., 2007
	Pb^{2+}, Hg^{2+}, Cu^{2+}	*Vibrio fischeri*	Biolumiescence	Komaitis et al., 2010
	As, Cd^{2+}, Cu^{2+}, Pb^{2+}, Hg^{2+}	*Escherichia coli - lucFF*	Bioluminescence	Hakkila et al., 2004
	Hg, Cu, Zn, Ni,	*Escherichia coliv*	Amperometric	Wang et al., 2008
	KCN, As_2O_3, Hg^{2+}	*Escherichia coli* DHα	Amperometric	C. Liu et al., 2009
	Fe	*Pseudomonas putida fepA-fes::luxCDABE*	Bioluminescence	Mioni et al., 2003 and 2005
(Oxidative stress)	Cd^{2+}, Cu^{2+}, Pb^{2+}, Zn^{2+} selenite, arsenite, H_2O_2 menadione, triphenyltin and naphthalene	*Escherichia coli DH5α pRSET::roGFP2).*	Fluorimetric	Arias-Barreiro et al., 2010

Application Area	Analyte	Biosensing method	Transduction	References
Antibiotics	Penicillin G Cephalosporin Chloramphenicol	*Escherichia coli pUC18 (ß-lactamase, penicillinase)* *Pseudomonas aeruginosa MTCC647* *Escherichia coli JM105*	Not specified Potenziometric Amperometric	Chao and Lee, 2000 Kumar et al., 2008 Mann and Mikkelsen, 2008
Endocrine Disrupting Compounds	17 β-estradiol E2	*Saccharomices cerevisiae Y190 medER::lacZ*	Amperometry	Ino et al., 2009
Genotoxicants	Atrazine, DCMU, Formaldehyde	*Chlorella vulgaris* *Chlorella vulgaris / Pseudokirchneriella subcapitata/ Chlamydomonas reinhardtii*	Amperometric	Shitanda et al., 2009; Tatsuma et al., 2009
	Mitomycin C	*Photobacterium phosphoreum (A2)*	Bioluminescence	Sun et al., 2004
	Mitomycin C, p-chlorophenol	*Escherichia coli DPD2794 recA::luxCDABE* *Escherichia coli TV1061 grpE::luxCDABE*	Bioluminescence	Eltzov et al., 2009
	Mitomycin C, phenol, H_2O_2	*Escherichia coli DPD2511::luxCDABE* (Oxidative damage) *Escherichia coli DPD2540:: luxCDABE* (Membrane damage) *Escherichia coli DPD2794:: luxCDABE* (DNA damage) *Escherichia coli TV1061:: luxCDABE* (Cytotixicity damage)	Bioluminescence	Kim et al. 2003
	Mitomicyn C, pentachlorophenol, H_2O_2 Mitomycin C, ethidium bromide,H_2O_2, toluene, pyrene, benzo[*a*]pyrene, MMS	*Escherichia coli K12 recA ::luxCDABE* and *ColD::luxCDABE* (heat shock, oxidative stress) A*cinetobacter baylyi ADP1 recA::luxCDABE*	Bioluminescence	Kotova et al., 2010 Song et al., 2009
	Mitomycin C, nalidixic acid, MNNNG, 4-NQQ	*Escherichia coli RFM443 with recA, NrdA, dinI, sbmC, recN, sulA or alkA promoters and luxCDABE reporter*	Bioluminescence	Ahn et al., 2009
	Nalidixic acid, IQ	*Escherichia coli RFM443 sulA ::phoA Salmonella typhimurium TA1535 umuC::lacZ*	Potenziometric	Ben-Yoav et al., 2009
	Nalidixic acid, mitomycin C, H_2O_2	*Escherichia coli RFM443 nrdA ::luxCDABE*	Bioluminescence	Hwang et al., 2008
Herbicides	Paraquat (N,N'-dimethyl-4,4'-bipyridinium dichloride), H_2O_2	*Various E. coli strains with hmp::luxCDABE and malK::luxCDABE* (Oxidative stress)	Bioluminescence	Lee et al., 2007

DCMU: 3-(3,4-dichlorophenyl)-1,1-diethylurea, IQ : 2-amino-3-methylimidazo[4,5-f]quinoline, MNNG: 1-methyl-1-nitroso-N-methylguanidine, MMS: Methyl methanesulfonate , 4-NQQ: 4-nitroquinoline N-oxide

5.4.3.2 Electrochemical Cell Biosensors

Electrochemical transducers are undoubtedly the most common transducers used in whole-cell biosensors. Detection is realized by monitoring electroactive species that are

produced or consumed by the cell during the exposure and can be performed with conductimetric, potentiometric and amperometric methods. The most common reporter gene used in electrochemical whole-cell biosensors is *lacZ* gene, encoding for β-galactosidase; once produced, the enzyme catalyzes the oxidative hydrolisis of its substrate *p*-aminophenyl-β-D-galactopyranoside (PAPG), releasing p-aminophenol that is detected at the amperometric electrode. Different microbial strains exhibiting a wide range of substrates have been used in electrochemical biosensors for the determination of the Biological Oxygen Demand (BOD) (Ponomareva et al., 2011). However, BOD index have been usually measured by biosensors in deareated conditions; a recent proposal is based on a non-deareated bioassay method, using ferricyanide reduction by *Escherichia c*oli strain DH5a, already exploited in BOD biosensors (Liu et al., 2010). Since BOD is an index of the amount of degradable organic matter present in samples, amperometric biosensors have been constructed with bacteria that specifically degrade pollutants via aerobic metabolism to detect different analytes as surfactants, phenolic compounds, organophosphorous pesticides and alcohols (Lagarde and Jaffrezic-Renault, 2011). Alternatively, the wild-type organophosphate-degrading bacterium *Flavobacterium* have been exploited in potenziometric biosensors for detecting organophosphates (Gäberlein et al., 2000). Amperometric and conductometric whole-cell biosensors have been employed also to detect heavy metals in samples by assessment of the inhibition of AchE or AP activity (Chouteau et al., 2005; Guedri and Durrieu, 2008; Chong et al., 2008). A potenziometric biosensor coupled with bacterial strain *P. aeruginosa* JI104 was constructed for monitoring trichloroethylene in waters (Han et al., 2002); more recently, to detect the same pollutants a miniaturized conductometric biosensor was realized with

cells of *P. putida* immobilized on the surface of the gold microelectrode through a three-dimensional alkanethiol/carbon nanotubes architecture functionalized with *Pseudomonas* antibodies (Hnaien et al., 2011). This kind of re-conceptualization of a previous project with new technologies and nanomaterials for performance improving is quite remarkable. Amperometric biosensors for heavy metals detection was developed also exploiting eukariotic cells, particularly *S. cerevisiae* 19.3 C and SEY6210 strains (Tag et al., 2007). In both cases the Cu^{2+}-inducible promoter of the CUP1 gene from *S. cerevisiae* was fused to the *lacZ* gene from *E. coli*; the expressed lactose is used as a carbon source, altering the cellular oxygen consumption that is detected.

Also microalgae cells have been used in the construction of electrochemical whole-cell biosensors. Cadmium and zinc levels of parts per billion (ppb) have been measured with a biosensor constituted of *Chlorella vulgaris* microalgae immobilized in self-assembled monolayers (Guedri and Durrieu, 2008); limits ten times lower (0,1 ppb) have been obtained for the same metal ions with a cell-based diamond biosensor (Chong et al., 2008). Similarly, Cd^{2+}, Co^{2+}, Ni^{2+} and Pb^{2+} have been assessed in wastewaters with a biosensor of microalgae immobilized in a sol-gel matrix (Berezhestkyy et al., 2007). A bienzymatic biosensor have also been developed with *C. vulgaris*, that expresses alkaline phosphatase, sensitive to heavy metal ions, and acethylcolinesterase, sensible to organophosphorous pesticides (Chouteau et al., 2005).

5.4.3.3 Optical Cell Biosensors

During the last decade, thanks to the availability of genetically-engineered light-producing bacteria, bioluminescence methods have started to be employed in whole cell

biosensors technology. Recombinant bioluminescent *E. coli* strains that have plasmids bearing a fusion of *luxCDABE* operon and its promoter are the cell types most used in bioluminescent biosensors design (Kim et al., 2003). However, naturally emitting bacteria, like the marine luminous bacterium *Photobacterium phosphoreum* have also been used in BOD determination (Sakaguchi et al., 2007) or to detect pollutants that are difficult to detect with conventional analytical chemical methods (Yin et al., 2005).

As previously mentioned, the availability of metal fraction in biological systems is a relevant issue, as analytical methods detect the total amount of metals in samples. Bioluminescent bacteria for monitoring heavy metals amounts where developed using *E. coli* strains genetically engineered to sensitively respond to the presence of heavy metal ions (As, Cd^{2+}, Cu^{2+}, Pb^{2+}, Hg^{2+}) by emitting luminescence through the expression of the *luc* gene for firefly luciferase (Hakkila et al., 2004). The relation between metal biovailability and detectability was confirmed also by another biotoxicological assay: when bioluminescent bacteria where put in contact with mollusk tissue extracts that had been exposed to high metal concentrations, the bacterial luminescence was inhibited, suggesting a minor availability of metal ions bond to bio-organic compounds (Girotti et al., 2006). One interesting application of optical cell biosensors is the employment of a heterotrophic, halotolerant bacteria reporter that quantitatively respond to amounts of bioavailable Fe by producing light. This reporter was constructed by fusion of the *E .coli* fepA-fes Fe-uptake promoter to the luxCDABE cassette and was integrated into the chromosome of the halotolerant *Pseudomonas putida*. The obtained cell bioreporter is able to detect the changes in Fe availability, thus being of potential utility in biosensors

design for studying the relation between Fe bioavailabilty and Fe chemistry in complex marine systems (Mioni et al., 2005).

Regarding genotoxicant compounds, various studies have been carried out on antibiotics and antitumorals, like mitomycin C and nalidixic acid, and their combinations with other toxic compounds like aromatic and chlorinated compounds using optical biosensors. Mitomycin C is used as a chemotherapeutic agent against tumoral development and is a potent DNA cross-linker: it causes DNA-damage by covalent binding to the minor DNA groove and preventing the double helix separation during DNA replication. The combined biological effects of mytomicin C and p-chlorophenol (which causes damages to membrane and proteins), have been studied using a biosensor containing two immobilized *E. coli* strains (DPD2794 and TV1061) containing fusions of *recA* (DNA damage) and *grpE* (encoding a heat-shock protein sensible to cytotoxic substances) promoters to the *lux* operon (Eltzov et al., 2009). Similarly, other studies have been carried out on nalidixic acid (Hwang et al, 2008). Curiously, the toxic effects resulting from exposure to mytomicin C have been assessed also by a biosensor made with a stable dark variant separated from *Photobacterium phosphoreum* (A2) fixed in agar-gel membrane and immobilized onto an exposed end of a fiber-optic linked with a bioluminometer with optimal response (Sun et al., 2004). In another application, a microfluidic whole cell biosensor integrating four biochips with four microchambers each was developed to detect the effects of two more genotoxic compounds, nalidixic acid and 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) (Ben-Yoav et al., 2009). Also the effects of polycyclic aromatic hydrocarbons on bioluminescent recombinan*t E. coli* have been

assessed (Lee et al., 2003), as well as the damage to proteins and membranes induced by phenol exposure (Popovtzer et al., 2006).

It is noteworthy reporting the development of a biosensor for assessing chemically-induced cellular oxidation, realized using a genetically-modified strain of *E. coli* that constitutively expresses the redox-sensitive green fluorescent protein roGFP2 (*E. coli DH5α pRSET::roGFP2*). Using a double-wavelenght ratiometric approach to detect the disulfide bonds formation in the protein roGFP2, it was possible to assess the oxidative stress induced by the combined exposure to heavy metals (Cd^{2+}, Cu^{2+}, Pb^{2+}, Zn^{2+}), selenite, arsenite, H_2O_2 and various other organic compounds that are known to have toxic effect (menadione, triphenyltin and naphthalene) (Arias-Barreiro et al., 2010).

Lastly, fluorescence biosensors with the GFP reporter, also in a microfluidic chip form, were applied to the assessment of toluene, benzene and xylenes (Li et al., 2008), as well as heavy metals like mercury (Biran et al., 2003), lead (Chakraborty et al., 2008) and arsenic (Theytaz et al., 2009). The presence of this last heavy metal ion can be detected also by a colorimetric biosensor exploiting the photosynthetic activity of the bacterium *Rhodopeudomonas palustris* 7, that changes its color from green-yellow to red in a dose-dependent manner in response to arsenite (Yoshida et al., 2008).

5.5 Biosensors and Biomarkers of Environmental stress

Progress in the biosensors technology is expected to depend on the discovery of new biomarkers capable to adequately and specifically respond to the exposure to a stressor. But how a biomarker for environmental stress can be defined?

According to previous definitions, stress must be considered as "the physiological cascade of events that occurs when an organism is attempting to resist death or re-establish homeostatic conditions in face of an 'insult'" (Piñeiro et al., 2010). In the environment, living systems are exposed to a mixture of chemicals and harmful agents; depending on the biological and ecological conditions, each toxic compound can exist in forms with different biological availability and toxicity and interact with other compounds with diverse biological outcomes. That is, the exerting stress is determined by the overall biological effects of exposure (Lam, 2009), and the physiological stress response of an organism can be polymorphic with regard to species and stage of maturity of the individual.

In the past decades, environmental monitoring programs have concentrated on the measurements of several physical and chemical variables; these programs, albeit gave information on the levels of contaminants, did not provide information on the effects of contaminants on biological systems. There have been a change of perspective in the biomonitoring approach. Nowadays, biological monitoring is focused at assessing the biological effects induced by a toxicant rather than quantifying the presence of chemical residues in the tissues of an organism. In this perspective, a biomarker is considered as a variation in the biochemical or physiological condition that is registered in an organism at the endpoint of exposure to a toxicant or another stressor, and that may be indicative of

an early warning signal of biological effects that may occur at higher levels of biological organization (Connell et al., 1999). At a molecular or physiological level, the biomarker is assessed by evaluating specific cellular biochemical parameters that respond, with different specificity but same reproducibility, as a consequence of the toxic action of the environmental stress (Amelina et al., 2007). A variety of biochemical molecules have been selected as biomarkers for toxic compounds or classes of compounds. Usually these biomarkers are related to cellular antioxidant systems, such as the cellular levels of the molecular scavenger glutathione (GSH), its metabolic enzymatic system glutathione S transferase (GST), glutathione peroxidase (GPx) and glutathione reductase (GR) (Fitzpatrick et al., 1997), as well as metallothioneins, superoxide dismutase (SOD), catalase (CAT), cholinesterase (Engenheiro et al., 2005), lipid peroxidation (LPO) system, cytochrome P450 oxygenase system, lysosomal membrane destabilization, peroxisome proliferation, hormones (Lemos et al., 2009; Zaccaroni et al., 2009) and DNA damage (Siu et al., 2004).

One of the issues related to biomarkers is the degree of sensitivity and specificity. Some biomarkers are highly specific, that is, they respond only to one chemical or group of chemicals: for instance, the enzyme aminolevulinic acid dehydtratase (ALAD) is highly inhibited by lead (Selander and Cramer, 1970). Oxidative biomarkers are non-specific and can identify a general condition of environmental stress not necessarily correlating with the presence of toxicants in the environment (Sheehan and Power, 1999); their response may be influenced by the presence of other chemical compounds that are not investigated at the moment; or can be confounded by factors such as food availability, water temperature (Nyiogi et al., 2001) and reproductive activity (Viarengo et al., 1991);

provided that, the interpretation of data may be complicated. Postulating that highly specific biomarkers are fewer than non-specific ones, and that biomarkers providing a satisfactory information on the effects or the exposure to pools of different pollutants do not exist, it is clear that orientating towards the use of multiple biomarkers is appropriate in environmental monitoring purposes.

Biochemical indicators of stress and metabolism and the definition of new biosensors for marine control have been widely studied. Various antioxidant scavengers such as glutathione transferase (GST) and reductase (GSR), catalase (CAT) and superoxide dismutase (SOD) well correlate with concentrations of PAH (Cheung et al., 2001), PCB (Cheung et al., 2002), as well as benzo(*a*)pyrene and Cu, individually or in mixture (Maria and Bebianno, 2011). Also heat shock proteins (Hsps) have been proving to be a valuable biomarker of stress: recent studies have reported changes in Hsp expression levels resulting from temperature variations (Brun et al., 2008), long-term water acidification (Hernroth et al., 2011) and cadmium exposure (Ivanina et al., 2009). The increase in Hsps levels effecting to high temperature exposure also increases energy demand and influences the expression of some metabolic allozymes, thus changing the relatives allele frequencies (Brokordt et al., 2009). Moreover, different genotypic variations occur in the period of initial response, while others occur during chronic temperature stress.

The major advances in the application of biomarkers in biomonitoring is the automation of these procedures: thanks to the progression in digital microfluidic and nanotechnology, biomarkers can be integrated in “lab-on-a-chip” devices and biosensors (high-affinity or whole-cell biosensors) for the *in situ* environmental and food monitoring.

Many examples of biomarkers applications are provided in sections 1.- 3. of the present review.

5.6 The role of Proteomics in Ecotoxicology Biomarkers Discovery.

In toxicology, proteins are the first functional stage interested by the action of the toxicant; thus it is easy to understand how proteomics, the study of the complete profile of proteins in a given cell, tissue or biological system, may enormously contribute in ecotoxicology studies when the organism is affected by a environmental stressor (Kovacevich et al., 2009). The growing interest in the application of proteomic technologies to solve toxicology issues and its relevance in ecotoxicology research has resulted in the emergence of ‘‘ecotoxicoproteomics’’ (Gomiero et al, 2006).

Proteomics uses a set of high-throughput methodologies for protein analysis with a wide dynamic range, from 2D-PAGE to protein identification by MALDI-TOF mass fingerprinting, to peptide sequencing by tandem mass spectrometry, to shotgun proteomics, that is capable of detecting subtle changes in the expression of individual proteins and amino acid sequence modifications in response to an altered environment (Unwin et al., 2006). After its current applications in human medicine for the discovery of novel biomarkers of disease (Arnouk et al., 2009; Colquhoun et al., 2009), the ultimate goal in the application of proteomics to environmental issues is the discovery of new biomarkers of environmental toxicity and contributing to elucidate the mode of action of environmental stressors.

Proteomics, together with transcriptomics and metabolomics, constitutes the high-throughput platform of “omics” techniques that is giving great contribution in the

identification of molecular biomarkers and biomarkers patterns to be deployed in the construction of low-cost, high-speed and multi-analyte screening devices for environmental and food toxicology. Proteomics approach is much closer to physiology rather than to genomics or transcriptomics. In fact, the cellular metabolism of proteins, particularly the post-translational modification and degradation, implies that the cellular expressed proteome considerably differs from what is expected by the analysis of the transcriptome (Barrett et al., 2005). In this sense, proteomics information is complementary to that of transcriptomics regarding gene expression and regulation. The proteome, more than the transcriptome, provides an adherent physiological snapshot of the cell under a specific condition of chemical stress (Hogstrand et al., 2002).

The approach of proteomics in environmental toxicology may be developed from two perspectives. From the one hand, contributing to better understand the mechanisms of action (MoA) of toxic substances by identifying their molecular targets (Poynton et al., 2008); on the other hand, identifying stressor-specific biomarkers to employ in a predictive screening of toxic compounds action (Nesatyy and Suter, 2008). Some recent reviews about proteomic strategies are explanatory (Piñeiro et al., 2010; Lemos et al., 2010).

A big advantage for the application of proteomics in ecotoxicology lays in its approach: contrarily to experimental biology coupled with traditional biochemical analysis methods, proteomics is not hypothesis-driven: identifying and quantifying hundreds to thousands of proteins is possible without any prior assumption on the nature of the biomarker nor on the mechanism of action (Monsinjon and Knigge, 2007; Kovacevic, 2009). Therefore, ecotoxicoproteomics findings contribute to discover unexpected relationships and

revealing associations at superior levels that have not been described earlier and that in their turn can lead to the development of new hypothesis.

Regarding biomarker discovery, the effects of environmental chemicals have traditionally been detected by monitoring biomarkers of exposure or biomarkers of effect. According to the traditional approach, environmental monitoring is based on the perspective of a single-parameter measurement. However, there are some issues regarding the single-parameter biomarker-based assessment that must be considered. The stress response produced in living organism by pollutants and other stressing agents can have a wide range of protein expression patterns, depending on the species, the stage of maturity of the individual and the type and severity of the stressor. Although single parameter biomarkers may be highly specific, their robustness is limited, as they cannot discriminate changes induced by toxicant exposure from "natural" occurring variations, or their parameter variations may be induced by inter-individual differences (Amelina et al., 2007). The direct corollary is that a single biomarker cannot be capable of adequately inform about the effects of the cumulative exposure to different environmental stressors, so a multiple biomarker-based approach is more indicated in environmental monitoring (Lam, 2009). Proteomics-based methods allow the global analysis of cellular constituents, providing molecular signatures that could overcome the disadvantages of single-parameter biomarkers. The high-throughput approach of proteomics allows the evaluation of the effects of exposure to a chemical or a stressor in the whole expression pattern; the detection of cumulative effects of multiple contaminants on individual biological pathways is also possible. The selection of proteins identified from altered (over-expressed or under-expressed) molecular expression

patterns with respect to the pattern identifying the normal condition, constitute the so-called “protein expression signatures” (PES) and represent the new candidate molecular biomarkers patterns of ecotoxicity that, once validated for their robustness, can be used to develop high-throughput screening tests like biosensors and *in situ* bioassays to predict toxicity for environmental monitoring and quality seafood assessment (Aardema and MacGregor, 2002). One feature of PES is that they can be used as biomarkers even without the need of protein identification (Monsinjon and Knigge, 2007); this permits to obviate the problem in ecotoxicoproteomics that a reliable identification of novel proteins is rather difficult in some cases, because of the lacking of protein primary structure and genome information for some organisms. However, a selection of biochemical indicators of stress and metabolism running for new candidate biomarkers have been already identified (Apraiz and Cristobal, 2006; Brun et al., 2008; Chora et al., 2010; Berg et al, 2011).

Most of the studies on ecotoxicology carried out so far were focused only on the acute response that organisms settle after short-term exposure. Nevertheless, there is a clear need for better tools to study subchronic concentrations, long-term toxic exposure and to evaluate toxic mixtures. there are still a lot of uncertainties linked to the toxicological assessment of contaminants, and even worst, synergistic or antagonistic effects of different contaminants.

The information deriving from the protein expression signatures (PES) provides valuable help to understand the mechanism of action (MoA) of the stressor, because stress-induced changes at molecular levels occur at toxicant concentrations usually lower than those causing effects at physiological level of an organism; this helps to understand

the early molecular events occurring in toxicant responses. Moreover, proteomics offers also the opportunity to evaluate cellular molecular response to exposure to toxicant at sub-pathological levels, thus making studies on long-term exposure and predictions of toxicity on a large-time scale. Partial understanding of the mechanism of action have been reached with a proteomic approach in study cases with endocrine disruptors like steroids (Martyniuk et al., 2009) and cadmium (Ling et al., 2009). In some cases, understanding the mechanism by which an organism adapts to long-term exposure and the molecular basis for the reduced toxicity of chronic exposure has been possible (Silvestre et al., 2006). It is remarkable, considering that most of the ecotoxicology studies carried out so far were focused only on the acute response that organisms settle after short-term exposure and there is a need for better tools to study subchronic concentrations or long-term toxic exposure. Interestingly, in these studies some altered PES described the expected protein products according to the model of differential genetic expression inferred by previous transcriptomics investigations, confirming and validating their conclusions (Momose and Iwahashi, 2001; Poynton et al., 2008).

In classical ecotoxicology, the effects of the stressor on the organisms and at higher levels of biological organization have been evaluated at physiologic, behavioural and community levels. Different behavioural aspects such as feeding rates, growth performance, reproduction and survival have been monitored as endpoints for the effects of the environmental stressor. Similarly, also several biochemical parameters like hormones, enzymes, regulatory and effecting proteins have been monitored as biomarkers in affected organisms. The results deriving from the combinatory approach of proteomics, transcriptomics and metabolomics provide a complete information on the

molecular determinants (genes) that are induced or suppressed by the stressor exposure, the expression levels of their products (proteins) plus the type and amounts of degradation products deriving from protein action (metabolites) (Bundy et al., 2009). Taking together the molecular data and the knowledge of the phenotypic effect of stress in an holistic approach, this will help understand the complete mechanism of action of a stressor, the intensity of its impact over organisms and make predictions on the dynamics of the communities and populations exposed (Heckman et al., 2008).

5.7 EcotoxicoProteomics Applications

The use of proteomics in environmental toxicology has been pioneered by a number of groups (Adams et al., 1989; Witzmann et al., 1995; Shepard and Bradley, 2000; Shepard et al., 2000; Vido et al., 2001), and the number of publications devoted to the subject in a variety of different settings is steadily increasing (Dowling and Sheehan, 2006; López, 2007; Sheehan, 2007; Nesatyy and Suter, 2008; Piñeiro et al., 2010).

Proteomics technologies have been 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 (*M. edulis*, *D polymorpha*) (Apraiz and Cristobal, 2006; Mc Donagh and Sheehan, 2007 and 2008; Riva et al., 2011), clams (Dowling et al., 2006) and oysters (meiller and Bradley, 2002) for their peculiarity of being “sentinel species” from both environmental and seafood point of view. But there are also other works highlighting that other species may be chosen as sentinel species, such as rainbow trout (*Onchorynchus mykis*s) (Albertsson et al., 2008) and salmon (*Salmo salar*) (Søfteland et al., 2011), Atlantic cod (*Gadus morhua*) (Pérez-Casanova et al., 2008; Beg et al., 2011), flatfish (*Limanda limanda*) (Ward et al, 2006), and

the fish model zebrafish (*Danio rerio*) (Kültz et al., 2007; Riva et al., 2011). A compilation of the most recent studies on the use of proteomics as a tool for evaluating climate change and environmental ecotoxicity is reported in Table 5.4.

Protein expression patterns (PES) have been determined for exposures to metals (Shepard and Bradley, 2000; Shepard et al., 2000; Meiller and Bradley, 2002); polychlorinated biphenils (Shepard et al., 2000; Berg et al., 2011); polyaromatic hydrocarbons (Knigge et al., 2004), physic conditions like hyperoxia (Gardestrom et al., 2007), salinity and temperature (Kimmel and Bradley, 2001); .

Recent environmental proteomics studies have been focused on the effects of environmental contaminants as brominated flame retardants hexabromocyclododecane (HBCD) and tetrabromobisphenol A (TBBPA) (Kling and Förlin, 2009), organophosphate (OP) and carbamate (CM) pesticides (Alves de Almeida et al, 2008) and lastly, growth promoters (trenbelone) employed in aquaculture (Schultz et al., 2008). Interestingly, some gender-specific responses emerged during the treatment with HBCD and TBBPA, peroxiredoxin 6 being up-regulated in males and iron regulatory protein 1 down-regulated in females (Kling et al., 2008). Another work studying the effects of the exposure to benzo(α)pyrene on zebra mussel (*D. polymorpha*), reported a complex response pattern in protein expression signatures with marked gender differences between exposed male and female mussels (Riva et al., 2011). Similarly, gender differences were reported also in males and females of rare minnow (*Gobiocypris rarus*) exposed to perfluorooctanoic acid (Wei at al., 2008). This finding are remarkable, considering that there is a lack of knowledge regarding the role of gender in proteome response in the ecotoxiproteomic field.

Most of proteins and enzymes affected by environmental pollutant and stressing conditions belong to the following compartments or biochemical pathways: cytoskeleton (actin, α2- and β-tubulin), carbohydrate, aminoacid and lipid metabolism (enolase, aldehyde dehydrogenase, phenylalanine hydroxylase), detoxification (glutathione peroxidase, alcohol dehydrogenase), energetic and respiratory pathways (ATP synthase α and β, cytochrome c oxidase), chaperone proteins (heat shock proteins 70 and 71, hsp gp96), protein degradation and iron-metabolism (transferrin, ferritin). In general, ecotoxicproteomics approaches are mainly based on identifying post-translational modifications (PTMs) of proteins, such as phosphorylation and glycosylation, considering that the main biological molecular targets of many ecotoxic compounds like pesticides and organotin compounds or toxins are mainly hydrolase (esterase, phosphatase) enzymes (Kröger et al., 2002; Campàs and Marty, 2007), as well as the investigation of redox-based post-translational protein modifications like ubiquitination and carboxylation (Chora et al., 2008 and 2010). Exposure to pollutants or stressors not only causes changes in the protein expression patterns, but also significantly decreases the number of detected spots. Exposure to toxic compounds determines the activation of cellular labelling of protein via ubiquitination and carbonylation mechanisms and protein removal from cells by proteolysis via the ubiquitin–proteasome pathway (UPP). Two interesting works on *Ruditapes decussatus* focused on the changes in protein ubiquitination and carbonylation patterns induced in clams *Ruditapes decussatus* by exposition to toxic substances like cadmium (Chora et al., 2008) and nonylphenol (NP) (Chora et al., 2010). In both cases, gills and digestive gland showed different ubiquitinated and carbonylated protein expression patterns in treated and control individuals, showing that some gill and

digestive gland proteins are specifically targeted for either ubiquitination or carbonylation in response to NP or Cd exposure and that ubiquitination and carbonylation are independent processes.

Also the extensive use of nanomaterials and nanoparticles and their release in the environment have caused many concerns about their potential hazards to the aquatic environment. At present, little is known about the toxicity of NPs to the aquatic environment and organisms. A proteomics approach can be successfully employed to shed light over the mechanisms of toxicity of nanoparticles in biological systems. It is worth reporting the study on the effects of Au-NPs combined with menadione in mussels using proteomics tools (Tedesco et al., 2008). This study confirmed at protein level that au-NPs may cause oxidative stress with production of reactive oxygen species (ROS) and activation of the process of targeting specific proteins for degradation (ubiquitination and carbonylation). Also, the combined exposition to Au-NPs-menadione alters the levels of ubiquitination and carbonylation with treatment and tissue-specific differences.

Table 5.4 Overview of the studies reporting alterations in metabolic pathways determined by stressing agents and pollutants and the corresponding chemical compound class.

Stressing agent or condition	Alterated enzymatic processes	Studied animal species	References
Exposure to marine pollutants (BisphenolA, Diallyl phtalate, 2,2'-4,4'-tetrabromodiphenyl ether)	α-oxidation (hydroxiacid oxidase), β-oxidation (catalase, enoyl-CoA hydratase), Cytoskeleton (β-tubulin), Detoxication (alcohol dehydrogenase, GST, Mn-SOD), Energetic and respiratory pathways (ATP synthase β subunit, cytochrome C oxidase subunit II), Metabolic pathways (aldehyde dehydrogenase 4A1, carbonic anhydrase, cytochrome P450 2A6, phospholipase A).	Mussel (M*ytilus. edulis*) Peroxisomes	Apraiz et al. (2007)
	Detoxification (peroxysome glutathione S-transferase), Protein degradation(cathepsin B like cysteine proteinase), Chaperone proteins (heat shock proteins 70 and 71), Metabolic pathways (acyl-CoA dehydrogenase, phosphoglycerate kinase, aldehyde dehydrogenase 1A2), Others (AMP binding protein, fascin-like protein, peroxisome biogenesis factor 1).	Mussel (*Mytilus edulis*) Digestive gland	Amelina et al. (2007)
Benzo(α)pyrene	Cell signaling pathways (dual specificity phosphatase DUPD1), metabolic process (N-acetyltransferase 8-like protein, aspartate aminotransferase), cytoskeleton (actin cytoplasmic 1, actin), cell redox homeostasis (alcohol dehydrogenase class-3, peroxiredoxin-6).	Zebra mussel (*Dreissena polymorpha*) Gill tissue cytosolic fraction	Riva et al. (2011)
Polychlorinated biphenyls (PCB 153)	Carbohydrate, aminoacid and lipid metabolism (enolase, fructose-bisphosphate aldolase class I, l-Lactate dehydrogenase, malate dehydrogenase, ribose-phosphate pyrophosphokinase, creatine kinase, tryptophanyl-tRNA synthetase, glutaminase, acylglycerol lipase), cofactors and energy metabolism (nicotinamide phosphoribosyltransferase, pyridoxine kinase, NADH dehydrogenase (ubiquinone) Fe–S protein 1, NADH dehydrogenase (ubiquinone) Fe–S protein 3,) cytoskeleton (protein phosphatase 1 catalytic subunit, α-tubulin, villin 2 (ezrin) (ezrin/radixin/moesin, ERM-family), actin beta/gamma 1, β-tubulin), cellular growth, cycle and death (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (=14-3-3 protein), protein synthesis and degradation (ubiquitin carboxyl-terminal hydrolase L5, 26S proteasome regulatory subunit N9, proteasome activator subunit 3 (PA28 gamma)), axon guidance (dihydropyrimidinase-like 2 (=collapsin response mediator protein 2, CRMP-2)), neurotransmitters synthesis (pyridoxal kinase), protection against axonal degeneration (nicotinamide phosphoribosyl-transferase), synaptic plasticity control (protein phosphatase 1), Notch signaling pathways and endosomal functions (deltex, Ras-related protein Rab14 and sorting nexin 6).	Atlantic cod (*Gadus morhua*) Brain	Berg et al. (2011)
Perfluorooctanoic acid	Intracellular fatty acid transport (fatty acid binding protein 10 and 3, muscle fatty acid binding protein), lipid metabolism (phytanoyl-CoA dioxygenase), aminoacid metabolism (phenylalanine hydroxylase), protein synthesis and metabolism ((prosome, macropain) 26S subunit ATPase 2), muscle contraction (guanidinoacetate *N*-methyltransferase), oxidative stress (glutathione peroxidase 1, peroxiredoxin Zgc:92891), macromolecule catabolism (galactokinase 1, 6-pyruvoyl	Rare minnow (*Gobiocypris rarus*) Liver	Wei et al. (2008)

	tetrahydropterin synthase isoform), oxidative phosphorylation (ATP synthase H^+ transporting mitochondrial F0), cytoskeleton (β-actin), cell cycle (vertebrate cyclin G associated kinase, putative translationally controlled tumor protein), response to oxidative stress (methionine sulfoxide reductase B, glutathione *S*-transferase), maintenance of intracellular Ca^{2+} homeostasis (regucalcin), mitochondrial function, others (ribosomal protein large P0).		
Stressing agent or condition	**Alterated enzymatic processes**	**Studied animal species**	**References**
Flame retardants (BDE47, BDE153 and BDE154)	Glucose homeostasis (glyoxylate reductase/hydroxypyruvate reductase (GRHPR), dihydrolipoyl dehydrogenase (DLD)), cell cycle control (glucose-regulated protein 94 (GRP 94)), proliferation signal pathways (calmodulin 2 (CaM2)).	Atlantic salmon (*Salmo salar* L.) Hepatocytes	Søfteland et al. (2011)
Sewage effluent	Betaine aldehyde dehydrogenase, lactate dehydrogenase, mitochondrial ATP synthase α-subunit and carbonyl reductase/20b-hydroxysteroid dehydrogenase.	Rainbow trout (*Onchorynchus mykiss*) Liver	Albertsson et al. (2008)
Oxidative Stress (Menadione)	Proteins with free-thiols: proteins folding and re-folding (protein disulphide isomerase (PDI), calreticulin), chaperone proteins (hsp gp96), heavy metal binding protein, cellular control (protease serine 1). Proteins with disulphide bonds: cytoskeleton (α2-tubulin, β-tubulin, gelsolin), proteins folding (protein disulphide isomerase (PDI)), vesicular trafficking (GDP-dissociation inhibitor), detoxification (glutathione transferase Pi (GST P1-1)), cellular control (RNA binding protein), enolase and transferring.	Blue mussel (*Mytilus edulis*) Gill tissue	McDonagh and Sheehan (2008) McDonagh and Sheehan (2007)
Chronic Hypoxia	Energy metabolism and electron transport chain (Cytochrome c oxidase I (Ccox1), cytochrome c oxidase subunit 2 (Ccox2), cytochrome c oxidase III (Ccox3), cytochrome b (cytb), ferritin).	Grass shrimp (*Palaemonetes pugio*) Hepatopancreatic mitochondria	Brouwer et al. (2008)
Cyanobacteria toxins (microcystin-LR)	Phosphatase PP1 and PP2A, Selenium binding protein 1, Keratin 18 type 1, Raf kinase inhibitor protein, Acidic ribosomal phosphoprotein P0, Natural killer enhancing factor, Hypoxanthine guanine phosphoribosyl transferase, F-actin capping protein A, Actin capping protein B subunit, Enolase, 14-3-3 protein zeta/delta (RKIP-1). Protein translation and maturation (protein disulfide isomerase A4 and A6, glucose regulated protein 78 kDa, heat shock cognate protein 71 kDa, ribosomal protein SA), Metabolism (phenylalanine hydroxilase, fumarylacetoacetase), Detoxification (thiosulfate sulfurtransferase, 40S cytochrome b5), Oxidative stress response (prohibitin, ATP synthase mitochondrial β and d subunit, aldehyde dehydrogenase 2), Cytoskeleton (β-tubulin 4), Immunity (complement C3-1), Others (transferrin, Apolipoprotein A1).	Medaka fish (*Oryzias latipes*) Liver membrane and organelle fractions Medaka fish (*Oryzias latipes*) Liver membrane and organelle fractions	Mezhoud et al, (2007) Malècot et al. (2009)

5.8 Perspectives and Conclusions

Protection of human health is guaranteed only through assessment of environmental quality and food safety: we are what we eat and, I dare say, where we live. Assuring protection and restoration of habitats and ecosystem is possible through adequate actions of detection and monitoring of hazardous substances and the adequate understanding of their toxicology for the prevention and mitigation of adverse effects. It is clear that the future of ecological risk assessment depends, in a scale ranging from molecular to ecosystemic, from a interdisciplinary approach. A collaboration is needed among different disciplines to obtain the expected results: ecology, toxicology, oceanography, biology, molecular biology, genetics, analytical chemistry, material science, electrochemical and electronic engineering; all of them coordinated by a system biology approach.

From the one hand, the human community will soon have at her disposal a great variety of selective, sensitive, robust, rapid, easy-to-use and low-cost monitoring tools for a wide range of applications in environmental and food monitoring. Biosensors field is taking advance, both for miniaturization and performance improvement, from the continuous progress made in the recent years in several disciplines, such as science, technology and systems engineering, and from the integration of these developments. The advances in the frontier of molecular biology allows the availability of different bioreporting systems with more specificity, sensitivity and reversibility. Undoubtedly, biosensor design is receiving a boost from the use not only of DNA microarrays, but also of protein chips. These are high-throughput measurement devices that can simultaneously determine the protein presence and amount, the last expressed as

relative quantity of thousands of proteins in biological samples (McBeath and Schreiber, 2000). Protein chips have been developing (Lee et al., 2011) or testing in biomedical (Sauer et al., 2011) and biochemical or molecular biology (Martiny-Baron et al., 2011) applications. Recently, blood-derived proteins immunochips-based and proteinchip-based biosensors coupled with SPR have been tested (Yuk et al., 2007). Also the progression made in the sector in nanotechnology will allow the development of novel transducers with greater sensitivity, as well as advances in the technology fields or nano- and micro-fluidic, and nano-and micro-electronics will help reducing transducers and biosensors dimensions.

From the other hand, the rapid development of the high-throughput technologies proteomics, transcriptomics and metabolomics have changed the way ecotoxicology is practiced. In a top-down approach, organisms are considered as interacting networks (Weston and Hood, 2004). The ‘‘omics” technologies clearly provide coverage of information about genes, proteins and metabolites, that are interpreted under a integrated and interacting network perspective, which is unsurpassed compared with traditional targeted and bottom-up approaches (Hood and Perlmutter, 2004). Together with biosensors, they provide a novel high-throughput platform of data acquisition. The problem is, however, the management of these data: the use of advanced techniques coupled with bioinformatics tools, biological models and computational platforms is necessary. During the workshop “DNA microarray and Proteomics. Application to Ecotoxicology” (Joint Research Center-JRC of the European Commission in Ispra, Italy) gathering European and USA researchers, experts in DNA microarray and proteomics, ecotoxicology, molecular biology and bioinformatics discussed on how to integrate

proteomics and transcriptomics data and use them in improved risk assessment procedures (Calzolai et al., 2007). In order to develop robust biomarkers of ecotoxicology detection and assessment, the transcriptomic and proteomic communities are making significant effort to establish standards for experimental quality and data handling for gene and protein expression – the MIAME (Minimum Information About a Microarray Experiment) and MIAPE (Minimum Information About a Proteomics Experiment) standards, respectively (Davies, 2010). I daresay, this is what we are.

5.9 References

Aardema M.J., MacGregor J.T. (2002). Toxicology and genetic toxicology in the new era of ‘‘toxicogenomics’’: impact of ‘‘-omics’’ technologies. Mutation Research, 499: 13–25.

Abdullah J., Ahmad M., Karuppiah N., Heng L.W., Sidek H. (2006). Immobilization of tyrosinase in chitosan film for an optical detection of phenol. Sensors and Actuators B-Chemicals, **114:** 604–609.

Adams S.M., Shepard K.L., Greeley Jr M.S., Jimenez B.D., Ryon M.G., Shugart L.R., McCarthy J.F. (1989). The use of bioindicators for assessing the effects of pollutant stress on fish. Marine Environmental Research, 28: 459-464.

Adeloju S.B. and Sohail M. (2011). Polypyrrole-based bilayer nitrate amperometric biosensor with an integrated permselective poly-ortho-phenylenediamine layer for exclusion of inorganic interferences. Biosensors and Bioelectronics, 26: 4270-7275.

Ahmad A., Paschero A., Moore E. (2011). Amperometric immunosensors for screening of polycyclic aromatic hydrocarbons in water. Journal of Physics. Conference Series, 307: 12023-12026.

Ahn J.M., Hwang E.T., Youn C.H., Banub D.L., Kim B.C., Niazi J.H., Gu M.B. (2009). Prediction and classification of the modes of genotoxic actions using bacterial biosensors specific for DNA damages. Biosensors and Bioelectronics, 25: 767–772.

Albertsson E., Kling P., Gunnarsson L., Larsson J.D.G., Förlin L. (2008). Induction of hepatic CR20b-HSD B in rainbow trout exposed to sewage effluent. Fourteenth International Symposium on Pollutant Responses in Marine Organisms (PRIMO 14) – Environmental proteomics. Marine Environmental Research, 66: 101–103.

Alizadeh T. (2010). Comparison of different methodologies for integration of molecularly imprinted polymer and electrochemical transducer in order to develop a paraoxon voltammetric sensor. Thin Solid Films, 518: 6099-6106.

Allan I.J., Vrana B., Greenwood R., Mills G.A., Roig B., Gonzalez, C. (2006). A "toolbox" for biological and chemical monitoring requirements for the European Union's Water Framework Directive. Talanta, 69,: 302–322.

Alves de Almeida E., Vioque-Fernandez A., López-Barea J. (2008). Proteomic alterations and biomarker responses in Procambarus clarkii after controlled exposure to chlorpyrifos and carbaryl. Fourteenth International Symposium on Pollutant Responses in Marine Organisms (PRIMO 14) – Environmental proteomics. Marine Environmental Research, 66: 101–103.

Amelina H., Apraiz I., Sun W., Cristobal S. (2007). Proteomics-based method for the assessment of marine pollution using liquid chromatography coupled with two-dimensional electrophoresis. Journal of Proteome Research, 6: 2094–2104.

Apraiz I., Mi J., Cristobal S. (2006). Identification of proteomic signatures of exposure to marine pollutants in mussels (*Mytilus edulis*). Molecular and Cellular Proteomics, 5: 1274–1285.

Arduini F., Amine A., Moscone D., Palleschi G. (2010). Biosensors based on cholinesterase inhibition for insectidices, nerve agents and aflatoxin B1 detection (review). Microchimica Acta, 170: 193-214.

Arias-Barreiro C.R., Okazaki K., Koutsaftis A., Inayat-Hussain S.H., Tani A., Katsuhara M., Kimbara K., Mor I.C. (2010). A bacterial biosensor for oxidative stress using the constitutively expressed redox-sensitive protein roGFP2. Sensors, 10: 6290-6306.

Arnouk H., Merkley M.A., Podolsky R.H., Stoppler H., Santos C., Álvarez M., Mariategui J., Ferris D., Lee J.R, Dynan W.S. (2009). Characterization of molecular markers indicative of cervical cancer progression. Proteomics Clinical Applications, 3: 516–527.

Aune T., Larsen S., Aasen J.A.B., Rehmann N., Satake M., Hess P. (2007). Relative toxicity of dinophysistoxin-2 (DTX-2) compared with okadaic acid, based on acute intraperitoneal toxicity in mice. Toxicon, 49: 1-7.

Badihi-Mossberg M., Buchner V., Rishpon J. (2007). Electrochemical biosensors for pollutants in the environment. Electroanalysis, 19: 2015-2028.

Banik R.M., Mayank; Prakash R. and Upadhyay S.N. (2008). Microbial biosensor based on whole cell of *Pseudomonas* sp. for online measurement of p-nitrophenol. Sensors Actuators B, Vol.131, No.1: 295-300.

Barrett J., Brophy P.M., Hamilton J.V. (2005). Analysing proteomic data. International Journal of Parasitology, 35: 543–553.

Behzadian F., Barjeste H., Hosseinkhani S., Zare A.R. (2011). Construction and characterization of *Escherichia coli* whole-cell biosensors for toluene and related compounds. Current Microbiology, 62: 690-696.

Ben-Yoav H., Biran A., Pedahzur R., Belkin S., Buchinger S., Reifferscheid G., Shacham-Diamand Y. (2009). A whole cell electrochemical biosensor for water genotoxicity bio-detection. Electrochimica Acta, 54: 6113-6118.

Berezhetskyy A.L., Durrieu C., Nguyen-Ngoc H., Chovelon J.M., Dzyadevych S.V., Tran-Minh C. (2007). Conductometric biosensor based on whole-cell microalgae for assessment of heavy metals in wastewater. Biopolymers and Cell, 23: 511–518.

Berg K., Puntervoll P., Klungsøyr J., Goksøyr A. (2011). Brain proteome alterations of Atlantic cod (*Gadus morhua*) exposed to PCB 153. Aquatic Toxicology, 105: 206– 217.

BioCop, Integrated Project Funded by the European Commission under the Food Quality and Safety Priority Thematic Area, www.biocop.org/theprojetpackages.html.

Biran I., Rissin D.M., Ron E.Z., Walt D.R. (2003). Optical imaging fiber-based live bacterial cell array biosensor. Analytical Biochemistry, 315: 106–113.

Bratov A., Abramova N., Ipatov A. (2010). Recent trends in potentiometric sensor arrays – a review. Analytica Chimica Acta, 678: 149-159.

Brokordt K., Leiva N., Jeno K., Martínez G., Winkler F. (2009). Effect of allozyme heterozygosity on basal and induced levels of heat shock protein (Hsp70), in juvenile

Concholepas concholepas (Mollusca). Journal of Experimental Marine Biology and Ecology, 370: 18-26.

Brouwer M., Brown-Peterson N.J., Hoexum-Brouwer T., Manning S., Denslow N. (2008). Changes in mitochondrial gene and protein expression in grass shrimp, *Palaemonetes pugio*, exposed to chronic hypoxia. Marine Environmental Research, 66: 143–145.

Brun N.T., Bricelj M.V., MacRae T.H., Ross N.W. (2008). Heat shock protein responses in thermally stressed bay scallops, *Agropecten irradians*, and sea scallop, *Placopecten magellanicus*. Journal of experimental marine biology and ecology, 358: 151-162.

Bucur B., Fournier D., Danet A., Marty J.L. (2006). Biosensors based on highly sensitive acetylcholinesterases for enhanced carbamate insecticides detection. Analytica Chimica Acta, 562: 115-121.

Bundy J.G., Davey M.P., Viant M.R. (2009). Environmental metabolomics: a critical review and future perspectives. Metabolomics, 5: 3–21.

Calzolai L., Ansorge W., Calabrese E., Denslow N., Part P., Lettieri T. (2007). Transcriptomics and proteomics. Applications to ecotoxicology. Comparative Biochemistry and Physiology, Part D: Genomics and Proteomics, 2: 245-249.

Campàs M., Marty J.L (2007). Enzyme sensor for the electrochemical detection of the marine toxin okadaic acid. Analytica Chimica Acta, 12: 87-93.

Campàs M., De la Iglesia P., Le Berre M., Kane M., Diogéne J., Marty J.L. (2008). Enzymatic recycling-based amperometric immunosensor for the ultrasensitive detection of okadaic acid in shellfish. Biosensors and Bioelectronics, 24: 716-722.

Campbell K., Stewart L.D., Doucette G.J., Fodey T.L., Haughey S.A., Vilarino N., Kawatsu K., Elliot C.T. (2007). Assessment of specific binding proteins suitable for the detection of paralytic shellfish poisons using optical biosensor technology. Analytical Chemistry, 79: 5906-5914.

Campbell K., Rawn D.F.K., Niedzwiadek B., Elliott C.T., (2011a). Paralytic shellfish poisoning (PSP) toxins binders for optical biosensor technology: problems and possibilities for the future: a review. Food Additives and Contaminants, 28: 711-725.

Campbell K., McGrath T., Sjölander S., Hanson T., Tidare M., Jansson Ö., Moberg A., Mooney M., Elliott C.T., Buijs J. (2011b). Use of a novel micro-fluidic device to create arrays for multiplex analysis of large and small molecular weight compounds by surface plasmon resonance. Biosensors and Bioelectronics, 26: 3029-3036.

Campbell K., Vilariño N., Botana L.M., Elliott C.T. (2011c). A European perspective on progress in moving away from the mouse bioassay for marine-toxin analysis. TrAC Trends in Analytical Chemistry, 30: 239-253.

Can F., Ozoner S.K., Ergenekon P., Erhan E. (2011). Amperometric nitrate biosensor based on Carbon nanotube/Polypyrrole/Nitrate reductase biofilm electrode. Material Science and Engineering: C, Accepted manuscript, In Press.

Carralero V., Mena M.L., Gonzalez-Cortes A., Yanez-Sedeno P., Pingarron J.M. (2006). Development of a high analytical performance-tyrosinase biosensor based on a composite graphite–Teflon electrode modified with gold nanoparticles. Biosensors and Bioelectronics, 22: 730–736.

Chakraborty T., Babu P.G., Alam A., Chaudhari A. (2008). GFP expressing bacterial biosensor to measure lead contamination in aquatic environment. Current Science, 94: 800–805.

Chandra Boro R., Kaushal J., Nangia Y., Wangoo N., Bhasin A., Suri C.N. (2011). Gold nanoparticles catalyzed chemiluminescence immunoassay for detection of herbicide 2,4-dichlorophenoxyacetic acid. Analyst, 136: 2125-2130.

Chao H.P. and Lee W.C. (2000). A bioelectrode for penicillin detection based on gluten-membrane-entrapped microbial cells. Biotechnology and Applied Biochemistry, 32: 9–14.

Chen J. and Jin Y. (2010). Sensitive phenol determination based on co-modifying tyrosinase and palygorskite on glassy carbon electrode. Microchimica Acta, 169: 249–254.

Chen L., Zeng G., Zhang Y., Tang L., Huang D., Liu C., Pang Y., Luo J. (2010). Trace detection of picloram using an electrochemical immunosensor based on three-dimensional gold nanoclusters. Analytical Biochemistry, 407: 172–179.

Chen P.J., Su C.H., Tseng C.Y., Tan S.W., Cheng C.H. (2011). Toxicity assessments of nanoscale zerovalent iron and its oxidation products in medaka (*Oryzias latipes*) fish. Marine Pollution Bulletin, 63: 339-346.

Cheung C.C.C., Zheng Z.J., Li A.M.Y, Richardson B.J., Lam P.K.S. (2001). Relationships between tissue concentration of polycyclic hydrocarbons and antioxidative responses of marine mussels, *Perna viridis*. Aquatic Toxicology, 52: 189-203.

Cheung C.C.C., Zheng G.J., Lam P.K.S., Richardson B.J. (2002). Relationships between tissue concentrations of chlorinated hydrocarbons (polychlorinated biphenyls and chlorinated pesticides) and antioxidative responses of marine mussels, *Perna viridis*. Marine Pollution Bulletin, 45: 181–91.

Chianella I., Piletsky S.A., Tothill I.E., Chen B., Turner A.P.F. (2003). MIP-based solid phase extraction cartridges combined with MIP-based sensors for the detection of microcystin-LR. Biosensors and Bioelectronics, 18: 119-127.

Chiappini S.A., Kormes D.J., Bonetto M.C.; Sacco N. and Cortona E. (2010). A new microbial biosensor for organic water pollution based on measurement of carbon dioxide production. Sensors and Actuators B, Vol.148, No.1: 103-109.

Chong K.F.,Loh K.P., Ang K. ,Ting Y.P. (2008). Whole cell environmental biosensor on diamond. Analyst, 133: 739–743.

Chora S., McDonagh B., Sheehan D., Starita-Geribaldi M., Roméo M., Bebianno M.J. (2008). Ubiquitination and carbonylation as markers of oxidative-stress in *Ruditapes decussatus*. Marine Environmental Research, 66: 95–97.

Chora S., McDonagh B., Sheehan D., Starita-Geribaldi M., Roméo M., Bebianno M.J. (2010). Ubiquitination and carbonylation of proteins in the clam *Ruditapes decussatus*, exposed to nonylphenol using redox proteomics. Chemosphere, 81: 1212–1217.

Chouteau C., Dzyadevych S., Chovelon J.M., Durrieu C. (2004). Development of novel conductimetric biosensors based on immobilized *Chlorella vulgaris* microalgae. Biosensors and Bioelectronics, 19: 1089-1096.

Chouteau C., Dzyadevych S., Durrieu C., Chovelon J.M. (2005). A bi-enzymatic whole cell conductometric biosensor for heavy metal ions and pesticides detection in water samples. Biosensors and Bioelectronics, 21: 273–281.

Colquhoun D.R., Goldman L.R., Cole R.N., Gucek M., Mansharamani M., Witter F.R., Apelberg B.J., Halden R.U. (2009). Global screening of human cord blood proteomes for biomarkers of toxic exposure and effect. Environmental Health Perspectives, 117: 832–838.

Commission of the European Communities (2000). White paper on food safety, Brussels, 12 January 2000, COM (1999) 719 final. http://ec.europa.eu/dgs/health_consumer/library/pub/pub06_en.pdf

Connel D.W., Lam P.K.S., Richardson B.J., Wu R.S.S. (1999). Introduction to ecotoxicology. Blackwell Science, London .

Cosnier S., Innocent C., Jouanneau Y. (1994). Amperometric detection of nitrate via a nitrate reductase immobilized and electrically wired at the electrode surface. Analytical Chemistry, 66: 3198-3201.

Cosnier S., Da Silva S., Shan D., Gorgy K. (2008). Electrochemical nitrate biosensor based on poly(pyrrole–viologen) film–nitrate reductase–clay composite. Bioelectrochemistry, 74: 47-51.

Davies H. (2010). A role for “omics” technologies in food safety assessment. Food Control, 21: 1601-1610.

Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000, establishing a framework for the Community action in the field of water policy.

Directive 2008/56/EC of the European Parliament and of the Council of 17 June 2008, establishing a framework for community action in the field of marine environmental policy.

Dowling V.A. and Sheehan D. (2006). Proteomics as a route to identification of toxicity targets in environmental toxicology. Proteomics, 6: 5597–5604.

Dowling V., Hoarau P.C., Romeo M., O'Halloran J., Van Pelt F., O'Brien N., Sheehan D. (2006). Protein carbonylation and heat shock response in *Ruditapes decussatus* following p,p'-dichlorodiphenyldichloroethylene (DDE) exposure: a proteomic approach reveals that DDE causes oxidative stress. Aquatic Toxicology, 77: 11-18.

Du D., Chen S., Cai J., Zhang A. (2008a). Electrochemical pesticide sensitivity test using acetylcholinesterase biosensor based on colloidal gold nanoparticle modified sol-gel interface. Talanta, 74: 766-772.

Du D., Chen S., Songa D., Li H., Chen X. (2008b). Development of acetylcholinesterase biosensor based on CdTe quantum dots/gold nanoparticles modified chitosan microspheres interface. Biosensors and Bioelectronics, 24: 475-479.

Du D., Chen W., Zhang W., Liu D., Li H., Lin Y. (2010). Covalent coupling of organophosphorus hydrolase loaded quantum dots to carbon nanotube/Au nanocomposite for enhanced detection of methyl parathion. Biosensors and Bioelectronics, 25: 1370–1375.

Dumont V., Huet A.C., Traynor I., Elliott C., Delahaut P. (2006). A surface plasmon resonance biosensor assay for the simultaneous determination of thiamphenicol, florefenicol, florefenicol amine and chloramphenicol residues in shrimps. Analytica Chimica Acta, 567: 179-183.

Durand M.J., Thouand G., Dancheva-Ivanova T., Vachon P., DuBow M. (2003). Specific detection of organotin compounds with a recombinant luminescent bacteria. Chemosphere, 52: 103-111.

Eguilaz M., Moreno-Guzman M., Campuzano S., Gonzalez-Cortes A., Yanez-Sedeno P., Pingarron J.M. (2010). An electrochemical immunosensor for testosterone using functionalized magnetic beads and screen-printed carbon electrodes. Biosensors and Bioelectronics, 26: 517–522.

Eltzov E., Marks R.S., Voost S., Wullings B.A., Heringa M.B. (2009). Flow-through real time bacterial biosensor for toxic compounds in water. Sensors and Actuators B-Chemicals, 142: 11–18.

Eltzov E. and Marks R.S. (2011). Whole cell aquatic biosensors. Analytical and Bioanalytical Chemistry, 400: 895-913.

Engbloom S.O. (1998). The phosphate sensor. Biosensors and Bioelectronics, 13: 981-994.

Engenheiro E.L., Hankar, P.K., Sousa J.P., Lemos M.F., Weeks J.M., Soares A.M.V.M. (2005). Influence of dimethoate on acetylcholinesterase activity and locomotor function in terrestrial isopods. Environmental Toxicology and Chemistry, 24: 603–609.

Farre M., Martínez E., Ramon J., Navarro A., Radjenovic J., Mauriz E., Lechuga L., Marco M.P., Barcelo D. (2007). Part per trillion determination of atrazine in natural water samples by a surface plasmon resonance immunosensor. Analytical and Bioanalytical Chemistry, 388: 207–214.

Fernández M.L., Richard D.J., Cernbella A.D. (2003). *In vivo* bioassays for phycotoxins. In: Manual of harmful marine microalgae. Monographs of Oceanic methodology. Editors: Hallegraeff G.M., Anderson D.M., Cernbella A.D., 11: 947-380.

Fiorentino D., Gallone A., Fiocco D., Palazzo G., Mallardi A. (2010). Mushroom tyrosinase in polyelectrolyte multilayers as an optical biosensor for *o*-diphenols. Biosensors and Bioelectronics, 25: 2033-2037

First Joint Ministerial Meeting of the Helsinki and OSPAR Commissions (JMM), Bremen: 25 - 26 june 2003. Agenda item 6, Annex 5: Statement on the Ecosystem Approach to the Management of Human Activities “Towards an Ecosystem Approach to the Management of Human Activities”.

Fitzpatrick P.J., O'Halloran J., Sheehan D., Walsh A.R. (1997). Assessment of a glutathione S-transferase and related proteins in the gill and digestive gland of *Mytilus edulis* (L.), as potential organic pollution biomarkers. Biomarkers, 2: 51–56.

Fonfría E.S., Vilariño N., Campbell K., Elliott C., Haughey S.A., Ben-Gigirey B., Vieites J.M., Kawatsu K., Botana L.M. (2007). Paralytic shellfish poisoning detection by surface plasmon resonance-based biosensors in shellfish matrixes. Analytical Chemistry, 79: 6303-6311.

Food and Agriculture Organization (FAO), FAO expert workshop on climate change implications for fisheries and aquaculture. Rome, 7−9 April 2008. FAO Fisheries Report No. 870.

Food and Agriculture Organization (FAO) (2009). Climate change: implications for food safety.

Gäberlein S., Spener F., Zaborosch C.(2000). Microbial and cytoplasmic membrane-based potentiometric biosensors for direct determination of organophosphorus insecticides. Applied Microbiology and Biotechnology, 54: 652–658.

Gan N., Yang X., Xie D., Wu Y., Wen W. (2010). A disposable organophosphorus pesticides enzyme biosensor based on magnetic composite nano-particles modified screen printed carbon electrode. Sensors, 10: 625-638.

Ganesana M., Istarnboulie G., Marty J.L., Noguer T., Andreescu S. (2011). Site-specific immobilization of a (His)6-tagged acetylcholinesterase on nickel nanoparticles for highly sensitive toxicity biosensors. Biosensors and Bioelectronics, In Press, Corrected Proof.

Gardestrom J., Elfwing T., Lof M., Tedengren M., Davenport J.L., Davenport J. (2007). The effect of thermal stress on protein composition in dogwhelks (*Nucella lapillus*) under non-normoxic and hyperoxic conditions. Comparative Biochemistry and Physiology-Part A: Molecular and Integrative Physiology, 148: 869–875.

Gavlasova P., Kuncova G., Kochankova L., Mackova M. (2008). Whole cell biosensor for polychlorinated byphenil analysis based on optical detection. International Biodeterioration and Biodegradation, 62: 304-312.

Gilbert L., Browning S., Jenkins A.T.A. and Hart J.P. (2010). Studies towards an amperometric phosphate ion biosensor for urine and water analysis. Microchimica Acta, Vol.170, No.3-4: pp.331–336.

Girotti S., Bolelli L., Fini F., Monari M., Andreani G., Carpene E. (2006). Trace metals in arcid clam *Scapharca inaequivalvis*: effects of molluskan extracts on bioluminescent bacteria. Chemosphere, 65: 627-633.

Gomiero A., Pampanin D.M., Bjornstad A., Larsen B.K., Provan F., Lyng E., Andersen O.K. (2006). An ecotoxicoproteomic approach (SELDI-TOF mass spectrometry) to biomarker discovery in crab exposed to pollutants under laboratory conditions. Aquatic Toxicology, 78: S34–S41.

Gong J., Wang L., Zhang L. (2009). Electrochemical biosensing of methyl parathion pesticide based on acetylcholinesterase immobilized onto Au–polypyrrole interlaced network-like nanocomposite. Biosensor Bioelectronic, 24: 2285-2288.

Gu M.B., Mitchell R.J., Kim B.C. (2004). Whole-cell-based biosensors for environmental biomonitoring and application. Advances in Biochemical Engineering Biotechnology, 87:269-305.

Guedri H and Durrieu C. (2008). A self-assembled monolayers based conductometric algal whole cell biosensor for water monitoring. Microchimica Acta, 163: 179–184.

Gueuné H., Thouand G., Durand M.J. (2009). A new bioassay for the inspection and identification of TBT-containing antifouling paint. Marine Pollution Bulletin, 58: 1734-1738.

Habauzit D., Chopineau J., Roig B. (2007). SPR-based biosensors: a tool for biodetection of hormonal compounds. Analytical and Bioanalytical Chemistry, 387: 1215-1223.

Hakkila K., Green T., Leskinen P., Ivask A., Marks R., Virta M. (2004). Detection of bioavailable heavy metals in EILATox-Oregon samples using luminescent whole-cell bacterial sensors in suspension or immobilized onto fiber-optic tips. Journal of Applied Toxicology, 24: 333-342.

Hamada-Sato N., Minamitani N., Inaba Y., Nagashima Y., Kobayashi T., Imada C., Watanabe E. (2004). Development of Amperometric Sensor System for Measurement of Diarrheic Shellfish Poisoning (DSP) Toxin, Okadaic Acid (OA). Sensor Materials, 16: 99-107**.**

Han T.S., Sasaki S., Yano K., Ikebukuro K., Atsushi K., Nagamune T., Karube I. (2002). Flow injection microbial trichloroethylene sensor. Talanta, 57: 271–276.

Haughey S.A., Campbell K., Yakes B.J., Prezioso S.M., DeGrasse S.L., Kawatsu K., Elliott C.T. (2011). Comparison of biosensor platforms for surface plasmon resonance based detection of paralytic shellfish toxins. Talanta, 85: 519-526.

Hayat A., Barthelmebs L., Marty J.L. (2011a). Enzyme-linked immunosensor based on super paramagnetic nanobeads for easy and rapid detection of okadaic acid. Analytica Chimica Acta, 690: 248-252.

Hayat A., Barthelmebs L., Sassaolas A., Marty J.L. (2011b). An electrochemical immunosensor based on covalent immobilization of okadaic acid onto screen printed carbon electrode via diazotization-coupling reaction. Talanta, 85: 513-518.

Heckmann L.H., Sibly R.M., Connon R., Hooper H.L., Hutchinson T.H., Maund S.J, Hill C.J, Bouetard A., Callaghan A. (2008). Systems biology meets stress ecology: linking molecular and organismal stress responses in *Daphnia magna*. Genome Biology, 9: R40.

Heineman W.R. and Jensen W.B. (2006). Leland C. Clark Jr. (1918–2005). Biosensors and Bioelectronics, 21: 1403-1404.

Hernroth B., Baden S., Thorndyke M., Dupont S. (2011). Immune suppression of the echinoderm *Asterias rubens* (L.) following long-term ocean acidification. Aquatic Toxicology, 103: 222-224.

Hervas Perez J.P., Sanchez-Paniagua Lopez M., Lopez-Cabarcos E., Lopez-Ruiz B. (2006). Amperometric tyrosinase biosensor based on polyacrylamide microgels. Biosensors and Bioelectronics, 22: 429–439.

Hleli S., Martelet C., Abdelghani A., Bessueille F., Errachid A., Samitier J., Burais N., Jaffrezic-Renault N. (2006). Atrazine analysis using an impedimetric immunosensor based on mixed biotinylated self-assembled monolayer. Sensors Actuators B, 113: 711-717.

Hnaien M., Lagarde F., Bausells J., Errachid A., Jaffrezic-Renault N. (2011). A new bacterial biosensor for trichloroethylene detection based on a three dimensional carbon nanotubes bioarchitecture. Analytical and Bioanalytical Chemistry, 400: 1083-1092.

Hogstrand C., Balesaria S., Glover C.N. (2002). Application of genomics and proteomics for study of the integrated response to zinc exposure in a non-model fish species, the rainbow trout. Comparative Biochemistry and Physiology-Part B: Biochemistry and Molecular Biology, 133: 523–535.

Homola J., Yee S.S., Gauglitz G. (1999). Surface plasmon resonance sensors: review. Sensors and Actuators B-Chemicals, 54, 3-15.

Hood L. and Perlmutter R.M. (2004). The impact of systems approaches on biological problems in drug discovery. Nature Biotechnology, 22: 1215–1217.

Horry H., Charrier T., Durand M.J., Vrignaud B., Picart P., Daniel P., Thouand G. (2007). Technological conception of an optical biosensor with a disposable card for use with bioluminescent bacteria. Sensors and Actuators B: Chemicals, 122: 527-534.

Hou Y., Tlili C., Jaffrezic-Renault N., Zhang A., Martelet C., Ponsonnet L., Errachid A., Samitier J., Bausells J. (2004). Study of mixed Langmuir–Blodgett films of immunoglobulin G/amphiphile and their application for immunosensor engineering. Biosensors and Bioelectronics 20: 1126-1133.

Huet A.C., Charlier C., Singh G., Godefroy S.B., Leivo J., Vehniainen M., Nielen M.W., Weigel S., Delahaut P. (2008). Biosensor immunoassay for (fluoro)quinolone antibiotics in fish, egg and poultry meat. In: van Ginkel L.A., Bergwerff A.A. (eds) EuroResidue VI, Egmond aan Zee, Netherlands, 19–21 May 2008, p 651.

Huet A.C., Charlier C., Weigel S., Godefroy S.B., Delahaut P. (2009). Validation of an optical surface plasmon resonance biosensor assay for screening (fluoro)quinolones in egg, fish and poultry. Food Addititional Contaminant, 26: 1341-1347.

Hwang E.T., Ahn J.M., Kim B.C., Gu M.B. (2008). Construction of a *nrdA: luxCDABE* Fusion and its use in *Escherichia coli* as a DNA Damage Biosensor. Sensors, 8: 1297-1307.

Im J.E., Han J.A., Kim B.K., Han J.H., Park T.S., Hwang S., Cho S.I., Lee W.Y., Kim Y.R. (2010). Electrochemical detection of estrogen hormone by immobilized estrogen receptor on Au electrode. Surface and Coatings Technology, 205: S275–S278.

Ino K., Kitagawa Y., Watanabe T., Shiku H., Koide M., Itayama T., Yasukawa T., Matsue T. (2009). Detection of hormone active chemicals using genetically engineered yeast cells and microfluidic devices with interdigitated array electrodes. Electrophoresis, 30: 3406-3412.

Islam M. D. and Tanaka M. (2004). Impacts of pollution on coastal and marine ecosystems including coastal and marine fisheries and approach for management: A review and synthesis. Marine Pollution Bulletin, 48: 624−649.

Ivanina A.V., Taylor C., Sokolova I.M. (2009). Effects of elevated temperature and cadmium exposure on stress protein response in eastern oysters *Crassostrea virginica* (Gmelin). Aquatic toxicology, 91: 245-254.

Jang E., Son K.J., Kimb B., Koh W.G. (2010). Phenol biosensor based on hydrogel microarrays entrapping tyrosinase and quantum dots. Analyst, 135: 2871–2878.

Jiang X., Li D., Xu X., Ying Y., Li Y., Ye Z., Wang J. (2008). Immunosensors for detection of pesticide residues. Biosensors and Bioelectronics, 23: 1577-1587.

Jebrail M.J., Wheeler A.R. (2010). Let's get digital: digitizing chemical biology with microfluidics. Current Opinion in Chemical Biology, 14: 574–581.

Kim B.C., Gu M.B. (2003). A bioluminescent sensor for high troughput toxicity classification. Biosensors and Bioelectronics, 18: 1015–1021.

Kim S.J., Gobi K.V., Iwasaka H., Tanaka H., Miura N. (2007). Novel miniature SPR immunosensor equipped with all-in-one multi-microchannel sensor chip for detecting low-molecular-weight analytes. Biosensors and Bioelectronics, 23: 701-707.

Kim S.J., Gobi K.V., Tanaka H., Shoyama Y., Miura N. (2008). A simple and versatile self-assembled monolayer based surface plasmon resonance immunosensor for highly sensitive detection of 2,4-D from natural water resources. Sensors and Actuators B: Chemical, 130: 281-289.

Kimmel D. G. and Bradley B. P. (2001). Specific protein responses in the calanoid copepod *Eurytemora affinis* (Poppe, 1880) to salinity and temperature variation. Journal of Experimental Marine Biology and Ecology, 266: 135–149.

Kling P., Norman A., Andersson P.L., Norrgren L., Förlin L. (2008). Gender-specific proteomic responses in zebrafish liver following exposure to a selected mixture of brominated flame retardants. Ecotoxicology and environmental safety, 71: 319-327.

Kling P. and Förlin L. (2009). Proteomic studies in zebrafish liver cells exposed to the brominated flame retardants HBCD and TBBPA. Ecotoxicology and environmental safety, 72: 1985-1993.

Knigge T., Monsinjon T., Andersen O.K. (2004). Surface-enhanced laser desorption/ionization-time of flight-mass spectrometry approach to biomarker discovery in blue mussels (*Mytilus edulis*) exposed to polyaromatic hydrocarbons and heavy metals under field conditions. Proteomics, 4: 2722–2727.

Komaitis E., Vasiliou E., Kremmydas G., Georgakopoulos D.G., Georgiou C. (2010). Development of a fully automated flow injection analyzer implementing bioluminescent biosensors for water toxicity assessment. Sensors, 10: 7089-7098.

Kotova V.Y., Manukhov I.V., Zavilgelskii G.B. (2010). Lux-Biosensors for Detection of SOS-response, heat shock, and oxidative stress. Applied Biochemistry and Microbiology, 46: 781–788.

Kovacevic R., Teodorovic I., Kaisarevic S., Planojevic I., Fa S., Dakic V., Pogrmic K., Virijevic S. (2009). First REP-LECOTOX Workshop—ecotoxicogenomics: the challenge of integrating genomics/proteomics/metabolomics into aquatic and terrestrial ecotoxicology. Environmental Science and Pollution Research, 16: 130–131.

Kröger S., Piletsky S., Turner A.P.F. (2002). Biosensors for marine pollution research, monitoring and control. Marine Pollution Bulletin, 45: 24-34.

Kröger S., Parker E.R., Metcalfe J.D., Greenwood N., Forster R.M., Sivyer D.B., Pearce D.J. (2009). Sensors for observing ecosystem status. Ocean Science, 5: 523-535.

Kültz D., Fiol D., Valkova N., Gomez-Jimenez S., Chan S.Y., Lee J. (2007). Functional genomics and proteomics of the cellular osmotic response in "non-model" organisms. Journal of Experimental Biolgy, 210: 1593-1601.

Kumar S., Kundu S., Pakshirajan K., Dasu V.V. (2008). Cephalosporins determination with a novel microbial biosensor based on permeabilized *Pseudomonas aeruginosa* whole cells. Applied Biochemistry and Biotechnology, 151: 653–664.

Kwan R.C.H., Leung H.F., Hon P.Y.T., Barford J. P., Renneberg R. (2005) A screenprinted biosensor using pyruvate oxidase for rapid determination of phosphate in synthetic wastewater Applied Microbiology and Biotechnology, Vol. 66, No.4: 377–383.

Lagarde F., Jaffrezic-Renault N. (2011). Cell-based electrochemical biosensors for water quality assessment. Analytical and Bioanalytical Chemistry, special issue "Microorganisms in Analysis", 400: 947-964.

Lam P.K.S. (2009). Use of biomarkers in environmental monitoring. Ocean and Coastal Management, 52: 348-354.

Lanyon Y.H., Tothill I.E., Mascini M. (2006). An amperometric bacterial biosensor based on gold screen-printed electrodes for the detection of benzene. Analytical Letters, 39: 669-1681.

Larsen L.H., Kjaer T., Revsbech N.P. (1997). A microscale NO_3- biosensor for environmental applications. Analytical Chemistry, 69: 3527-3531.

Lawton L.A., Edwards C., Codd G.A. (1994). Extraction and high-performance liquid chromatographic method for the determination of microcystins in raw and treated waters. Analyst, 119: 1525–1530.

Lee H.J., Villaume J., Cullen D.C., Kim B.C., Gu M.B. (2003). Monitoring and classification of PAH toxicity using an immobilized bioluminescent bacteria. Biosensors and Bioelectronics 18: 571–577.

Lee J.H., Youn C.H., Kim B.C., Gu M.B. (2007). An oxidative stress-specific bacterial cell array chip for toxicity analysis. Biosensors and Bioelectronics 22: 2223–2229.

Lee Y.J., Lyu Y.K., Choi H. N., Lee W.Y. (2007). Amperometric tyrosinase biosensor based on carbon nanotube – titania – nafion composite film. Electroanalysis, 19: 1048-1054.

Lee J.H., Park J.Y., Min K., Cha H.J., Choi S.S., Yoo, Y.J. (2010). A novel organophosphorus hydrolase-based biosensor using mesoporous carbons and carbon black for the detection of organophosphate nerve agents, Biosensors and Bioelectronics, 25: 1566–1570.

Lee D.W., Kim H.J., Choi C.H., Shin J.H., Kim E.H. (2011). Development of a protein chip to measure PKCβ activity. Applied Biochemistry and Biotechnology, 163: 803-812.

Lei Y., Mulchandani P., Chen W., Mulchandani A. (2005). Direct determination of p-nitrophenyl substituent organophosphorus nerve agents using a recombinant *Pseudomonas putida* JS444-modified clark oxygen electrode. Journal of Agricultural and Food Chemistry, 53: 524-527.

Lei Y., Chen W., Mulchandani A. (2006). Microbial Biosensors. Analytica Chimica Acta, 568: 200–210.

Lei Y., Mulchandani P., Chen W. and Mulchandani A. (2007). Biosensor for direct determination of fenitrothion and EPN using recombinant *Pseudomonas putida* JS444 with surface-expressed organophosphorous hydrolase. 2. Modified carbon paste electrode. Applied Biochemistry and Biotechnology, Vol.136, No.3: 243-250.

Lemos M.F.L., Soares A.M.V.M., Correia A.C., Esteves A.C. (2010). Proteins in ecotoxicology – How, why and why not?. Proteomics, 10: 873-887.

Li Y.F., Li F.Y., Ho C.L., Liao V.H.C. (2008). Construction and comparison of fluorescence and bioluminescence bacterial biosensors for the detection of bioavailable toluene and related compounds. Environmental Pollution, 152: 123–129.

Lin L., Xiao L.L., Huang S., Zhao L., Cui, J. S., Wang X. H. and Chen X. (2006). Novel BOD optical fiber biosensor based on co-immobilized microorganisms in ormosils matrix. Biosensors and Bioelectronics , Vol.21, No.9: 1703–1709.

Ling X.P., Zhu J.Y., Huang L., Huang H.Q. (2009). Proteomic changes in response to acute cadmium toxicity in gill tissue of *Paralichthys olivaceus*. Environmental Toxicology and Pharmacology, 27: 212–218.

Liu S., Yuan L., Yue X., Zheng Z., Tang Z. (2008). Recent advances in nanosensors for organophosphate pesticide detection. Advanced Poowder Technology, 19: 419-441.

Liu C., Suna T., Xub X. , Dong S. (2009). Direct toxicity assessment of toxic chemicals with electrochemical method. Analytica Chimica Acta, 641: 59-63.

Liu L., Shang L., Liu C., Liu C., Zhang B., Dong S. (2010). A new mediator method for BOD measurement under non-deaerated condition. Talanta, 81: 1170-1175.

Llamas N.M., Stewart L., Fodey T., Higgins H.C., Velasco M.L.R., Botana L.M., Elliott C.T. (2007). Development of a novel immunobiosensor method for the rapid detection of okadaic acid contamination in shellfish extracts. Analytical Bioanalytical Chemistry, 389: 581-587.

Long F., He M.,. Shi H.C , Zhu A.N. (2008). Development of evanescent wave all-fiber immunosensor for environmental water analysis. Biosensors and Bioelectronics, 23: 952–958.

López J.L. (2007). Applications of proteomics in marine ecology. Marine Ecology Progress Series, 332: 275-279.

Mann T.S. and Mikkelsen S.R. (2008). Antibiotic susceptibility testing at a screen-printed carbon electrode array. Analytical Chemistry, 80: 843-848.

Marchesini G.R., Meulenberg E., Haasnoot W., Irth H. (2005). Biosensor immunoassays for the detection of bisphenol A. Analytica Chimica Acta, 528: 37-45.

Maria V.L. and Bebianno M.J. (2011). Antioxidant and lipid peroxidation responses in *Mytilus galloprovincialis* exposed to mixtures of benzo(*a*)pyrene and copper. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology, 154: 56-63.

Martiny-Baron G., Haasen D., D'Dorazio D., Voshol J., Fabbro D. (2011). Characterization of kinase inhibitors using reverse-phase protein arrays. Methods in Molecular Biology, 785: 79-107.

Martyniuk C.J., Alvarez S., McClung S., Villeneuve D.L., Ankley G.T., Denslow N.D. (2009). Quantitative proteomic profiles of androgen receptor signaling in the liver of Fathead Minnows (*Pimephales promelas*). Journal of Proteome Research, 8: 2186–2200.

Masó M. and Garcés E. (2006). Harmful microalgae blooms (HAB); problematic and conditions that induce them. Marine Pollution Bulletin, 53: 520-530.

Mauriz E., Calle A., Manclus J.J., Montoya A., Hildebrandt A., Barcelo D., Lechuga L.M. (2007). Optical immunosensor for fast and sensitive detection of DDT and related compounds in river water samples. Biosensors and Bioelectronics, 22: 1410-1418.

McBeath G., Schreiber S.L. (2000). Printing proteins as microarrays for high-thoughput function determination. Science, 289: 176-1763.

McDonagh B. and Sheehan D. (2007). Effect of oxidative stress on protein thiols in the blue mussel *Mytilus edulis*: Proteomic identification of target proteins. Proteomics, 7: 3395–3403.

McDonagh B. and Sheehan D. (2008). Effects of oxidative stress on protein thiols and disulphides in *Mytilus edulis* revealed by proteomics: Actin and protein disulphide isomerase are redox targets. Marine Environmental Research, 66: 193–195.

Meiller J.C. and Bradley B.P. (2002). Zinc concentration effect at the organismal, cellular and subcellular levels in the eastern oyster. Marine Environmental Research, 54: 401–404.

Michelini E., Leskinen P., Virta M., Karp M., Roda A. (2005). A new recombinant cell-based bioluminescence assay for sensitive androgen-like compounds detection. Biosensors and Bioelectronics, 20: 2261-2267.

Mioni C.E., Howard A.M., DeBruyn J.M., Bright N.G., Twiss M.R., Applegate B.M., Wilhelm S.W. (2003). Characterization and field trials of a bioluminescent bacterial reporter of iron availability. Marine Chemistry, 83:31–46.

Mioni C.E., Handy S.M., Ellwood M.J., Twiss M.R., McKay R.M.L., Boyd P.W., Wilhelm S.W. (2005). Tracking changes in bioavailable Fe within high-nitrate low-chlorophyll oceanic waters: a first estimate using a heterotrophic bacterial bioreporter. Global Biogeochemical Cycles 19, Special Section: GB4S25.

Mitchell J. (2010). Small Molecule Immunosensing Using Surface Plasmon Resonance. Sensors, 10: 7323-7346.

Momose Y. and Iwahashi H. (2001). Bioassay of cadmium using a DNA microarray: Genome-wide expression patterns of *Saccharomyces cerevisiae* response to cadmium. Environmental Toxicology and Chemistry, 20: 2353–2360.

Monsinjon T. and Knigge T. (2007). Proteomic applications in ecotoxicology. Proteomics, 7: 2997–3009.

Moore M.N. (2006). Do nanoparticles present ecotoxicological risks for the health of the aquatic environment?. Environmental International 32: 967–976.

Nakamura H. (2010). Recent organic pollution and its biosensing methods. Analytical Methods, Vol.2, No.5: pp.430–444.

Nesatyy V.J. and Suter M.J. (2008). Analysis of environmental stress response on the proteome level. Mass Spectrometry Reviews, 27: 556-574.

Niyogi S., Biswas S., Sarker S., Datta A.G. (2001). Seasonal variation of antioxidant and biotransformation enzymes in barnacle, *Balanus balanoides*, and their relation with polyaromatic hydrocarbons. Marine Environment Research, 52: 13–26.

Nowicka A.M., Kowalczyk, A., Stojek Z., Hepel M. (2010). Nanogravimetric and voltammetric DNA-hybridization biosensors for studies of DNA damage by common toxicants and pollutants. Biophysical Chemistry, 146: 42-53.

Ottova A.L. and Tien H.T. (1997). Self-assembled bilayer lipid membranes: from mimicking biomembranes to practical applications. Bioelectrochemistry and Bioenergetics, 42: 141-152.

Paitan Y., Biran I., Shechter N., Biran D., Rishpon J., Ron E.Z. (2004). Monitoring aromatic hydrocarbons by whole cell electrochemical biosensors. Analytical Biochemistry, 335: 175-183.

Palchetti I., Mascini M. (2008). Nucleic acid biosensors for environmental pollution monitoring. Analyst, 133: 846-854.

Perez-Casanova J.C., Afonso L.O.B., Johnson S.C., Currie S., Gamperl A.K. (2008). The stress and metabolic responses of juvenile Atlantic cod (*Gadus morhua* L.) to an acute thermal challenge. Journal of Fish Biology, 72: 899-916.

Periasamy A.P., Umasankar Y., Chen S.M. (2009). Nanomaterials - acetylcholinesterase enzyme matrices for organophosphorus pesticides electrochemical sensors: a review. Sensors, 9: 4034-4055.

Petz M. (2009). Recent applications of surface plasmon resonance biosensors for analyzing residues and contaminants in food. Monatshefte Chemie, 140: 953–964.

Piñeiro C., Cañas B., Carrera M. (2010). The role of Proteomics in the study of the influence of climate change on seafood products. Food Research International, 43: 1791-1802.

Polyak B., Bassis E., Novodvorets A., Belkin S., Marks R.S. (2001). Bioluminescent whole cell optical fiber sensor to genotoxicant: system optimization. Sensors and Actuators B-Chemical, 74: 18-26.

Ponomareva O.N. Arlyapov V.A., Alferov, V.A. and Reshetilov A.N. (2011). Microbial biosensors for detection of biological oxygen demand (a review). Applied Biochemistry and Microbiology, 47: 1–11.

Popovtzer R., Neufeld T., Ron E.Z., Rishpon J., Shacham-Diamand Y. (2006). Electrochemical detection of biological reactions using a novel nano-bio-chip array. Sensors and Actuators B 19: 664-672.

Poynton H.C., Varshavsky J.R., Chang B., Cavigiolio G., Chan S., Holman P.S., Loguinov A.V., Bauer D.J., Komachi K., Theil E.C., Perkins E.J., Hughes O., Vulpe C.D. (2007). *Daphnia magna* ecotoxicogenomics provides mechanistic insights into metal toxicity. Environmental Science and Technology, 41: 1044–1050.

Pratscher J., Stichternoth C., Fichtl K., Schleifer K.H., Braker G. (2009). Application of recognition of individual genes-fluorescence in situ hybridization (RING-FISH) to detect nitrite reductase genes (*nirk*) of denitrifiers in pure cultures and environmental samples. Applied and Environmental Microbiology, 75: 802-810.

Premkumar R.J., Lev O., Marks R.S., Polyak B., Rosen R, Belkin S. (2001). Antibody-based immobilization of bioluminescent bacterial sensor cells. Talanta, 55: 1029-1038.

Prieto-Simón B., Miyachi H., Karube I., Saiki H. (2010). High-sensitive flow-based kinetic exclusion assay for okadaic acid assessment in shellfish samples. Biosensors and Bioelectronics, 25: 1395-1401.

Přibyl J., Hepel M., Skládal P. (2006). Piezoelectric immunosensors for polychlorinated biphenyls operating in aqueous and organic phases. Sensors and Actuators B: Chemical, 113: 900-910.

Prodromidis M.I. (2010). Impedimetric immunosensors—A review. Electrochimica Acta, 55: 4227–4233.

Proll G., Tschmelak J., Gauglitz G (2005). Fully automated biosensors for water analysis. Analytical Bioanalytical Chemistry, 381: 61-63.

Quan D., Nagarale R.K., Shin W. (2010). A nitrite biosensor based on coimmobilization of nitrite reductase and viologen-modified polysiloxane on glassy carbon electrode. Electroanalysis, 22: 2389-2398.

Regulation (EC) No 1907/2006 of The European Parliament and of The Council of 18 December 2006, concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC.

Regulation (EC) No 178/2002 of The European Parliament and of The Council of 28 January 2002, laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety.

Riva C., Binelli A., Rusconi F., Colombo G., Pedriali A., Zippel R., Provini A. (2011). A proteomic study using zebra mussels (*D. polymorpha*) exposed to benzo(α)pyrene: The role of gender and exposure concentrations. Aquatic Toxicology, 104: 14–22.

Roberto F.F., Barnes J.M., Bruhn D.F. (2002). Evaluation of a GFP reporter gene construct for environmental arsenic detection. Talanta, 58: 181-188.

Rodriguez-Mozaz S., López de Alda M.J., Marco M.-P., Barceló D. (2005). Biosensors for environmental monitoring. A global perspective. Talanta, 65: 291-297.

Rogers K.R. (2006). Recent advances in biosensor techniques for environmental monitoring. Analytica Chimica Acta, 568: 222-231.

Sakaguchi T., Morioka Y., Yamasaki M., Iwanaga J., Beppu K., Maedac H., Morita, Y.,Tamiya E. (2007). Rapid and onsite BOD sensing system using luminous bacterial cells-immobilized chip. Biosensors and Bioelectronics, 22: 1345–1350.

Samsonova J.V., Uskova N.A., Andresyuk A.N., Franek M., Elliott C.T. (2004). Biacore biosensor immunoassay for 4-nonylphenols: assay optimization and applicability for shellfish analysis. Chemosphere, 57: 975-985.

Sanchez-Acevedo Z.C., Riu J., Rius F.X. (2009). Fast picomolar selective detection of bisphenol A in water using a carbon nanotube field effect transistor functionalized with estrogen receptor-α. Biosensensors and Bioelectronics, 24: 2842-2846.

Sauer U., Domnanich P., Preininger C. (2011). Protein chip for the parallel quantification of high and low abundant biomarkers for sepsis. Analytical Biochemistry, 419: 46-52.

Scholin C.A., Doucette G.J., Cembella A.D. (2008). Prospects for developing automated systems for in situ detection of harmful algae and their toxins. In: Real-Time coastal observing systems for ecosystem dynamics and harmful algal blooms. Ed: Babin M, Roesler CS, Cullen JJ, 413-462, UNESCO Publishing, Paris, France.

Schultz I.R., Skillman A.D., Barry R., Wunschel D. (2008). Toxicokinetic and toxicodynamic aspects of trenbelone exposure in adult fathead minnows and rainbow trout. Fourteenth International Symposium on Pollutant Responses in Marine Organisms (PRIMO 14) – Environmental proteomics. Marine Environmental Research, 66: 101–103.

Selander S.and Cramer K. (1970). Interrelationships between lead in blood, lead in urine, and ALA in urine during lead work. British Journal of Industrial Medicine, 27: 28–39.

Shankaran D.R., Matsumoto K., Toko K., Miura N. (2006). Development and comparison of two immunoassays for the detection of 2,4,6-trinitrotoluene (TNT) based on surface plasmon resonance. Sensors and Actuators B, 114: 71-79.

Sheehan D. and Power A. (1999). Effects of seasonality on xenobiotic and antioxidant defence mechanisms of bivalve molluscs. Comparative Biochemistry and Physiology C: Pharmacology, Toxicology and Endocrinology, 123C: 193–199.

Sheehan D. (2007). The potential of proteomics for providing new insights into environmental impacts on human health. Reviews on Environmental Health, 22: 175-194.

Shepard J.L. and Bradley B.P. (2000). Protein expression signatures and lysosomal stability in *Mytilus edulis* exposed to graded copper concentrations. Marine Environmental Research, 50: 457-463.

Shepard J.L., Olsson B., Tedengren M., Bradley B.P. (2000). Protein expression signatures identified in *Mytilus edulis* exposed to PCBs, copper and salinity stress. Environmental Research, 50:337–340.

Shitanda I., Katamatsu S., Watanabe K., Itagaki M. (2009). Amperometric screen-printed algal biosensor with flow injection analysis system for detection of environmental toxic compounds. Electrochimica Acta, 54: 4933-4936.

Silvestre F., Dierick J.F., Dumont V., Dieu M., Raes M., Devos P. (2006). Differential protein expression profiles in anterior gills of *Eriocheir sinensis* during acclimation to cadmium. Aquatic Toxicology, 76: 46–58.

Siu W.H.L., Cao J., Jack R.W., Wu R.S.S., Richardson B.J., Xu L., Lam P.K. (2004). Application of the comet and micronucleus assays to the detection of B[a]P genotoxicity in haemocytes of the green-lipped mussel (*Perna viridis*). Aquatic Toxicology, 66: 381–392.

Song Y ., Li G., Thorton S.F., Thompson I.P., Banwart S.A., Lerner D.N., Huang W. (2009). Optimization of Bacterial Whole Cell Bioreporters for Toxicity Assay of Environmental Samples. Environmental Science and Technology, 43: 7931–7938.

Søfteland L., Petersen K., Stavrum A.K., Wu T., Olsvik P.A. (2011). Hepatic in vitro toxicity assessment of PBDE congeners BDE47, BDE153 and BDE154 in Atlantic salmon (*Salmo salar* L.). Aquatic Toxicology, 105: 246–263.

Stevens R.C., Soelberg S.D., Eberhart B.T.L., Spencer S., Wekell J.C., Chinowsky T.M., Trainer V.L., Furlong C.E. (2007). Detection of the toxin domoic acid from clam extracts using a portable surface plasmon resonance biosensor. Harmful Algae, 6: 166-174.

Stewart L.D., Hess P., Connolly L., Elliott C.T. (2009). Development and Single-Laboratory Validation of a pseudo-functional biosensor immunoassay for the detection of the Okadaic Acid group of toxins. Analytical Chemistry, 81: 10208–10214.

Sun Y., Zhou T., Guo J., Li Y. (2004). Dark variants of luminous bacteria whole cell bioluminescent optical fiber sensor to genotoxicants. Journal of Huazhong University of Science and Technology, Medical Science, 24: 507-509.

Suri C.R., Raje M., Varshney G.C. (2002). Immunosensors for pesticide analysis. Antibody production and sensor develpment. Critical Reviews in Biotechnology, 22: 15-32.

Suwansa-ard S., Kanatharana P., Asawatreratanakul P., Limsakul C., Wongkittisuksa B., Thavarungkul P. (2005). Semi disposable reactor biosensors for detecting carbamate pesticides in water. Biosensors and Bioelectronics, 21: 445-454.

Tag K., Riedel K., Bauer H.J., Hanke G., Baronian K.H.R. , Kunze G. (2007). Amperometric detection of Cu^{2+} by yeast biosensors using flow injection analysis (FIA). Sensors and Actuators B, 122: 403–409.

Taranova L., Semenchuk I., Manolov T., Iliasov P., Reshetilov A. (2002). Bacteria-degraders as the base of an amperometric biosensor for detection of anionic surfactants. Biosens Bioelectron, 17:635–640.

Tatsuma T., Yoshida Y., Shitanda I., Notsu H. (2009). Algal biosensor array on a single electrode. Analyst, 134: 223–225.

Tedeschi L., Domenici C., Ahluwalia A., Baldini F., Mencaglia A. (2003). Antibody immobilization on fiber optic TIRF sensors. Biosensors and Bioelectronics, 19: 85-93.

Tedesco S., Doyle H., Redmond G., Sheehan D. (2008). Gold nanoparticles and oxidative stress in *Mytilus edulis*. Marine Environmental Research, 66: 131–133.

Theytaz J., Braschler T., Van Lintel H., Renaud P., Diesel E., Merulla D., Van der Meer J. (2009). Biochip with *E. coli* bacteria for detection of arsenic in drinking water. Procedia Chemistry, 1: 1003–1006.

Thouand G., Horry H., Durand M.J., Picart P., Bendriaa L., Daniel P., DuBow M.S. (2003). Development of a biosensor for on-line detection of tributyltin with a recombinant escherischia coli strain, Applied Microbial Biotechnologies, 62: 218-225.

Timur S., Anik U., Odaci D., Gorton L. (2007). Development of a microbial biosensor based on carbon nanotube (CNT) modified electrodes. Electrochemical Communications, 9: 1810-1815.

Traynor I.M., Plumpton L., Fodey T.L., Higgins C., Elliott C.T. (2006). Immunobiosensor detection of Domoic Acid as a screening test in bivalve molluscs: comparison with liquid chromatography-based analysis. Journal of AOAC International, 89: 868-872.

Tschmelak J., Proll G., Riedt J., Kaiser J., Kraemmer P., Bárzaga L., Wilkinson J.S., Hua P., Hole J.P., Nudd R., Jackson M., Abuknesha R, Barceló D., Rodríguez-Mozaz S., López de Alda M.J., Sacher F., Stien J., Slobodník J., Oswald P., Kozmenko H., Korenková E., Tóthová L., Krascsenits Z., Gauglitz G. (2005). Automated Water Analyser Computer Supported System (AWACSS) Part I: Project objectives, basic technology, immunoassay development, software design and networking. Part II: Intelligent, remote-controlled, cost-effective, on-line, water-monitoring measurement system. Biosensors and Bioelectronics 20: 1499-1508 (Part I); 1509-1519 (Part II).

Tsutsumi T., Miyoshi N., Sasaki K., Maitani T. (2008). Biosensor immunoassay for the screening of dioxin-like polychlorinated biphenyls in retail fish. Analytica Chimica Acta, 617: 177-183.

Turner A.P.F. (2000). Biosensors – Sense and Sensitivity. Science, 290: 1315-1317.

Unwin R.D., Evans C.A., Whetton A.D. (2006). Relative quantification in proteomics: New approaches for biochemistry. Trends in Biochemical Science, 31: 473–484.

Viarengo A., Canesi L., Pertica M., Livingstone D.R. (1991). Seasonal variations in the antioxidant defence systems and lipid peroxidation of the digestive gland of mussels. Comparative Biochemistry and Physiology C: Pharmacology, Toxicology and Endocrinology, 100C: 187–190.

Vidal J.C., Bonel L., Castillo J. R. (2008) A modulated tyrosinase enzyme-based biosensor for application to the detection of dichlorvos and atrazine pesticides. Electroanalysis, Vol. 20, No.8: 865-873.

Vido K., Spector D., Lagniel G., Lopez S., Toledano M.B., Labarre J. (2001). A proteome analysis of the cadmium response in *Saccharomyces cerevisiae*. Journal of Biological Chemistry, 276: 8469–8474.

Viswanathan S., Radecka H. and Radecki J. (2009). Electrochemical biosensor for pesticides based on acetylcholinesterase immobilized on polyaniline deposited on vertically assembled carbon nanotubes wrapped with ssDNA. Biosensors and Bioelectronics, Vol.24, No9: 2772-2777.

Volpe G., Cotroneo E., Moscone D., Croci L., Cozzi L., Ciccaglioni G., Palleschi G. (2009). A bienzyme electrochemical probe for flow injection analysis of okadaic acid based on protein phosphatase-2a inhibition: an optimization study. Analytical Biochemistry, 385: 50-56.

Wang H., Wang X.J., Zhao J.F. and Chen L. (2008). Toxicity assessment of heavy metals and organic compounds using CellSense biosensor with *E. coli*. Chinese Chemical Letters, 19: 211-214.

Wang S., Tan, Y., Zhao D., Liu G. (2008). Amperometric tyrosinase biosensor based on Fe3O4 nanoparticles–chitosan nanocomposite. Biosensors and Bioelectronics, 23: 1781–1787.

Wang L., Ran Q., Tian Y., Ye S., Xu J., Xian Y., Peng R., Jin L. (2010). Covalent grafting tyrosinase and its application in phenolic compounds detection. Microchimica Acta, 171: 217–223.

Ward D.G., Wei W., Cheng Y., Billingham L.J., Martin A., Johnson P.J., Lyons B.P., Feist S.W., Stentiford G.D. (2006). Plasma proteome analysis reveals the geographical origin and liver tumor status of dab (*Limanda limanda*) from UK marine waters. Environmental Science and Technology, 40: 4031–4036.

Wei Y., Chan L.L., Wang D., Zhang H., Wang J., Dai J. (2008). Proteomic analysis of hepatic protein profiles in rare minnow (*Gobiocypris rarus*) exposed to perfluorooctanoic acid. Journal of Proteome Research, 7: 1729–1739.

Weigel S., Pikkemaat M.G., Elferink J.W.A., Mulder P.P.J., Huet A.C., Delahaut P., Schittko S., Flerus R., Nielen M. (2009). Comparison of a fluoroquinolone surface plasmon resonance biosensor screening assay with established methods. Food Additives and Contaminants. A. 26, 441-452.

Weston A.D. and Hood L. (2004). Systems biology, proteomics, and the future of health care: Toward predictive, preventative, and personalized medicine. Journal of Proteome Research, 3:179–196.

Witzmann F.A., Fultz C., Lipscomb J. (1995). Comparative 2D-electrophoretic mapping of human and rodent hepatic stress proteins as potential biomarkers. Applied and Theoretical Electrophoresis, 5: 113–117.

Wulff G. (1995). Molecular imprinting in cross-linked materials with the aid of molecular templates – a way towards artificial antibodies. Angewandte Chemie – International edition in English, 34: 1812-1832.

Xia W., Li Y., Wan Y., Chen T., Wei J., Lin Y., Xu S. (2010). Electrochemical biosensor for estrogenic substance using lipid bilayers modified by Au nanoparticles. Biosensors and Bioelectronics, 25: 2253–2258.

Yakes B.J., Prezioso S., Haughey S.A., Campbell K., Elliott C.T., DeGrasse S.L. (2011). An improved immunoassay for detection of saxitoxin by surface plasmon resonance biosensors. Sensors and Actuators B: Chemicals, 156: 805-811.

Yin J.Q., Li X.Z., Zhou C., Zhang Y.H. (2005). Luminescent bacterial sensors made from immobilized films of *Photobacterium phosphoreum.* Chemical Research in Chinese Universities, 21: 44-47.

Yoshida K., Inoue K., Takahashi Y., Ueda S., Isoda K., Yagi K., Maeda I. (2008). Novel carotenoid-based biosensor for simple visual detection of arsenite: characterization and preliminary evaluation for environmental application. Applied and Environmental Microbiology, 74: 6730–6738.

Yuan C.J., Wang C.L., Wu T. Y., Hwang K.C., Chao W.C. (2011). Fabrication of a carbon fiber paper as the electrode and its application toward developing a sensitive unmediated amperometric biosensor. Biosensors and Bioelectronics, 26: 2858–2863.

Yu J.C.C. and Lai E.P.C. (2010). Molecularly imprinted polymers for Ochratoxin A extraction and analysis. Toxins 2: 1536-1553.

Yu Q., Chen S., Taylor A.D., Homola J., Hock B., Jiang S., (2005). Detection of low-molecular-weight domoic acid using surface plasmon resonance sensor. Sensors and Actuators B: Chemical, 107: 193-201.

Yuk J.S., Lee M.J., Kim U.R., Ha K.S. (2007). Analysis of blood proteins on protein arrays with a spectral Plasmon surface resonance biosensor. Current Applied Physics, 7: 102-107.

Zaccaroni A., Gamberoni M., Mandrioli L., Sirri R., O. Mordenti, Scaravelli D., Sarli G., Parmeggiani A. (2009). Thyroid hormones as a potential early biomarker of exposure to 4-nonylphenol in adult male shubunkins (*Carassius auratus*). Science of the Total Environment, 407: 3301–3306.

Zhang A., Jaffrezic-Renault N. , Wan J., Hou Y., Chovelon J.M. (2002). Optimization of the mixed urease/amphiphile Langmuir–Blodgett film and its application for biosensor development. Materials Science and Engineering C, **21**: 91-96.

Zhang Z., Jaffrezic-Renault N., Bessueille F., Léonard D., Xia S., Wang X., Chen L., Zhao J. (2008). Development of a conductometric phosphate biosensor based on trilayer maltose phosphorylase composite films. Analytica Chimica Acta, 615: 73-79.

Zhang Z., Xia S., Leonard D., Jaffrezic-Renault N., Zhang J., Bessueille F., Goepfert Y., Wang X., Chen L., Zhu Z., Zhao J., Almeida M.G., Silveira C.M. (2009). A novel nitrite biosensor based on conductometric electrode modified with cytochrome c nitrite reductase composite membrane. Biosensors and Bioelectronics, 24: 1574-1579.

Zhao J., Zhi J., Zhou Y., Yan W. (2009). A tyrosinase biosensor based on ZnO nanorod clusters / nanocrystalline diamond electrodes for biosensing of phenolic compounds. Analytical Sciences, 25: 1083-1088.

Zhou J.H. (2003). Microarrays for bacterial detection and microbial community analysis, Current Opinion in Microbiology, 6: 288–294.

Zielinski O., Busch J.A., Cembella A.D., Daly K.L., Engelbrektsson J., Hannides A.K., Schmidt H. (2009). Detecting marine hazardous substances and organisms: sensors for pollutants, toxins, and pathogens. Ocean Science Discussions, 6: 953–1005.

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References

Adams S.M., Shepard K.L., Greeley Jr M.S., Jimenez B.D., Ryon M.G., Shugart L.R., McCarthy J.F. (1989). The use of bioindicators for assessing the effects of pollutant stress on fish. Marine Environmental Research, 28: 459-464.

Addis M.F., Cappuccinelli R., Tedde V., Pagnozzi D., Porcu M.C., Bonaglini E., Roggio T., Uzzau S. (2010a). Proteomic analysis of muscle tissue from gilthead sea bream (*Sparus aurata*, L.) farmed in offshore floating cages. Aquaculture, 309: 245-252.

Addis M.F., Cappuccinelli R., Tedde V., Pagnozzi D., Viale I., Meloni M., Salati F., Roggio T., Uzzau S. (2010b). Influence of Moraxella sp. colonization on the kidney proteome of farmed gilthead sea bream (*Sparus aurata*, L.). Proteome Science, 8: 50-58.

Aebersold R. and Goodlett D.R. (2001). Mass spectrometry in Proteomics. Chemical Reviews, 101: 269-295.

Aebersold R.H., Leavitt J., Saavedra R.A:, Hood L.E., Kent S.B. (1987). Internal amino acid sequence analysis of proteins separated by one- or two-dimensional gel electrophoresis after in situ protease digestion on nitrocellulose. Proceedings of the National Academy of Science of the United States of America, 84 (20): 6970-6974.

Agaton C., Uhlen M., Hobe S. (2004). Genome-based proteomics. Electrophoresis, 25: 1280-1288.

Albertsson E., Kling P., Gunnarsson L., Larsson J.D.G., Förlin L. (2008). Induction of hepatic CR20b-HSD B in rainbow trout exposed to sewage effluent. Fourteenth International Symposium on Pollutant Responses in Marine Organisms (PRIMO 14) – Environmental proteomics. Marine Environmental Research, 66: 101–103.

Altschul SF, Gish W., Miller W., Myers E.W., Lipman D.J. (1990). Basic local alignment search tool. Journal of Molecular Biology, 215: 403–410.

Alves de Almeida E., Vioque-Fernandez A., López-Barea J. (2008). Proteomic alterations and biomarker responses in Procambarus clarkii after controlled exposure to chlorpyrifos and carbaryl. Fourteenth International Symposium on Pollutant Responses in Marine Organisms (PRIMO 14) – Environmental proteomics. Marine Environmental Research, 66: 101–103.

Alves R. N., Cordeiro O., Silva T. S., Richard N., De Vareilles M., Marino G., Di Marco P., Rodrigues P. M., Conceição L.E.C. (2010). Metabolic molecular indicators of chronic stress in gilthead sea bream (*Sparus aurata*) using comparative proteomics. Aquaculture, 299: 57-66.

Amatruda J. F., Patton E. (2008). Genetic Models of Cancer in Zebrafish, in: International Review of Cell and Molecular Biology, Jeon K. W. (Ed.), Academic Press, San Diego, USA, 1–34.

Amelina H., Apraiz I., Sun W., Cristobal S. (2007). Proteomics-based method for the assessment of marine pollution using liquid chromatography coupled with two-dimensional electrophoresis. Journal of Proteome Research, 6: 2094–2104.

Anderson A.S., Bilodeau A.L., Gilg M.R., Hilbish T.J. (2002). Routes of introduction of the Mediterranean mussel (*Mytilus galloprovincialis*) to Puget Sound and Hood Canal. Journal of Shellfish Research, 21: 75-79.

Anderson L. and Seilhamer J. (1997). A comparison of selected mRNA and protein abundances in human liver. Electrophoresis, 18: 533–537.

Appel R.D., Bairoch A., Sanchez J.C., Vargas J.R., Golaz O., Pasquali C., Hochstrasser D.F. (1996). Federated 2-DE database: a simple means of publishing 2-DE data. Electrophoresis, 17: 1996, 540-546.

Appel R.D., Sanchez J.C., Bairoch A., Golaz O., Miu M., Vargas J.R., Hochstrasser D.F. (1993). SWISS 2D-PAGE: a database of two-dimensional gel electrophoresis images. Electrophoresis, 14: 1232-38.

Apraiz I., Mi J., Cristobal S. (2006). Identification of proteomic signatures of exposure to marine pollutants in mussels (*Mytilus edulis*). Molecular and Cellular Proteomics, 5: 1274–1285.

Apte S., Holland B.S., Godwin L.S., Gardner J.P. (2000). Jumping ships: a stepping stone event mediating transfer of a non-indigenous species via a potentially unsuitable environment. Biological Invasions, 2: 75-79.

Apweiler R., Bairoch A., Wu C.H. (2004). Protein sequence databases. Current Opinion in Chemical Biology, 8: 76–80.

Arellano J. M., Ortiz J. B., Capeta Da Silva, D., González De Canales M. L., Sarasquete C., Blasco J. (1999). Levels of copper, zinc, manganese and iron in two fish species from salt marshes of Cadiz Bay (southwest Iberian Peninsula). Boletín del Instituto Español de Oceanografía, 15: 485−532.

Bairoch A. (2000). Serendipity in bioinformatics, the tribulations of a Swiss bioinformatician through exciting times!. Bioinformatics, 16: 48-64.

Banks R.E., Dunn M.J., Hochstrasser D.F., Sanchez J.C., Blackstock W., Pappin D.J., Selby P.J. (2000). Proteomics: new perspectives, new biochemical opportunities. Lancet, 356: 1749-1756.

Banks R.E., Dunn M.J., Hochstrasser D.F., Sanchez J.C., Blackstock W., Pappin D.J., Selby P.J. (2000). Proteomics: new perspectives, new biochemical opportunities. Lancet, 356: 1749-1756.

Barabási A.L. and. Oltvai Z.N. (2004). Network biology: understanding the cell's functional organization. Nature Reviews Genetics, 5: 101-113.

Barnes R.D. (1980). Invertebrate Zoology, 4th edition. W.B. Saunders Co., Philadelphia.

Barsotti G, Meluzzi C. (1968). Osservazioni su *Mytilus edulis* L. e *Mytilus galloprovincialis* Lamarck. Conchiglie, 4: 50-58.

Bayne B.L. (1976). Marine Mussels: their ecology and physiology. Cambridge University Press, Cambridge.

Beadle G.W. and Tatum E.L. (1941). Genetic control of biochemical reactions in Neurospora. Proceedings of the National Academy of Sciences of the of the United States of America, 27: 499–506.

Beaumont A.R., Hawkins M.P., Doig F.L., Davies I.M., Snow M. (2008). Three species of Mytilus and their hybrids identified in a Scottish Loch: relicts, endemics and invaders? Journal of Experimental Marine Biology and Ecology, 367: 100-110.

Benton D. (1990). Recent changes in the GenBank on-line service. Nucleic Acid Research, 18: 1517-1520.

Berg K., Puntervoll P., Klungsøyr J., Goksøyr A. (2011). Brain proteome alterations of Atlantic cod (Gadus morhua) exposed to PCB 153. Aquatic Toxicology, 105: 206– 217.

Berrini A., Tepedino V., Borromeo V., Secchi C. (2006). Identification of freshwater fish commercially labelled ‘‘perch’’ by isoelectric focusing and two-dimensional electrophoresis. Food Chemistry, 96: 163–168.

Bertanlaffy L.V. (1950). An outline of general system theory. British Journal for the Philosophy of Science, 1: 134-164.

Best J. D., Alderton W. K. (2008). Zebrafish: an in vivo model for the study of neurological diseases. Journal of Neuropsychiatric Disease and Treatment, 4: 567–576.

Bini L., Sanchez-Campillo M., Santucci A., Magi B., Marzocchi B., Comanducci M., Christiansen G., Birkelund S., Cevenini R. Vretou E., Ratti G., Pallini V. (1996). Mapping of *Chlamydia trachomatis* proteins by immobiline-polyacrylamide two-dimensional electrophoresis: spot identification by N-terminal sequencing and immunoblotting. Electrophoresis, 17: 185-190.

Biron D.G., Loxdale H.D., Ponton F., Moura H., March L., Brugidou C., Thomas F. (2006). Population proteomics: an emerging discipline to study metapopulation ecology. Proteomics, 6: 1712-1715.

Bjørnstad A., Larsen B.K., Skadsheim A., Jones M.B., Andersen O.K. (2006). The potential of ecotoxicoproteomics in environmental monitoring: biomarker profiling in mussel plasma using ProteinChip array technology. Journal of Toxicology and Environmental Health A, 69: 77-96.

Blackstock W.P., Weir M.P. (1999). Proteomics: quantitative and physical mapping of cellular proteins. Trends in Biotechnology, 17: 121-127.

BLAST Basic Local Alignment Search Tool algorithm of the National Institute of Health, USA: http://blast.ncbi.nlm.nih.gov/Blast.cgi.

Booth N. J. and Bilodeau-Bourgeois L. (2009). Proteomic analysis of head kidney tissue from high and low susceptibility families of channel catfish following challenge with Edwardsiella ictaluri. Fish Shellfish Immunology, 26: 193–196.

Borchers C., Peter J.F., Hall M. C., Kunkel T.A., Tomer K.B. (2000). Identification of in-gel digested proteins by complementary peptide mass fingerprinting and tandem mass spectrometry data obtained on an electrospray ionization quadrupole time-of-flight mass spectrometer. Analytical Chemistry, 72: 1163-1168.

Bossier P. and Cooreman K. (2000). A databank able to be used for identifying and authenticating commercial flatfish (*Pleuronectiformes*) products at the species levels using isoelectric focusing of native muscle proteins. International Journal of Food Science and Technology, 35: 563-568.

Bossio R.E. and Marshall A.G. (2002). Baseline resolution of isobaric phosphorylated and sulfated peptides and nucleotides by electrospray ionization FTICR MS: another step toward mass spectrometry-based proteomics. Analytical Chemistry, 74: 1674-1679.

BosworthI V.C.A., Chou C.W., Cole R.B., Rees B. (2005). Protein expression patterns in zebrafish skeletal muscle: initial characterization and the effects of hypoxic exposure. Proteomics, 5: 1362–1371.

Bower S.M. (1992). Diseases and parasites of mussels. In: The Mussel *Mytilus*: ecology, physiology, genetics and culture. Gosling E. (Ed.), pp. 543-563. Elsevier Science Publisher B.V., Amsterdam.

Braby C.E and Somero G.N. (2006). Following the heart: temperature and salinity effects on heart rate in native and invasive species of blue mussels (genus *Mytilus*). Journal of Experimental Biology, 209: 2554-2566.

Branch G.M., Odendaal F., Robinson T.B. (2010). Competition and facilitation between the alien mussel *Mytilus galloprovincialis* and indigenous species: Moderation by wave action. Journal of Experimental Marine Biology and Ecology, 383: 65-78.

Branch G.M. and Steffani C.N. (2004). Can we predict the effects of alien species? A case-history of the invasion of South Africa by *Mytilus galloprovincialis* (Lamarck). Journal of Experimental Marine Biology and Ecology, 300: 189-215.

Breci L., Hattrup E., Letarte J, Johnson R., Haynes P.A. (2005). Comprehensive proteomics in yeast using chromatographic fractionation, protein gel electrophoresis and isoelectric focusing. Proteomics, 5: 2018-2028.

Brouwer M., Brown-Peterson N.J., Hoexum-Brouwer T., Manning S., Denslow N. (2008). Changes in mitochondrial gene and protein expression in grass shrimp, *Palaemonetes pugio*, exposed to chronic hypoxia. Marine Environmental Research, 66: 143–145.

Brown P.O. and Botstein D. (1999). Exploring the new world of the genome with DNA microarrays. Nature Genetics, 21: 33-37.

Brun N.T., Bricelj V.M., MacRae T.H., Ross N.W. (2008). Heat shock protein responses in thermally stressed bay scallops, *Argopecten irradians*, and sea scallop, *Placopecten magellanicus*. Journal of Experimental Marine Biology and Ecology, 358: 151-162.

Brunt J., Hansen R., Jamieson D. J., Austin B. (2008). Proteomic analysis of rainbow trout (*Oncorhynchus mykiss*, Walbaum) serum after administration of probiotics in diets. Veterinary Immunology and Immunopathology, 121: 199–205.

Bussani M. (1983). Guida pratica di mitilicoltura. Edagricole, Bologna.

Caceres-Martinez J., Figueras A. (1998). Distribution and abundance of mussel (*Mytilus galloprovincialis* Lmk) larvae and post-larvae in the Ria de Vigo (NW Spain). Journal of Experimental Marine Biology and Ecology, 229: 277-287.

Calvo-Ugarteburu G., McQuaid C.D. (1998). Parasitism and introduced species: epidemiology of trematodes in the intertidal mussels *Perna perna* and *Mytilus galloprovincialis*. Journal of Experimental Marine Biology and Ecology, 220: 47-65.

Cañas B., López-Ferrer D., Ramos-Fernández A., Camafeita E., Calvo E. (2006). Mass Spectromtry technologies for proteomics. Briefings in Functional Genomics and Proteomics, 4: 1-26.

Candiano G., Bruschi M., Musante L., Santucci L., Ghiggeri G.M., Carnemolla B., Orecchia P., Zardi L., Righetti P.G. (2004). Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis, 25: 1327-1333.

Cannas A. (2001). Gli stagni sardi. In: Acquacoltura responsabile. Verso le produzioni acquatiche del terzo millennio. Cataudella S., Bronzi P. (Ed.), pp. 300-303. Unimar-Uniprom, Roma.

Carlton J.T. (1992). Introduced marine and estuarine mollusks of North America: an end-of-the-20th-century perspective. Journal of Shellfish Research, 11 (2): 489-505.

Carlton J.T. (1999). The scale and ecological consequences of biological invasions in the world's oceans. In: Invasive Species and Biodiversity Management. Sandlund, O. T., P. Johan Schei and Å. Viken (eds), Kluwer Academic Publishers, Dordrecht: 195-212.

Carlton J.T. (2003). Community assembly and historical biogeography in the North Atlantic Ocean: the potential role of human-mediated dispersal vectors. Hydrobiologia, 503: 1-8.

Carraro U. and Catani C. (1983). A sensitive SDS-PAGE method separating myosin heavy chains isoforms of rat skeletal muscles reveals the heterogeneous nature of the embryonic myosin. Biochemical and Biophysical Research Communications, 116: 793-802.

Carrera M., Cañas B., Piñeiro C., Vázquez J., Gallardo J.M. (2006). Identification of commercial hake and grenadier species by proteomic analysis of the parvalbumin fraction. Proteomics, 6: 5278–5287.

Carrera M., Cañas B., Piñeiro C., Vázquez J., Gallardo J.M. (2007). De novo mass spectrometry sequencing and characterization of species-specific peptides from nucleoside diphosphate kinase b for the classification of commercial fish species belonging to the family Merlucciidae. Journal of Proteome Research, 6: 3070-3080.

Ceccherelli V.U. and Rossi, R. (1984). Settlement, growth and production of the mussel *Mytilus galloprovincialis*. Marine Ecology Progress Series, 16: 173-184.

Chora S., McDonagh B., Sheehan D., Starita-Geribaldi M., Roméo M., Bebianno M.J. (2008). Ubiquitination and carbonylation as markers of oxidative-stress in *Ruditapes decussatus*. Marine Environmental Research ,66: 95–97.

Chora S., McDonagh B., Sheehan D., Starita-Geribaldi M., Roméo M., Bebianno M.J. (2010). Ubiquitination and carbonylation of proteins in the clam *Ruditapes decussatus*, exposed to nonylphenol using redox proteomics. Chemosphere, 81: 1212–1217.

Coghlan B. and Gosling E. (2007). Genetic structure of hybrid mussel populations in the west of Ireland: two hypotheses revisited. Mar. Biol., 150: 841-852.

Cole R.B. (1997). Electrospray Ionization Mass Spectrometry: Fundamentals, Instrumentation and Applications. Wiley, New York.

Colledge M. and Scott J.D. (1999). AKAPs: from structure to function. Trends in Cell Biology, 9: 216-221.

Collier T.S., Sarkar P., Rao B., Muddiman D.C. (2010). Quantitative Top-down Proteomics of SILAC labeled human embryonic stem cells. Journal of the American Society for Mass Spectrometry, 21: 879-889.

Commission Regulation (EC) No 1881/2006 of 19th December 2006 setting maximum levels for certain contaminants in foodstuffs.

Craig R. and Beavis R.C. (2004). TANDEM: matching proteins with tandem mass spectra. Bioinformatics, 20: 1466-1467.

D'Albis A., Pantaloni C., Bechet J. (1979). An electrophoretic study of native myosin isoenzymes and their subunit content. European Journal of Biochemistry, 99: 261-272.

Dame R.F. (1996). Ecology of marine bivalves: an ecosystem approach. CRC Press LLC, Boca Raton, Florida.

Damodaran S., Dlugos C. A., Wood T.D., Rabin R.A. (2006). Effects of chronic ethanol administration on brain protein levels: a proteomic investigation using 2-D DIGE system. European Journal of Pharmacology, 547: 75-82.

Davies M.J. (2005). The oxidative environment and protein damage. Biochimica and Biophysica Acta, 1703: 93–109.

Davis M.T., Lee T.D. (1997). Variable flow liquid chromatography-tandem mass spectrometry and the comprehensive analysis of complex protein digest mixtures. Journal of The American Society for Mass Spectrometry, 10: 1059.

de Godoy L.M., Olsen J.V., Cox J., Nielsen M.L., Hubner N.C., Fröhlich F., Walther T.C., Mann M. (2008). Comprehensive mass spectrometry-based proteome quantification of haploid versus diploid yeast. Nature, 455: 12511-1254.

de Godoy L.M., Olsen J.V., de Souza G.A., Li G., Mortensen P., Mann M. (2006). Status of complete protein analysis by mass spectrometry: SILAC labelled yeast as a model system. Genome Biology, 7: R50.

De Souza A. G., Maccormack T. J., Wang N., Li L., Goss G.G. (2009). Large-scale proteome profile of the Zebrafish (Danio rerio) gill for physiological and biomarker discovery studies. Zebrafish, 6: 229–238.

De Souza L., de Godoy L.M., Mann M. (2006). Identification of 491 proteins in the tear fluid proteome reveals a large number of proteases and protease inhibitors. Genome Biology, 7: R72.

De Vienne D., Burstin J., Gerber S., Leonardi A., Le Guilloux M., Murigneux A., Beckert M., Bahrman N., Damerval C., Zivy M. (1996). Two-dimensional electrophoresis of proteins as a source of monogenic and codominant markers for population genetics and mapping the expressed genome. Heredity, 76: 166-177.

De Zwan A., Mathieu M. (1992). Cellular biochemistry and endocrinology. In: The Mussel *Mytilus*: Ecology, Physiology, Genetics and Culture. Gosling E. (Ed.), pp. 223-307. Elsevier Science Publisher B.V., Amsterdam.

Decreto del Presidente della Repubblica 2 ottobre 1968, n. 1639. Regolamento per l'esecuzione della Legge 14 luglio 1965, n. 963, concernente la disciplina della pesca marittima. Supplemento ordinario alla Gazzetta Ufficiale, 25 luglio 1968, n. 188.

Devlin R.H., Yesaki T.Y., Donaldson E.M., Du S.J., Hew C.L (1995). Production of germline transgenic Pacific salmonids with dramatically increased growth performance. Canadian Journal of Fisheries and Aquatic Sciences., 52: 1376-1384.

Di Luccia A., Alviti G., Lamacchia C., Faccia M., Gambacorta G., Liuzzi V., Spagna Musso S. (2005). Effects of the hydration process on water-soluble proteins of preserved cod products. Food Chemistry, 93: 385–393.

Dias P.J., Sollelis L., Cook E.J., Piertney S.B., Davies I.M., Snow M. (2008a). Development of a real-time PCR assay for detection of *Mytilus* species specific alleles: Application to a

sampling survey in Scotland. Journal of Experimental Marine Biology and Ecology, 367: 253-258.

Dias P.J., Bland M., Shanks A.M., Piertney S.B., Davies I.M., Snow M. (2008b). Mytilus species under rope culture in Scotland: implications for management. Aquaculture International, 16: doi:10.1007/s10499-008-9214-6.

Directive 91/492/EEC of the Council of the European Economic Community of 22 July 1991, laying down the health conditions for the production and the placing on the market of fishery products. Official Journal of the European Communities L 268, Volume 34, 24 September 1991, pp. 15-34.

Diz A.P. and Skibinski D.O.F. (2007). Evolution of 2-DE protein patterns in a mussel hybrid zone. Proteomics, 7: 2111–2120.

Diz A.P., Dudley E., MacDonald B.W., Piña B., Kenchington E.L.R., Zouros E., Skibinski D.O.F. (2009a). Genetic variation underlying protein expression in eggs of the marine mussel Mytilus edulis. Molecular and Cellular Proteomics, 8: 132-144.

Diz A. P., Truebano M., Skibinski D.O.F. (2009b). The consequences of sample pooling in proteomics: an empirical study. Electrophoresis, 30: 2967-2975.

Domann P.J., Akashi S., Barbas C., Huang L., Lau W., Legido-Quigley C., McClean S., Neusüß C., Perrett D., Quaglia M., Rapp E., Smallshaw L., Smith N.W., Smyth W F., Taylor Chris F. (2010). Guidelines for reporting the use of capillary electrophoresis in proteomics. Nature Biotechnology, 28 (7): 654-655.

Dryer W.J. and Bennett J. (1965). The molecular basis of antibody formation: a paradox. Proceedings of the National Academy of Sciences of the of the United States of America, 54: 864-869.

Du S.J., Gong, Z., Tan C.H., Fletcher G.L., Hew C.L. (1992a). Development of an “all fish” gene cassette for gene transfer in aquaculture. Molecular Marine Biology and Biotechnology, 1: 290-300.

Du S.J., Gong Z., Fletcher G.L., Shears M.A., King M.J., Idler D.R., Hew C.L. (1992b). Growth enhancement in transgenic Atlantic salmon by the use of an "all fish" chimeric growth hormone gene construct. Nature Biotechnology, 10: 176-181.

Ducret A., Van Oostveen I., Eng J.K., Yates J.R. III, Aebersold R. (1998). High throughput protein characterization by automated reverse-phase chromatography/electrospray tandem mass spectrometry. Protein Science, 7: 706-719.

Edman P. and Begg J. (1967). A protein sequenator. European Journal of Biohemistry, 1: 80-91.

Edwards C.A. and Skibinski D.O.F. (1985). Genetic variation of mitochondrial DNA in mussel (*Mytilus edulis* and *M. galloprovincialis*). Populations from South West England and South Wales. Marine Biology, 94: 547-556.

Eng J.K., McCormack A.L., Yates III J.R. (1994). An Approach to Correlate Tandem Mass Spectral Data of Peptides with Amino Acid Sequences in a Protein Database. Journal of The American Society for Mass Spectrometry, 5: 976-989.

European Council Regulation (EC) No. 104/2000 of 17 December 1999 on the common organization of the markets in fishery and aquaculture products. Official Journal of the European Communities, 21 January 2000, L 17, 22-52.

ExPASy Bioinformatics Resource Portal of the Swiss Institute of Bioinformatics (SIB): http://expasy.org/

FASTA protein sequence alignment software package of the European Bioinformatics Institute (EMBL-EBI): http://www.ebi.ac.uk/Tools/sss/fasta/.

Fazekas de St. Groth S., Webster R.G., Datyner A. (1963). Two new staining procedures for quantitative estimation of proteins on electrophoretic strips. BiochimIca et Biophysica Acta, 71: 377–385.

Fenn J.B., Mann M., Meng C.K., Wong S.F., Whitehouse C.M. (1989). Analytical instrumentation: electrospray ionization for mass spectrometry of large biomolecules. Science, 246: 64–71.

Fey S.J. and Larsen P.M. (2001). 2D or not 2D. Two-dimensional gel electrophoresis. Current OpinIon in Chemical Biology, 5: 26-33.

Fianchini A. and Gravina M.F. (2001). Cenni di Biologia dei Molluschi Bivalvi. In: Acquacoltura Responsabile. verso le produzioni acquatiche del terzo millennio. Cataudella S., Bronzi P. (Ed.). Unimar – Uniprom, Roma.

Food and Agriculture Organization of the United Nations (FAO) (1986). Mediterranean Regional Aquaculture Project (MEDRAP). 13th training session "Nutrition in Marine Aquaculture", Lisboa, Portugal.

Food and Agriculture Organization of the United Nations (FAO) (2010). The state of World Fisheries and Aquaculture. Rome, Italy.

Forné I., Abián J., Cerdà J. (2010). Fish proteome analysis: Model organisms and non-sequenced species. Proteomics, 10: 858-872.

Forné I., Agulleiro M.J., Asensio E., Abián J., Cerdà J. (2009). 2-D DIGE analysis of Senegalese sole (*Solea senegalensis*) testis proteome in wild-caught and hormone-treated F1 fish. Proteomics, 9: 2171–2181.

Forner F., Kumar C., Luber C.A., Fromme T., Klingenspor M., Mann M. (2009). Proteome Differences between Brown and White Fat Mitochondria Reveal Specialized Metabolic Functions. Cell Metabolism, 10: 324-335.

Fournier M.L., Gilmore J.M., Martin-Brown S.A., Washburn M.P. (2007). Multidimensional separations-based shotgun proteomics. Chemical Reviews, 107: 3654–3686.

Francesconi K.A. (2007). Toxic metal species and food regulations-making a healthy choice. The Analyst, 132: 17−20.

Friguet B. (2006). Oxidized protein degradation and repair in ageing and oxidative stress. FEBS Letters, 580: 2910–2916.

Funabara D., Nakaya M., Watabe S. (2001). Isolation and characterization of a novel 45 kDa calponin-like protein from anterior byssus retractor muscle of the mussel Mytilus galloprovincialis. Fish Science, 67: 511-517.

Gangar V., Huang T.S., Wei C.I. (1996). Comparison of crabmeat protein patterns by isoelectric focusing. Food Control, 7: 295–307.

Garcia B.A., Shabanowitz J., Hunt D.F. (2007). Characterization of histones and their post-translational modifications by mass spectrometry. Current Opinion in Chemical Biology, 11: 66-73.

Gardestrom J., Elfwing T., Lof M., Tedengren M. et al. (2007). The effect of thermal stress on protein composition in dogwhelks (*Nucella lapillus*) under non-normoxic and hyperoxic conditions. Comparative Biochemistry and Physiology-Part A: Molecular and Integrative Physiology, 148: 869–875.

Gasteiger E., Gattiker A., Hoogland C., Ivanyi I., Appel R.D., Bairoch A. (2003). ExPASy: the proteomic server for in-depth protein knowledge and analysis. Nucleic Acids Research, 31: 3784-3788.

Gavin A.C., Bösche M., Krause R., Grandi P., Marzioch M., Bauer A., Schultz J., Rick J.M., Michon A.M., Cruciat C.M., Remor M., Höfert C., Schelder M., Brajenovic M., Ruffner H., Merino A., Klein K., Hudak M., Dickson D., Rudi T., Gnau V., Bauch A., Bastuck S., Huhse B., Leutwein C., Heurtier M.A., Copley R.R., Edelmann A., Querfurth E., Rybin V., Drewes G., Raida M., Bouwmeester T., Bork P., Seraphin B., Kuster B., Neubauer G., Superti-Furga G. (2002). Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature 415: 141-147.

Geiger T., Cox J., Ostasiewicz P., Wisniewski J.R., Mann M. (2010). Super-SILAC mix for quantitative proteomics of human tumor tissue. Nature Methods, 7: 383-385.

Gelfi C., De Palma S., Cerretelli P., Begum S., Wait R. (2003). Two-dimensional protein map of human vastus lateralis muscle. Electrophoresis, 24: 286-295.

Geller J.B. (1999). Decline of a native mussel masked by sibling species invasion. Conservation Biology, 13 (3): 661-664.

Geller J.B., Carlton J.T., Powers D.A. (1994). PCR-based detection of mtDNA haplotypes of native and invading mussels on the northeastern Pacific coast: latitudinal patterns of invasion. Marine Biology, 119: 243-249.

GenBank Genetic sequence database of the National Institute of Health (NIH) (USA): www.ncbi.nlm.nih.gov/genbank/.

GenPept Protein sequence database of the National Institute of Health (NIH) (USA): http://www.ncbi.nlm.nih.gov/protein.

Giribet G. and Wheeler W.C. (2002). On bivalve phylogeny: a high-level analysis of the Bivalvia (Mollusca) based on combined morphology and DNA sequence data. Invertebrate Biology, 121: 271-324.

Godiksen H., Morzel M., Hyldig G., Jessen F. (2009). Contribution of cathepsins B, L and D to muscle protein profiles correlated with texture in rainbow trout (*Oncorhynchus mykiss*). Food Chemistry, 113: 889–896.

Godwing L.S. (2003). Hull fouling of maritime vessels as a pathway for marine species invasions to the Hawaiian islands. Biofouling. 19: Supplement, 1: 123-131.

Gomiero A., Pampanin D. M., Bjornstad A., Larsen B. K., Provan F., Lyngg E., Andersen O.K. (2006). An ecotoxicoproteomic approach (SELDI-TOF mass spectrometry) to biomarker discovery in crab exposed to pollutants under laboratory conditions. Aquatic Toxicology, 78: S34–S41.

Gonzalez E.G., Krey G., Espiñeira M., Diez A., Puyet A., Bautista J.M. (2010). Population Proteomics of the European Hake (*Merluccius merluccius*). Journal of Proteome Research, 9: 6392–6404.

Goodacre R. (2007). Metabolomics of a superorganism. Journal of Nutrition, 137: 259–266.

Görg A., Obermaier C., Boguth G., Harder A., Schelbe B., Wildgruber R., Weiss W. (2000). The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis, 21: 1037-1053.

Gosling E. (Ed.) (1992). The Mussel *Mytilus*: ecology, physiology, genetics and culture. Development in Aquaculture and Fisheries Science, volume 25. Elsevier Science Publisher B.V., Amsterdam.

Gosling E. (Ed.) (2003). Bivalve molluscs. Biology, ecology and culture. Fishing New Books, Blackwell Science, Oxford.

Goulletquer F. and Herald M. (1997). Marine Molluscan Production Trends in France: From Fisheries to Aquaculture. In: U.S. Department of Commerce, NOAA Technical Report NMFS, 129: 137-164.

Grant W.S. and Cherry M.I. (1985). *M. galloprovincialis* Lmk. in southern Africa. Journal of Experimental Marine Biology and Ecology,90: 179-191.

Graves P.R. and Haystead T.A. (2002). Molecular biologist's guide to proteomics. Microbiology and Molecular Biology Reviews, 66: 39-63.

Griesmeier U., Vázquez-Cortés S., Bublin M., Radauer C., Ma Y., Briza P., Fernández-Rivas M., Breiteneder H. (2010). Expression levels of parvalbumins determine allergenicity of fish species. Allergy, 65: 191-198.

Griffiths C. and Day J. (2004). Aquatic invasive species in South Africa- Environmental impacts and management responses. In Abstracts: 13th International Conference on Aquatic Invasive Species, September 20-24, 2004. Lynch West County Hotel, Ennis, County Clare, Ireland.

Gündel U., Benndorf D., von Bergen M., Altenburger R., Küster E. (2007). Vitellogenin cleavage products as indicators for toxic stress in zebra fish embryos: a proteomic approach. Proteomics, 7: 4541-4554.

Gygi S.P. and Aebersold R. (2000). Mass spectrometry and proteomics. Current Opinions in Chemical Biology, 4: 489-494.

Gygi S.P., Rist B., Gerber S.A., Turecek F., Gelb M.H., Aebersold R. (1999). Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nature Biotechnology, 17: 994-999.

Han D.K., Eng J., Zhou H., Aebersold R. (2001). Quantitative profiling of differentiation-induced microsomal proteins using isotope-coded affinity tags and mass spectrometry. Nature Biotechnology, 19: 946-951.

Hanekom N. and Nel P. (2002). Invasion of sandflats in Langebaan Lagoon, South Africa, by the alien mussel *Mytilus galloprovincialis*: Size, composition and decline of the populations, African Zoology, 37(2): 197-208.

Harper E., Taylor J.D., Crame J.A. (2000). The Evolutionary Biology of the Bivalvia. Geological Society of London, Bath, UK.

He F., Hendrickson C.L., Marshall A.G. (2001). Baseline mass resolution of peptide isobars: a record for molecular mass resolution. Analytical Chemistry, 73: 647–50.

Henzel W.J., Billeci T.M., Stults J.T., Wong S.C., Grimley C., Watanabe C. (1993). Identifying proteins from two-dimensional gels by molecular mass searching of peptide fragments in protein sequence databases. Proceedings of the National Academy of Sciences of the United States of America, 90 (11): 5011-5.

Hess J.L., Blazer L., Romer T., Faber L., Buller M.R., Boyle M.D.P. (2005). Immunoproteomics. Journal of Chromatography B, 815: 65-75.

Hew C.L. and Fletcher G.L. (2001). The role of aquatic biotechnology in aquaculture. Aquaculture, 197: 1991-204.

Hewick R.M., Hunkapiller M.W., Hood L.E., Dreyer W.J. (1981). A gas-liquid solid phase peptide and protein sequenator. Journal of Biological Chemistry, 256: 7990-7997.

Hilbish T.J., Carson E.W., Plante J.R., Weaver L.A., Gilg M.R. (2002). Distribution of *Mytilus edulis*, *M. galloprovincialis* and their hybrids in open-coast populations of mussels in southwestern England. Marine Biology, 140 (1): 137-142.

Hochstrasser D.F., Harrington M.G., Hochstrasser A.C., Miller M. J., Merril C.R. (1988). Methods for increasing the resolution of two-dimensional protein electrophoresis. Analytical Biochemistry, 173: 424-435.

Hockey P.A. and van Erkom Schurink C. (1992). The invasive biology of the mussel *Mytilus galloprovincialis* on the Southern African coast. Transactions of the Royal Society of South Africa, 48 (1):123-139.

Hoh J.F.Y., McGrath P.A., White R.I. (1976). Electrophoretic analysis of multiple forms of myosin in fast-twitch and slow twitch muscles of the chick. Biochemistry Journal, 157: 87-95.

Hughes G.J., Frutiger S., Paquet N., Pasquali C., Sanchez J.C., Tissot J.D., Bairoch A., Appel R.D., Hochstrasser D.F. (1993). Human liver protein map: update. Electrophoresis, 14: 1216-1222.

Hunt D.F., Henderson R.A., Shabanowitz J., Sakaguchi K., Michel H., Sevilir N., Cox A.L., Appella E., Engelhard V.H. (1992). Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. Science, 255: 1261-1263.

Ideker T., Galitski T., Hood L. (2001). A new approach to decoding life: systems biology. Annual Review of Genomics and Human Genetics, 2: 343-372.

Innes D.J. and Haley L.E. (1977). Inheritance of a shell-color polymorphism in the mussel. Journal of Heredity 68: 203–204.

Integrated Taxonomic Information System (ITIS), www.itis.gov.

Invasive Species Specialist Group (ISSG) database, www.issg.org.

Islam M.D. and Tanaka M. (2004). Impacts of pollution on coastal and marine ecosystems including coastal and marine fisheries and approach for management: a review and synthesis. Marine Pollution Bulletin, 48: 624–649.

Issaq H.J., Conrads T.P., Prieto D.A., Tirumalai R., Veenstra T.D. (2003). SELDI-TOF MS for diagnostic proteomics. Analytical Chemistry, 75: 148A-155A.

Istituto di Ricerche Economiche per la Pesca e Acquacoltura (IREPA) (2009). Osservatorio economico sulle strutture produttive della pesca marittima in Italia. Salerno. www.irepa.org.

Istituto di Servizi per il Mercato Agricolo Alimentare (ISMEA) (2009). Il settore ittico in Italia. Check-up 2009. ISMEA, Rome. www.ismea.it.

Istituto di Servizi per il Mercato Agricolo Alimentare (ISMEA) (2010). Il settore ittico in Italia. Check-up 2010. Rome. www.ismea.it.

Jansen M., de Moor C.H., Sussenbach J.S., van den Brande J.L. (1995). Translational control of gene expression. Pediatric Research, 37: 681-686.

Jeebhay M.F., Robins T.G., Lehrer S.B., Lopata A.L. (2001). Occupational seafood allergy: a review. Occupational and Environmental Medicine, 58: 553-562.

Jensen O.N. (2004). Modification-specific proteomics: characterization of post-translational modifications by mass spectrometry. Current Opinion in Chemical Biology, 8: 33-41.

Kanaya S., Ujiie Y., Hasegawa K., Sato Imada H., Kinouchi M., Kudo Y., Ogata T., Ohya H., Kamada H., Itamoto K., Katsura K. (2000). Proteome analysis of *Oncorhynchus* species during embryogenesis. Electrophoresis, 21: 1907–1913.

Karas M. and Hillenkamp F. (1988). Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Analytical Chemistry, 60: 2299–2301.

Karp N.A., Spencer M., Lindsay H., O'Dell K., Lilley K. (2005). Impact of replicates types on proteomic expression analysis. Journal of Proteome Research, 4: 1867-1871.

Kendrick K.G. and Margolis J. (1970). Isoelectric focusing and gradient gel electrophoresis. A 2-dimensional technique. Analytical Biochemistry, 33: 204-207.

Kimmel D. G. and Bradley B.P. (2001). Specific protein responses in the calanoid copepod Eurytemora affinis (Poppe, 1880) to salinity and temperature variation. Journal of Experimental Marine Biology and Ecology, 266: 135–149.

Kirkpatrick D.S., Gerber S.A., Gygi S.P. (2005). The absolute quantification strategy: a general procedure for the quantification of proteins and post-translational modifications. Methods, 25: 265-273.

Kirschner M. (1999). Intracellular proteolysis. Trends in Cell Biology, 9: M42-M45.

Kitano H. (2002). Systems biology: a brief overview. Science, 295: 1662-1664.

Kjærsgård I.V.H., Nørrelykke M.R., Baron C.P., Jessen F. (2006). Identification of carbonylated protein in frozen rainbow trout (*Oncorhynchus mykiss*) fillets and development of protein oxidation during frozen storage. Journal of Agricultural and Food Chemistry, 54: 9437-9446.

Kling P. and Förlin L. (2009). Proteomic studies in zebrafish liver cells exposed to the brominated flame retardants HBCD and TBBPA. Ecotoxicology and Environmental Safety, 7: 1985–1993.

Kling P., Norman A., Andersson P.L., Norrgren L., Förlin L. (2008). Gender-specific proteomic responses in zebrafish liver following exposure to a selected mixture of brominated flame retardants. Ecotoxicology and environmental safety, 71: 319-327.

Klose J. (1975). Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues – novel approach to testing for induced point mutations in mammals. Humangenetik, 26: 231-243.

Knigge T., Monsinjon T., Andersen O.K. (2004). Surface-enhanced laser desorption/ionization-time of flight-mass spectrometry approach to biomarker discovery

in blue mussel (*Mytilus edulis*) exposed to polyaromatic hydrocarbons and heavy metals under field conditions. Proteomics, 4: 2722-2727.

Knoll-Gellida A., Andre M., Gattegno T., Forgue J., Admon A., Babin P.J. (2006). Molecular phenotype of zebrafish ovarian follicle by serial analysis of gene expression and proteomic profiling, and comparison with the transcriptomes of other animals. BMC Genomics, 7: 46.

Kültz D., Fiol D., Valkova N., Gomez-Jimenez S., Chan S.Y., Lee J. (2007). Functional genomics and proteomics of the cellular osmotic response in "non-model" organisms. Journal of Experimental Biology, 210: 1593-1601.

Laemmli U.K., (1970). Cleavage of structural proteins during the assembly of the head of bacteriofphage T4. Nature, 227: 680-685.

Lane D.J.W. and Nott J.A. (1975). A study of the morphology, fine structure and histochemistry of the foot of the pediveliger of *Mytilus edulis*. Journal of the Marine Biological Association of the United Kingdom, 55: 477-495.

Laughlin R.B. Jr. and French W. (1988). Concentration dependence of bis(tributyltin) oxide accumulation in the mussel M. edulis. Environmental of Toxicology and Chemistry, 7: 1021-1026.

Lee H., Williams S. K., Giddings J. C. (1998). Particle Size Analysis of Dilute Environmental Colloids by Flow Field-Flow Fractionation Using an Opposed Flow Sample Concentration Technique. Analytical Chemistry, 70: 2495-2503.

Lee S.Y and Morton B.S. (1985). The introduction of the Mediterranean mussel *Mytilus galloprovincialis* into Hong Kong. Malacological Review, 18: 107-109.

Lemeer S., Jopling C., Gouw J., Mohammed S., Heck A.J., Slijper M., den Hertog J. (2008a). Comparative phosphoproteomics of zebrafish fyn/yes morpholino knockdown embryos. Molecular and Cellular Proteomics, 7: 2176–2187.

Lemeer S., Pinkse M.W.H., Mohammed S., van Breukelen B., den Hertog J., Slijper M., Heck A.J.R. (2008b). Online automated in vivo zebrafish phosphoproteomics: from large-scale analysis down to a single embryo. Journal of Proteome Reserch, 7: 1555–1564.

Lemos M.F.L., Soares A.M.V.M., Correira A.C., Esteves A.C. (2010). Proteins in ecotoxicology: How, why and why not? Proteomics, 10: 873-887.

Leong J.C., Johnson M., Jordan D., Kim C., Simon B., Thomann E. (1999). Genetic vaccination of rainbow trout. 8th International Symposium of Society of Chinese Bioscientists in America, 14 – 19 August, Hong Kong. S4-S24.

Link A.J., Eng J., Schieltz D.M., Carmack E.; Mize G. J.; Morris D. R.; Garvik B. M.; Yates III J. R. (1999). Direct analysis of proteins complexes using mass spectrometry. Nature Biotechnology, 17: 676-682.

Link V., Carvalho L., Castanon I., Stockinger P., Shevchenko A., Heisenberg C.P. (2006). Identification of regulators of germ layer morphogenesis using proteomics in zebrafish. Journal of Cell Science, 119: 2073–2083.

Lipman D.J. and Pearson W.R. (1985). Rapid and sensitive protein similarity searches. Science, 227 (4693): 1435–1441.

Liu J., Huang T., McLuckey S.A. (2009). Top-Down Protein Identification/Characterization of a Priori Unknown Proteins via Ion Trap Collision-Induced Dissociation and Ion/Ion Reactions in a Quadrupole/Time-of-Flight Tandem Mass Spectrometer. Analytical Chemistry, 81: 1433-1441.

Liu X., Afonso L., Altman E., Johnson S., Brown L., Li J. (2008). O-acetylation of sialic acids in N-glycans of Atlantic salmon (*Salmo salar*) serum is altered by handling stress. Proteomics, 8: 2849–2857.

Livingston D.R. and Pipe R.K. (1992). Mussels and environmental contaminants: molecular and cellular aspects. In: The mussel *Mytilus*: ecology, physiology, genetics and culture. Gosling E. (Ed.), pp. 425-464. Elsevier, Amsterdam.

Lockwoood B.L. and Somero G.N. (2011). Invasive and native blue mussels (genus Mytilus) on the California coast: The role of physiology in a biological invasion. Journal of Experimental Marine Biology and Ecology, 400: 167-174.

Lokaj K., Meierjohann S., Schultz C., Teutschbein J., Schartl M., Sickmann A. (2009). Quantitative differential proteome analysis in an animal model for human melanoma. Journal of Proteome Research, 8: 1818–1827.

López J.L., Mosquera E., Fuentes J., Marina A., Vázquez J., Álvarez G. (2001). Two-dimensional gel electrophoresis of *Mytilus galloprovincialis*: differences in protein expression between intertidal and cultured mussels. Marine Ecology Progress Series, 224: 149-156.

López J.L., Marina A., Vázquez J., Álvarez G. (2002a). Application of proteomics for fast identification of species-specific peptides from marine species. Proteomics, 2: 1658-1665.

López J.L., Marina A., Vázquez J., Álvarez G. (2002b). A proteomic approach to the study of the marine mussels *Mytilus edulis* and *M. galloprovincialis*. Marine Biology, 141: 217-223.

López J.L. (2005). Role of proteomics in taxonomy: the *Mytilus* complex as a model of study . Journal of Chromatography B, 815: 261-274.

Lottspeich F. (1999). Proteome analysis: a pathway to the functional analysis of proteins. Angewandte Chemie International Edition, 38: 2476-2492.

Love D. R., Pichler F. B., Dodd A., Copp B. R., Greeenwood D.R. (2004). Technology for high-throughput screens: the present and future using zebrafish. Current Opinion in Biotechnology, 15: 564–571.

Lowe S., Browne M., Boudjelas S., De Poorter M. (2000). 100 of the World's Worst Invasive Alien Species: A Selection from the Global Invasive Species Database. Invasive Species Specialist Group of the Species Survival Commission (SSC) of the World Conservation Union (IUCN), Auckland, New Zealand. 12 pp.

Luber C.A., Cox J., Lauterbach H., Fancke B., Selbach M., Tschopp J., Akira S., Wiegand M., Hochrein H., O'Keeffe M., Mann M. (2010). Quantitative proteomics reveals subset-specific viral recogniction in dendritic cells. Immunity, 32: 279-289.

Lucitt M.B., Price T.S., Pizarro A., Wu W, Yocum A.K., Seiler C., Pack M.A., Blair I.A., FitzGerald G.A., GrosserT. (2008). Analysis of the zebrafish proteome during embryonic development. Molecular and Cellular Proteomics, 7: 981–994.

Lutz R.A. (1976). Annual growth patterns in the inner shell layer of Mytilus edulis. Journal of the Marine Biology Associates of the United Kingdom, 56: 723-731.

Lutz R.A. and Kennish M.J. (1992). Ecology and morphology of larval and early post-larval mussels. In: The Mussel *Mytilus*: Ecology, Physiology, Genetics and Culture. Gosling E. (Ed.), 53-85. Elsevier Science Publisher, B.V., Amsterdam.

Ma Y., Griesmeier U., Susani M., Radauer C., Briza P., Erler A., Bublin M., Alessandri S., Himly M., Vazquez-Cortes S., de Arellano I.R.R., Vassilopoulou E., Saxoni-Papageorgiou P., Knulst A.C., Fernandez-Rivas M., Hoffmann-Sommergruber K., Breiteneder H. (2008). Comparison of natural and recombinant forms of the major fish allergen parvalbumin from cod and carp. Molecular Nutrition and Food Research. 52: S196–S207.

Mackie I., Craig A., Etienne M., Jérôme M., Fleurence J., Jessen F., Smelt A., Kruijt A., Malmheden Yman I., Ferm M., Martinez I., Pérez-Martín M., Piñeiro C., Rehbein H., Kündiger R. (2000). Species identification of smoked and gravad fish products by sodium dodecylsulphate polyacrylamide gel electrophoresis, urea isoelectric focusing and native isoelectric focusing: a collaborative study. Food Chemistry, 71: 1-7.

Mackie I.M. (1980). A review of some recent applications of electrophoresis and iso-electrofocusing in the identification of fish species in fish and fish products. In: Advances in fish science and technology. Ed.: Connell J. J., Fishing News Books, London, 444–450.

Maestrale C., Carta A., Attene S., Galistu A., Santucciu C., Cancedda M.G., Saba M., Sechi S., Patta C., Bandino E., Ligios C. (2009). p.Asn176Lys and p.Met137Thr dimorphisms of

the PRNP gene significantly decrease the susceptibility to classical scrapie in ARQ/ARQ sheep. Animimal Genetics, 40: 982-985.

Makarov A. (2000). Electrostatic axially harmonic orbital trapping: a high-performance technique of mass analysis. Analytical Chemistry, 72: 1156–62

Malécot M., Mezhoud K., Marie A., Praseuth D., Puiseux-Dao S., Edery M. (2009). Proteomic study of the effects of microcystin-LR on organelle and membrane proteins in medaka fish liver. Aquatic Toxicology, 94: 153-161.

Manduzio H., Cosette P., Gricourt L., Jouenne T., Lenz C., Andersen O.K., Leboulenger F., Rocher B. (2005). Proteome modifications of blue mussel (*Mytilus edulis* L.) gills as an effect of water pollution. Proteomics, 5: 4958-4963.

Mann M., Højrup P., Roepstorff P. (1993). Use of mass spectrometric molecular weight information to identify proteins in sequence databases. Biological Mass Spectrometry, 22: 338–345.

Mann M. and Jensen O.N. (2003). Proteomic analysis of post-translational modifications. Nature Biotechnology, 21: 255-261.

Mann M. and Wilm M.S. (1994). Error tolerant identification of peptides in sequence databases by peptide sequence tags. Analytical Chemistry, 66: 4390-4399.

Marshall A.G., Hendrickson C.L., Jackson G.S. (1998). Fourier transform ion cyclotron resonance mass spectrometry: a primer. Mass Spectrometry Review, 17: 1-35.

Martin S.A.M., Cash P., Blaney S., Houlihan D.F. (2001). Proteome analysis of rainbow trout (*Oncorhynchus mykiss*) liver proteins during short term starvation. Fish Physiology and Biochemistry, 24: 259– 270.

Martin S.A.M., Vilhelmsson O., Medale F., Watt P., Kaushik S. ; Houlihan D.F. (2003). Proteomic sensitivity to dietary manipulations in rainbow trout. Biochimica et Biophysica Acta, 165: 17–29.

Martin S.A.M., Mohanty B.P., Cash P., Houlihan D.F., Secombes C.J. (2007). Proteome analysis of the Atlantic salmon (*Salmo salar*) cell line SHK-1 following recombinant IFN-gamma stimulation. Proteomics, 7: 2275–2286.

Martinez I., Ofstad R., Olsen R. L. (1990a). Electrophoretic study of myosin isoforms in white muscles of some teleost fishes. Comparative Biochemistry and Physiology, 96B: 221–227.

Martinez I., Ofstad R., Olsen R. L. (1990b). Intraspecific myosin light chain polymorphism in the white muscle of herring (*Clupea harengus harengus*, L.). FEBS Letters, 265, 23-26.

Martinez I., Ofstad R., Olsen R. L. (1990c). Myosin isoforms in red and white muscle of some marine teleost fishes. Journal of Muscle Research and Cell Motility. 11: 489-495.

Martinez I., Christiansen J.S., Ofstad R., Olsen R. L. (1991). Comparison of myosin isoenzymes present in skeletal and cardiac muscles of the Arctic charr *Salvelinus alpinus* (L.). European Journal of Biochemistry, 195: 743-753.

Martinez I., Bang B., Hatlen B., Blix P. (1993). Miofibrillar proteins in skeletal muscle of parr, smolt and adult Atlantic salmon (*Salmo salar*, L.). Comparison with another salmonid the Artctic charr *Salvelinus Alpinus* (L.). Comparative Biochemistry and Physiology, 106B: 1021–1028.

Martinez I., Christiansen J.S. (1994). Myofibrillar proteins in developing white muscle of the Arctic charr, *Salvelinus alpinus* (L.). Comparative Biochemistry and Physiology, 107B: 11–20.

Martinez I. and Friis T.J. (2004). Application of proteome analysis for seafood authentication. Proteomics, 4: 347-354.

Martínez-Fernández M., Rodríguez-Piñeiro A. M., Oliveira E., Páez de la Cadena M., Rolán-Alvarez E. (2008). Proteomic comparison between two marine snail ecotypes reveals details about the biochemistry of adaptation. Journal of Proteome Research, 7: 4926–4934.

Mascot Protein search engine from peptide mass maps databases of Matrix Science Ltd.: http://www.matrixscience.com/.

Mc Lafferty F.W., Breuker K., Jin M., Han X., Infusini G., Jiang H., Kong X., Begley T.P. (2007) Top-down MS, a powerful complement to the high capabilities of proteolysis proteomics. FEBS Journal, 274: 6256-6268.

McDonagh B. and Sheehan D. (2007). Effect of oxidative stress on protein thiols in the blue mussel *Mytilus edulis*: Proteomic identification of target proteins. Proteomics, 7: 3395–3403.

McDonagh B. and Sheehan D. (2008). Effects of oxidative stress on protein thiols and disulphides in *Mytilus edulis* revealed by proteomics: Actin and protein disulphide isomerase are redox targets. Marine Environmental Research, 66: 193-195.

McDonald J.H., Koehn R.H., Balakirev E.S., Manchenco G.P., Pudovkin A.I., Sergiyevskii S.O., Krutovskii K.V. (1990). Species identity of the 'common mussel' inhabiting the Asiatic coasts of the Pacific Ocean. Biologiëiìa Morëiìa, 1: 13-22.

McDonald J.H. and Koehn R.K. (1988). The mussels *Mytilus galloprovincialis* and *M. trossulus* on the Pacific coast of North America. Marine Biology, 99: 111-118.

McDonald J.H., Seed R., Koehn R.H. (1991). Allozyme and morphometric characters of three species of *Mytilus* in the Northern and Southern Hemispheres. Marine Biology, 111: 323-335.

McLean N., Nam S., Williams D., Lavender L. (1995). Book of Abstracts: Molecular Biology in Fish, Fisheries and Aquaculture: an international symposium, Fish Society of British Isles, Plymouth, U. K.

McQuaid C.D. and Phillips T.E. (2000). Limited wind-driven dispersal of intertidal mussel larvae: in situ evidence from the plankton and the spread of the invasive species *Mytilus galloprovincialis* in South Africa. Marine Ecology Progress Series, 201: 211-220.

Meierjohann S., Schartl M., Volff J. N. (2004). Genetic, biochemical and evolutionary facets of Xmrk-induced melanoma formation in the fish *Xiphophorus*. Comparative Biochemistry and Physiology Part C: Pharmacology, 138: 281–289.

Meiller J. C. and Bradley B. P. (2002). Zinc concentration effect at the organismal, cellular and subcellular levels in the eastern oyster. Marine Environmental Research, 54: 401–404.

Merril C.R., Goldman D., Sedman S.A., Ebert M.H. (1981). Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. Science, 211: 1437-1438.

Merril C.R., Switzer R.C., Van Keuren M.L. (1979). Trace polypeptides in cellular extracts and human body fluids detected by two-dimensional electrophoresis and a highly sensitive silver stain. Proceedings of the National Academy of Sciences of the United States of America 76: 4335-4340.

Mezhoud K., Praseuth D., Puiseux-Dao S., François J.-C., Bernard C., Edery M. (2008). Global quantitative analysis of protein expression and phosphorylation status in the liver of the medaka fish (*Oryzias latipes*) exposed to microcystin-LR I. Balneation study. Aquatic Toxicology, 86: 166-175.

Minden J. (2007). Comparative Proteomics and Difference Gel Electrophoresis. BioTechniques, 43: 739-745.

Mitton J.B. (1977). Shell colour and pattern variation in *Mytilus edulis* and its adaptive significance. Chesapeake Science, 18: 387-390.

Monsinjon T. and Knigge T. (2010). Proteomic applications in ecotoxicology. Proteomics, 7: 2997-3009.

Morton B. (1992). The evolution and success of the heteromyarian form in the Mytiloida. In: The Mussel *Mytilus*: ecology, physiology and culture. Goslin E. (Ed.), pp. 21-52. Elsevier Science Publishers B.V., Amsterdam.

Morton B. (1996). The evolutionary history of the Bivalvia. In: Origin and Evolutionary Radiation of the Mollusca, Editor: J.D. Taylor, Oxford University Press, Oxford (1996), pp. 337–359.

Morton J.E. (1967). Molluscs. Hutchinson University Library, London.

Mørtz E., O'Connor P.B., Roepstorff P., Kelleher N.L., Wood T.D., McLafferty F.W., Mann M. (1996). Sequence tag identification of intact proteins by matching tanden mass spectral data against sequence data bases. Proceedings of the National Academy of Sciences of the United States of America, 93 (16): 8264-8267.

Mosquera E., López J.L., Alvarez G. (2003). Genetic variability of the marine mussel *Mytilus galloprovincialis* assessed using two-dimensional electrophoresis. Heredity, 90: 432-442.

Motoyama A., Yates J.R.III. (2008). Multidimensional separation in Shotgun Proteomics. Analytical Chemistry, 80: 7187-7193.

Munford J.G., DaRos L., Strada R. (1981). A study on the mass mortality of mussels in the Laguna Veneta. Journal of the World Mariculture Society, 12: 186-199.

Nakamura R., Satoh R., Nakajima Y., Kawasaki N., Yamaguchi T., Sawada J., Nagoya H., Teshima R. (2009). Comparative study of GH-transgenic and non-transgenic amago salmon (*Oncorhynchus masou ishikawae*) allergenicity and proteomic analysis of amago salmon allergens. Regulatory toxicology and pharmacology, 55: 300–308.

Nazarian J., Hathout Y., Vertes A., Hoffman E.P. (2007). The proteome survey of an electricity-generating organ (*Torpedo californica*) electric organ. Proteomics, 7: 617–627.

Nesatyy V.J. and Suter M.J.F. (2008). Analysis of environmental stress response on the proteome level. Mass Spectrometry Reviews, 27: 556–574.

Neuhoff V., Arold N., Taube D., Ehrhardt W. (1988). Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis, 9: 255–262.

Newman A. (1998). RNA splicing. Current Biology, 8: R903-R905.

O'Farrell, P.H. (1975). High resolution of two-dimensional electrophoresis of proteins. Journal of Biological Chemistry, 250: 4007-4021.

O'Donovan C., Martin M.J., Gattiker A., Gasteiger E., Bairoch A., Apweiler R. (2002). High-quality protein knowledge resource: SWISS-PROT and TrEMBL. Briefings in Bioinformatics, 3: 275–84.

Oh J.E., Krapfenbauer K., Fountoulakis M., Frisher T., Lubec G. (2004). Evidence for the existence of hypothetical proteins in human bronchial epithelial, fibroblast, amnion, lymphocyte, mesothelial and kidney cell lines. Amino Acids, 26: 9-18.

Old W.M., Meyer-Arendt K., Aveline-Wolf L., Pierce K.G., Mendoza A., Sevinsky J.R., Resing K.A., Ahn N.G. (2005). Comparison of label-free methods for quantifying human proteins by shotgun proteomics. Molecular and Cellular Proteomics, 4: 1487-1502.

Oltvai Z.N. and Barabasi A.L. (2002). Systems biology. Life's complexity pyramid. Science, 298: 763-764.

Ong S.E., Blagoev B., Kratchmarova I., Kristensen D.B., Steen H., Pandey A., Mann M. (2002). Stable isotope labelling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Molecular Cellular Proteomics, 5: 376-386.

Ong S.E., Foster L.J., Mann M. (2003). Mass spectrometric-based approaches in quantitative proteomics. Methods, 29: 124-130.

Ortea I., Cañas B., Calo-Mata P., Barros-Velázquez J., Gallardo J.M. (2010). Identification of commercial prawn and shrimp species of food interest by native isoelectric focusing. Food Chemistry, 121: 569–574.

Papa I., Alvarez C., Verrez-Bagnis V., Fleurence J., Benyamin Y. (1996). Post mortem release of fish white muscle α-actinin as a marker of disorganization. Journal of the Science of Food and Agriculture, 72; 63 – 70.

Papa I., Taylor R.G., Astier C., Ventre F., Lebart M.C., Roustan C., Ouali A., Benyamin Y. (1997). Dystrophin cleavage and sarcolemma detachment are early post mortem changes on bass (*Dicentrarchus labrax*) white muscle. Journal of Food Science, 5: 917–921.

Pappin D.J. (1997). Peptide mass fingerprinting using MALDI-TOF mass spectrometry. Methods in Molecular Biology, 64: 165-173.

Pappin D.J., Hojrup P., Bleasby A.J. (1993). Rapid identification of proteins by peptide-mass fingerprinting. Current Biology, 3: 327-32.

Patterson S. D. and Aebersold R. (1995). Protein Identification and Characterization by Mass Spectrometry. Electrophoresis, 16: 1791-1814.

Pérez-Casanova J.C., Afonso L.O.B., Johnson S.C., Currie S., Gamperl A.K. (2008). The stress and metabolic responses of juvenile Atlantic cod (*Gadus morhua* L.) to an acute thermal challenge. Journal of Fish Biology, 72: 899–916.

Perkins D.N, Pappin D.J.C., Creasy D.M., Cottrell J.S. (1999). Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis, 20: 3551-3567.

Pflieger D., Le Caer J.P., Lemaire C., Bernard B.A., Dujardin G. (2000). Systematic identification of mitochondrial proteins by LC-MS/MS. Analytical Chemistry, 74: 2400-2406.

Picotti P., Bodenmiller B., Mueller L.N., Domon B., Aebersold R. (2009). Full dynamic range proteome analysis of S. cerevisiae by targeted proteomics. Cell, 138: 795-806.

Piñeiro C., Barros-Velázquez J., Sotelo C.G., Pérez-Martín R.I., Gallardo J.M. (1998). Two-Dimensional electrophoretic study of the water-soluble protein fraction in white muscle of gadoid fish species. Journal of Agricultural and Food Chemistry, 46: 3991-3997.

Piñeiro C., Vázquez J., Marina A.I., Barros-Velázquez J., Gallardo J.M. (2001). Characterization and partial sequencing of species-specific sarcoplasmic polypeptides

from commercial hake species by mass spectrometry following two-dimensional electrophoresis. Electrophoresis, 22: 1545–1552.

Piñeiro C., Barros-Velázquez J., Vázquez J., Figueras A., Gallardo J.M. (2003). Proteomics as a tool for the investigation of seafood and other marine products. Journal of Proteome Research, 2: 127–135.

Piñeiro C., Cañas B., Carrera M. (2010). The role of proteomics in the study of the influence of climate change on seafood products. Food Research International, 43: 1791-1802.

Plazzi F. and Passamonti M. (2010). Towards a molecular phylogeny of Mollusks: Bivalves' early evolution as revealed by mitochondrial genes. Molecular Phylogenetics and Evolution, 57: 641-657.

Ponder W.F., Lindberg D. R. (Editors) (2008). Phylogeny and evolution of the Mollusca. University of California Press, Berkeley and Los Angeles, California.

Pratt J.M., Simpson D.M., Doherty M.K., Rivers J., Gaskell S.J., beynon R.J. (2006). Multiplexed absolute quantification for proteomics using concatenated signature peptides encoded by QconCAT genes. Nature Protocols, 1: 1029-1043.

Prioli G. (2004). Shellfish Farming: Technologies and Production. Veterinary Research Communications, 28: 51–56.

Prioli G. (2008). La molluschicoltura in Italia. In: Lovatelli A., Farías A., Uriarte I. (Eds.) Estado actual del cultivo y manejo de mollusco bivalvos y su proyección futura: factores que afectan su sustenibilidad en América Latina. Taller Técnico Regional de la FAO. 20-24 de agosto de 2007, Puerto Montt, Chile. FAO Actas de Pesca y Acuicultura, n° 12. Roma, FAO. pp. 159-176.

ProFound Protein search engine from peptide mass maps databases of the Rockefeller University of New York (USA): http://prowl.rockefeller.edu/prowl-cgi/profound.exe.

Provan F., Bjørnstad A., Pampanin D.M., Lyng E., Fontanillas R., Andersen O.K., Koppe W., Bamber S., (2006). Mass spectrometric profiling - a diagnostic tool in fish? Marine Environmental Research, 62: S105–S108.

Purchon R.D. (1957). The stomach in the Filibranchia and Pseudolamellibranchia. Proceedings of the Zoological Society of London, 129: 27-60.

Rabilloud T. (2002). Two-dimensional electrophoresis in proteomics: old, old-fashioned, but it still climbs up the mountains. Proteomics, 2: 3-10.

Rajewsky K. (1986). Clonal selection and learning in the antibody system. Nature, 751-758.

Ramagli L.S. and Rodriguez L. (1985). Quantitation of microgram amounts of protein in two-dimensional polyacrylamide gel electrophoresis sample buffer. Electrophoresis, 6: 559-563.

Ramirez S.C., Cáceres-Martínez J. (1999). Settlement of the blue mussel *Mytilus galloprovincialis* Lamark on artificial substrates in Bahia de Todos Santos, BC, Mexico. Journal of Shellfish Research, 18: 33-39.

Rehbein H., Etienne M., Jerome M., Hattula T., Knudsen L.B., Jessen F., Luten J.B., Bouquet W., Mackie I.M., Ritchie A.H., Martin R., Mendes R. (1995). Influence of variation in methodology on the reliability of the isoelectric focusing method of fish species identification. Food Chemistry, 52: 193-197.

Rehbein H., Etienne M., Jerome M., Hattula T., Knudsen L.B., Jessen F., Luten J.B., Bouquet W., Mackie I.M., Ritchie A.H., Martin R., Mendes R. (1995). Influence of variation in methodology on the reliability of the isoelectric focusing method of fish species identification. Food Chemistry, 52: 193-197.

Rehbein H., Kündiger R., Malmheden Yman I., Ferm M., Etienne M., Jérôme M., Craig A., Mackie I., Jessen F., Martinez I., Mendes R., Smelt A., Luten J., Piñeiro C., Pérez-Martín M., (1999). Species identfication of cooked fish by urea isoelectric focusing and sodium

dodecylsulfate polyacrylamide gel electrophoresis: a collaborative study. Food Chemistry, 67: 333-339.

Rehbein H., Kündiger R., Malmheden Yman Y., Ferm M., Etienne M., Jerome M., Craig A., Mackie I., Jessen F., Martinez I., Mendes R., Smelt A., Luten J., Piñeiro C., Perez-Martin R. (1999). Species identification of cooked fish by urea isolectric focusing and sodium dodecylsulfate polyacrylamide gel electrophoresis: a collaborative study. Food Chemistry, 67: 333-339.

Reid R.G.B. (1968). The distribution of digestive tract enzymes in lamellibranchiate bivalves. Comparative Biochemistry and Physiology, 24: 727-744.

Reinders J. and Sickmann A. (2007). Modificomics: Posttranslational modifications beyond protein phosphorylation and glycosylation. Biomolecular Engineering, 24: 169-177.

Rime H., Guitton N., Pineau C., Bonnet E., Bobe J., Jalabert B. (2004). Post-ovulatory ageing and egg quality: A proteomic analysis of rainbow trout coelomic fluid. Reproductive Biology and Endocrinology, 2: 26.

Riva C., Binelli A., Rusconi F., Colombo G., Pedriali A., Zippel R., Provini A. (2011). A proteomic study using zebra mussels (*D. polymorpha*) exposed to benzo(α)pyrene: The role of gender and exposure concentrations. Aquatic Toxicology, 104: 14–22.

Robinson T.B., Govender A., Griffiths C.L., Branch G.M. (2007). Experimental harvesting of *Mytilus galloprovincialis*: Can an alien mussel support a small-scale fishery? Fisheries Research, 88: 33-41.

Robinson T.B., Griffiths C.L. (2002). Invasion of Langebaan Lagoon, South Africa, by *Mytilus galloprovincialis*: Effects on natural communities, African Zoology, 37: 151-158.

Robinson T.B., Griffiths C.L., McQuaid C.D., Ruis M. (2005). Marine alien species of South Africa—status and impacts. African Journal of Marine Science, 27: 297-306.

Rodríguez-Ortega M.J., Grøsvik B.E., Rodríguez-Ariza A., Goksøyr A., López-Barea J. (2003). Changes in protein expression profiles in bivalve molluscs (*Chamaelea gallina*) exposed to four model environmental pollutants. Proteomics, 3: 1535-1543.

Ross P., Huang Y., Marchese J.N., Williamson B., Parker K., Hattan S., Khainovski N., Pillai S., Dey S., Daniels S., Purkayastha S., Juhasz P., Martin S., Bartlet-Jones M., He F., Jacobson A., Pappin D.J. (2004). Multiplexed protein quantitation in *Saccharomices cerevisiae* using amine-reactive isobaric tagging reagents. Molecular and Cellular Proteomics, 3: 1154-1169.

Rout M.P., Aitchison J.D., Suprapto A., Hjertaas K., Zhao Y., Chait B.T. (2000). The yeast nuclear pore complex: composition, architecture, and transport mechanism. The Journal of Cell Biology, 148: 635-651.

Ruiz G.M., Carlton J.T., Grosholz E.D., Hines A.H. (1997). Global invasions of marine and estuarine habitats by non-indigenous species: mechanisms, extent, and consequences. American Zoologist, 37: 621-632.

Sagert J. and Waite J.H. (2009). Hyperunstable matrix proteins in the byssus of *Mytilus galloprovincialis*. The Journal of Experimental Biology, 212: 2224-2236.

Sancho A.I. and Mills E.N.C. (2010). Proteomic approaches for qualitative and quantitative characterization of food allergens. Regulatory Toxicology and Pharmacology, 58: S42–S46.

Schirle M., Heurtier M.A., Kuster B. (2003). Profiling core proteomes of human cell lines by one-dimensional PAGE and liquid chromatography-tandem mass spectrometry. Molecular and Cellular Proteomics, 2: 1297-1305.

Schirle M., Heurtier M.A., Kuster B. (2003). Profiling core proteomes of human cell lines by one-dimensional PAGE and liquid chromatography-tandem mass spectrometry. Molecular and Cellular Proteomics, 2: 1297-1305.

Schmale M.C. (2004). Aquatic animal models of human disease. Comparative Biochemistry and Physiology Part C: Toxicology, 138: 229–231.

Schultz I.R., Skillman A.D., Barry R., Wunschel D. (2008). Toxicokinetic and toxicodynamic aspects of trenbelone exposure in adult fathead minnows and rainbow trout. Fourteenth International Symposium on Pollutant Responses in Marine Organisms (PRIMO 14) – Environmental proteomics. Marine Environmental Research, 66: 101-103.

Scigelova M. and Makarov A. (2006). Orbitrap mass analyzer - overview and applications in proteomics. Proteomics, 6 (S2): 16–21.

Sebastián Ruiz C., Steffani C.N., Branch, G.M. (2002). Homing and movement patterns of a South African limpet *Scutellastra argenvillei* in an area invaded by an alien mussel Mytilus galloprovincialis, Marine Ecology-Progress Series, 243: 111-122.

Seed R. (1971). A physiological and biochemical approach to the taxonomy of *M. edulis* L. and *M. galloprovincialis* Lmk. From S.W. England. Cah. Biology Marine, 12: 291-322.

Seed R. (1976). Ecology. In: Marine Mussels: Their ecology and physiology. Ed. Bayne, B. L., pp. 13-65. Cambridge University Press, Cambridge.

Seed R. (1992). Systematics, evolution and distribution of mussels belonging to the genus *Mytilus*: and overview. American Malacological Bulletin, 9: 123-137.

Seed R., Suchanek T.H. (1992). Population and community ecology of Mytilus. In: The Mussel *Mytilus*: ecology, physiology and culture. Ed. Goslin E., pp. 87-169. Elsevier Science Publishers B.V., Amsterdam.

SEQUEST search engine for peptide and nucleotide databases of the The Scripps Research Institute, La Jolla (USA): http://fields.scripps.edu/sequest/.

Shadforth I., Crowther D., Bessant C. (2005). Protein and peptide identification algorithms using MS for use in high-throughput, automated pipelines. Proteomics, 5: 4082-4095.

Sheehan D. (2007). The potential of proteomics for providing new insights into environmental impacts on human health. Reviews on Environmental Health, 22: 175-94.

Shepard J.L. and Bradley B.P. (2000). Protein expression signatures and lysosomal stability in *Mytilus edulis* exposed to graded copper concentrations. Marine Environmental Research, 50: 457-463.

Shepard J.L. and Bradley B.P. (2000). Protein expression signatures and lysosomal stability in *Mytilus edulis* exposed to graded copper concentrations. Marine Environmental Research, 50: 457-463.

Shepard J.L., Olsson B., Tedengren M., Bradley B.P. (2000). Protein expression signatures identified in *Mytilus edulis* exposed to PCBs, copper and salinity stress. Marine Environmental Research, 50: 337-340.

Shevchenko A., Jensen O.N., Podtelejnikov A.V., Sagliocco F., Wilm M., Vorm O., Mortensen P., Shevchenko A., Boucherie H., Mann M. (1996). Linking genome and proteome by mass spectrometry: large-scale identification of yeast proteins from two dimensional gels. Proceedings of the National Academy of Sciences of the United States of America, 93: 14440-14445.

Sicherer S.H. and Sampson H.A. (2010). Food allergy. Journal of Allergy and Clinical Immunology, 125: S116–S125.

Skibinski D.O.F., Ahmad M., Beardsmore J.A. (1978). Genetic evidence for naturally occurring hybrids between *Mytilus edulis* and *Mytilus galloprovincialis*. Evolution, 32: 354-364.

Skibinski D.O.F., Beardmore J.A., Cross T.F. (1983). Aspects of the population genetics of *Mytilus* (Mitilidae: Mollusca) in the British Isles. Biological Journal of the Linnean Society, 19: 137-183.

Smith R.W., Wood,C.M., Cash P., Diao L., Pärt P. (2005). Apolipoprotein AI could be a significant determinant of epithelial integrity in rainbow trout gill cell cultures: a study in functional proteomics. Biochimica et Biophysica Acta, 1749: 81–93.

Somero G.N. (2004). Adaptation of enzymes to temperature: searching for basic "strategies". Comparative Biochemistry and Physiology Prt B: Biochemistry and Molecular Biology, 139: 321-333.

Søfteland L., Petersen K., Stavrum A.K., Wu T., Olsvik P.A. (2011). Hepatic in vitro toxicity assessment of PBDE congeners BDE47, BDE153 and BDE154 in Atlantic salmon (Salmo salar L.). Aquatic Toxicology, 105: 246–263.

Soot-Ryen T. (1969). The Superfamily Mytilacea. In: Treatise on Invertebrate Paleontology. Moore, R.C. (Ed.). Geological Society of America and University of Kansas Press. Boulder, Colorado and Lawrence, Kansas, Vol.1: 271–280.

Steffani C.N. and Branch G.M. (2003a). Growth rate, condition, and shell shape of *Mytilus galloprovincialis*: responses to wave exposure. Marine Ecology Progress Series, 246: 197–209.

Steffani C.N. and Branch G.M. (2003b). Spatial comparisons of populations of an indigenous limpet *Scutellastra argenvillei* and the alien mussel *Mytilus galloprovincialis* along a gradient of wave energy. African Journal of Marine Science, 25: 195–212.

Steffani C.N. and Branch G.M. (2003c). Temporal changes in an interaction between an indigenous limpet *Scutellastra argenvillei* and an alien mussel *Mytilus galloprovincialis*: effects of wave exposure. African Journal of Marine Science, 25: 213–229.

Steffani C.N. and Branch G.M. (2005). Mechanisms and consequences of competition between an alien mussel, *Mytilus galloprovincialis*, and an indigenous limpet, *Scutellastra argenvillei*. Journal of Experimental Marine Biology and Ecology, 317: 127-142.

Tanksley S.D. (1993). Mapping polygenes. Annual Review. Genetics, 27: 205-233.

Tay T.L., Lin Q., Seow T.K., Tan K.H., Hew C.L., Gong Z. (2006). Proteomic analysis of protein profiles during early development of the zebrafish, *Danio rerio*. Proteomics, 6, 3176–3188.

Tedesco S., Doyle H., Redmond G., Sheehan D., (2008). Gold nanoparticles and oxidative stress in *Mytilus edulis*. Marine Environmental Research 66: 131–133.

Tichá M., Pacákova V., Stulik K. (2002). Proteomics of allergens. Journal of Chromatography B, 771: 343-353.

Tognotti E. (2002). La coltivazione delle cozze nel golfo di Olbia. In: Parliamo della Sardegna. Brigaglia M. (Ed.). Il Messaggero Sardo, Anno XXXIV, December 2002, Cagliari.

Tomanek L. and Zuzov M.J. (2010). The proteomic response of the mussel congeners *Mytilus galloprovincialis* and *M. trossolus* to acute heat stress: implications for thermal tolerance limits and metabolic costs of thermal stress. The Journal of Experimental Biology, 213: 3559-3574.

Turgeon D.D., Quinn J.F.JR., Bogan A.E., Coan E.V., Hochberg F.G.JR., Lyons W.G., Mikkelsen P.M., Neves R.J., Roper C.F.E., Rosenberg G., Roth B., Scheltema A., Thompson F.G., Vecchione M., Williams J.D. (1998). Common and scientific names of aquatic invertebrates from the United States and Canada: Mollusks. 2nd Edition. American Fisheries Society, Special Publication 26, Bethesda, Maryland. ix + 526 pp.

UniProt Universal Protein Resource catalog: www.uniprot.org.

Ünlü M., Morgan M.E., Minden J.S. (1997). Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis, 18: 2071-2077.

Unwin R.D., Griffiths J.R., Whetton A.D. (2009). A sensitive mass spectrometric method for hypothesis-driven detection of peptide post-translational modifications: multiple reaction monitoring-initiated detection and sequencing (MIDAS). Nature Protocols, 4: 870-877.

van der Wielen L.A.M. and Cabatingan L.K. (1999). Fishing products from the sea-rational downstream processing of marine bioproducts. Journal of Biotechnology, 70: 363-371.

Vario S.L., Koehn R.K., Vainola R. (1988). Evolutionary genetics of the *Mytilus edulis* complex in the North Atlantic region. Marine Biology, 98: 51-60.

Venter J.C., Adams M.D., Myers E.W. (2001). The sequence of the human genome. Science, 291: 1303-1351.

Verma R., Chen S., Feldman R., Schieltz D., Yates J., Dohmen J., Deshaies R.J. (2000). Proteasomal proteomics: identification of nucleotide-sensitive proteasome-interacting proteins by mass spectrometric analysis of affinity-purified proteasomes. Molecular Biology of the Cell, 11: 3425-3439.

Vermeij G. (1991). Anatomy of an invasion: the trans-Arctic interchange. Paleobiology, 17: 281–307.

Verrez-**Bagnis** V., Ladrat C., Morzel M., Noel J., Fleurence J. (2001). Protein changes in post mortem sea bass (*Dicentrarchus labrax*) muscle monitored by one- and two-dimensional gel electrophoresis. Electrophoresis, 22: 1539–1544.

Verrez-Bagnis V., Noel J., Sautereau C., Fleurence J. (1999). Desmin Degradation in Postmortem Fish Muscle. Jounal of Food Science, 64: 240–242.

Viale I. (2009). Report "Il comparto dell'acquacoltura in Sardegna alla luce dei risultati dell'indagine conoscitiva" Laore, Regional Agency for the development in Agriculture of Sardinia, Siamaggiore, 2nd October 2009.

Viswanathan S., Ünlü M., Minde J.S. (2006). Two-dimensional difference gel electrophoresis. Nature Protocols, 1: 1351-1358.

Wahl J. H.; Goodlett D.R., Udseth H.R., Smith H.R. (1993). Use of small-diameter capillaries for increasing peptide and protein detection sensitivity in capillary electrophoresis-mass spectrometry. Electrophoresis, 14: 448-457.

Wahl J., Gale D.C., Udseth H.R., Smith R.D. (1992). Attomole level capillary electrophoresis-mass spectrometry protein analysis using 5-µm-i.d. capillaries. Analytical chemistry, 64: 3194-3196.

Wahl J.H.; Gale D.C.; Smith R.D. (1994). Sheathless capillary electrophoresis-electrospray ionization mass spectrometry using 10 µm I.D. capillaries: Analyses of tryptic digests of cytochrome c. Journal of Chromatography A, 659: 217-222.

Wang H., Chang-Wong T., Tang H.-Y., Speicher D.W. (2010). Comparison of Extensive Protein Fractionation and Repetitive LC-MS/MS Analyses on Depth of Analysis for Complex Proteomes. Journal of Proteome Research, 9: 1032-1040.

Wang N., MacKenzie L., De Souza A.G., Zhong H., Goss G., Li L. (2006). Proteome profile of cytosolic component of zebrafish liver generated by LC-ESI MS/MS combined with trypsin digestion and microwave-assisted acid hydrolysis. Journal of Proteome Research, 6: 263–272.

Ward R.D., Skibinski D.O.F., Woodwark M. (1992). Protein heterozygosity, protein structure, and taxonomic differentiation. Evolutionary Biology, 26: 73-159.

Ward D.G., Wei W., Cheng Y., Billingham L.J., Martin A., Johnson P.J., et al. (2006). Plasma proteome analysis reveals the geographical origin and liver tumor status of dab (Limanda limanda) from UK marine waters. Environmental Science and Technology, 40: 4031–4036.

Washburn M.P., Wolters D., Yates III J.R. (2001). Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nature Biotechnology, 19: 242-247.

Wasinger V.C., Cordwell S.J., Cerpa-Poljak A., Yan J.X., Gooley A.A., Wilkins M.R., Duncan M.W., Harris R., Williams K.L., Humphery-Smith I. (1995). Progress with gene-product mapping of the Mollicutes: Mycoplasma genitalium. Electrophoresis, 16: 1090-1094.

Watson J.D. and Crick F.H.C. (1953). Molecular Structure of Nucleic Acids: A Structure for Deoxyribose Nucleic Acid. Nature, 171: 737-738.

Wei Y., Chan L.L., Wang D., Zhang H., Wang J., Dai J. (2008). Proteomic analysis of hepatic protein profiles in rare minnow (*Gobiocypris rarus*) exposed to perfluorooctanoic acid. Journal of Proteome Research, 7: 1729–1739.

Weinberg S.R., Viner R.I., Ho P. (2002). Tagless extraction-retentate chromatography: a new global protein digestion strategy for monitoring differential protein expression. Electrophoresis, 23: 3182-3192.

Wells J.M. and McLuckey S.A. (2005). Collision-Induced Dissociation (CID) of peptides and proteins. Methods in Enzymology, 402: 148-185.

Widdows J., Donkin P. (1992). Mussels and environmental contaminants: bioaccumulation and physiological aspects. In: The Mussel *Mytilus*: ecology, physiology and culture. Goslin E. (Ed.), pp. 383-424. Elsevier Science Publishers B.V., Amsterdam.

Wiens J. (2000). Reconstructing phylogenies from allozyme data: comparing method performance with congruence. Biological Journal of the Linnean Society, 70: 613–632.

Wilkins N.P., Fujino K., Gosling E.M. (1983). The Mediterranean mussel *Mytilus galloprovincialis* Lmk. in Japan. Biological Journal of the Linnean Society, 20: 365-374.

Wilkins M.R., Pasquali C., Appel R.D., Ou K., Golaz O., Sanchez J.-C., Yan J.X., Gooley A. A., Hughes G., Humphery-Smith I., Williams K.L., Hochstrasser D.F. (1996). From Proteins to Proteomes: Large Scale Protein Identification by Two-Dimensional Electrophoresis and Amino Acid Analysis. Nature Biotechnology, 14: 61–65.

Wilkins M.R., Williams K.L., Appel R.D., Hochstrasser D.F. (1997). Proteome research: new frontiers in functional genomics. Springer, Germany.

Wilkins M.R., Gasteiger E., Tonella L., Ou K., Tyler M., Sanchez J.C., Gooley A.A., Walsh B.J., Bairoch A., Appel R.D., Williams K.L., Hochstrasser D.F. (1998). Protein identification with N- and C- terminal sequence tags in proteome projects. Journal of Molecular Biology, 278: 599-608.

Wilkins M.R., Yan J.X., Gooley A.A. (1999). 2-DE spot amino acid analysis with 9-fluorenylmethyl chloroformate. Methods in Molecular Biology, 112: 445-460.

Wilm M., Mann M. (1996). Analytical Properties of the Nanoelectrospray Ion Source. Analytical Chemistry, 68: 1-8.

Wilm M., Shevchenko A., Houthaeve T., Breit S., Schweigerer L., Fotsis T., Mann M. (1996). Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. Nature, 379: 466-469.

Wilm M.S. and Mann M. (1994). Electrospray and Taylor-Cone theory, Dole's beam of macromolecules at last?. International Journal of Mass Spectrometry and Ion Processes, 136: 167-180.

World 2D-PAGE database: http://world-2dpage.expasy.org/list.

Wu C. H., Yeh L.S., Huang H., Arminski L., Castro-Alvear J., Chen Y., Hu Z., Kourtesis P., Ledley R.S., Suzek B.E., Vinayaka C.R., Zhang J., Barker W.C. (2003). The Protein Information Resource. Nucleic Acids Research 31: 345–347.

Wu C.C. and MacCos M.J. (2002). Shotgun proteomics: tools for the analysis of complex biological systems. Current Opinions in Molecular Therapeutics, 4: 242-250.

Wulff T., Jessen F., Roepstorff P., Hoffmann E.S. (2008). Long term anoxia in rainbow trout investigated by 2-DE and MS/MS. Proteomics, 8: 1009–1018.

Wulfkuhle J.D., Paweletz C.P., Steeg P.S:, Petricoin E.F. 3rd, Liotta L. (2003). Proteomic approaches to the diagnosis, treatment, and monitoring of cancer. Advances in Experimental Medicine and Biology, 532: 59-68.

X!Tandem open source search engine for MS database search: www.thegpm.org/TANDEM

Yao X., Freas A., Ramirez J., Demirev P.A., Fenselau C. (2001). Proteolytic 18O labelling for comparative proteomics: model studies with two serotypes of adenovirus. Analytical Chemistry, 73: 2836-2842.

Yates J.R. III, Gilchrist A., Howell K.E., Bergeron J.J. (2005). Proteomics of organelles and large cellular structures. Nature Reviews Molecular Cell Biology, 6: 702-714.

Yates J.R. III, Speicher S., Griffin P.R., Hunkapiller T. (1993). Peptide Mass Maps: A Highly Informative Approach to Protein Identification. Analytical Biochemistry, 214: 397-408.

Yonge C.M. and Thompson T.E. (1976). Living Marine Molluscs. William Collins, Glasgow.

Zardi G.I., McQuaid C.D., Nicastro K.I. (2007). Balancing survival and reproduction: seasonality of wave action, attachment strength and reproductive output in indigenous Perna perna and invasive *Mytilus galloprovincialis* mussels. Marine Ecology Progress Series, 334: 155-163.

Zhang W. and Chait B.T. (2000). ProFound: an expert system for protein identification using mass spectrometry peptide mapping information. Analytical Chemistry, 72: 2482-89.

Zhu W., Smith J.W., Huang C—M. (2010). Mass spectrometry-based label-free quantitative proteomics. Journal of Biomedicine and Biotechnology, volume 2010, Article ID: 840518, 6 pages.

Zhu Z., Xu K., Xie Y., Li G., He L. (1989). A model of transgenic fish. Scientia Sinica (Series B), 2: 147-155.

Zilli L., Schiavone R., Storelli C., Vilella S. (2008). Molecular mechanisms determining sperm motility initiation in two sparids (*Sparus aurata* and *Lithognathus mormyrus*). Biology of Reproduction, 79: 356–366.

Zilli L., Schiavone R., Zonno V., Rossano R., Storelli C., Vitella S. (2005). Effect of cryopreservation on sea bass sperm proteins. Biology of Reproducion, 72: 1262–1267.

Ziv T., Gattegno T., Chapovetsky V., Wolf H., Barnea E., Lubzens E., Admon A. (2008). Comparative proteomics of the developing fish (zebrafish and gilthead seabream) oocytes. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics, 3: 12–35.

Zupanc M.M., Wellbrock U.M., Zupanc G.K.H. (2006). Proteome analysis identifies novel protein candidates involved in regeneration of the cerebellum of teleost fish. Proteomics, 6: 677–696.