Potential Use of Proteomics in Shellfish Aquaculture: from Assessment of Environmental Toxicity to Evaluation of Seafood Quality and Safety

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Abstract

A growing percentage of aquatic production worldwide is derived from aquaculture, whose importance is increasing due to commercial overfishing and a rising consumer demand for seafood. This has led to an increased interest in quality improvement and certification of seafood products to guarantee their safety, quality, authenticity, and nutritional benefits. In this context, proteomics has arisen as a promising tool for unravelling the biological, physiological and ecological traits of seafood products, thus improving cost-effectiveness and sustainability of aquaculture. As one of the fastest growing segments of global seafood production, shellfish farming can directly benefit from the application of these high-throughput technologies in search for biomarkers for the detection of shellfish contamination, health, quality, safety and nutritional value. With this in mind, the goal of this review is to highlight the potential of advanced and high-throughput proteomic tools for the evaluation of environmental toxicity and assessment of quality and safety in shellfish species, relevant both as bioindicators and as seafood products. An overview of the different proteomic approaches will be presented, outlining the main advantages, disadvantages and

challenges intrinsic to these techniques. Furthermore, several studies where proteomic techniques were applied for a better understanding of the mechanisms underlying alterations in shellfish proteomes are also described.

Keywords: Aquaculture, Shellfish, Proteomics, Ecotoxicoproteomics, Protein expression signatures, Biomarkers, Quality, Safety.

Running title: Shellfish Toxicity, Quality and Safety Assessment

1. Introduction

Aquaculture worldwide has increased rapidly in the last half century to accommodate a growing demand for fish and shellfish designed for human consumption. This industry has been going through major challenges in its effort to respond to the continuous higher human demand for seafood, in addition to the depletion of traditional fishery stocks and the increasing interest in the transparency of food processing, production, safety and marketing [1,2]. For this reason, national food control systems based upon scientific principles and guidelines in all sectors of the food chain are being promoted by the Food Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) to achieve improved food safety, quality and nutrition of seafood [3].

Aquaculture and farmed seafood in general, are vulnerable to a wide range of factors that pose immediate threat to its sustainability and consequently lead to considerable economical repercussions. Anthropogenic contamination is one of the main threats to this industry, specifically contamination due to high levels of high metals, polyaromatic hydrocarbons (PAHs), endocrine disrupting chemicals, pesticides, pharmaceutical or industrial contaminants, oil spills or other persistent environmentally substances entering the aquatic food chain [4]. Furthermore, shifts in environmental factors as climate and salinity changes, can also directly affect farming culture habitats and conditions and pose immediate risk to the physiological status of farmed organisms. As a consequence, severe biologic effects can directly arise on organisms that can potentially lead to indirect effects further up the food chain for human consumption [5,6].

To guarantee clear market transparency and to protect consumers, accurate, sensitive and fast detection methods allowing the monitoring of potential risks in any seafood product are highly recommendable. Proteomics in particular has emerged as a powerful tool for a better scientific understanding of the biological traits of seafood farming, thus leading to a higher productivity and better quality product in aquaculture. The study of proteins can provide important information about physiological functions and environmental effects in all living systems that can affect seafood quality properties. For this reason, the study of the proteome's dynamic changes can contribute to the discovery of potential biomarkers for evaluation of seafood quality and safety [2,4,7].

Shellfish farming has gained a great worldwide importance, not only for the wide variety of different species cultured around the world, but also because it is stimulated to expand by legislative initiatives, such as the proposed U.S. Offshore Aquaculture Act, and by an ever-increasing consumer demand [8]. Indeed, shellfish consumption is generally encouraged due to a low content of saturated fat, considerable levels of omega-3 polyunsaturated fatty acids [9-11], high quality animal proteins, vitamins, such as B12 and C, and minerals, such as Fe, Mn, P, Se, and Zn [12,13]. Moreover, they may be more sustainable than fish, since the environmental impact of their production is relatively low [14]. However, increased population and human activities constitute a threat for the environmental quality of farming areas and the quality/quantity of shellfish culture [15].

In recent years, proteomics has been applied to shellfish species in search for differentially regulated proteins and the characterization of biologically active proteins, primarily to investigate the alterations in the proteome that impact shellfish quality and safety (*e.g.* impact of contaminants, climate change, algal toxins, pathogens, allergens, processing and storage), with further repercussion to human health [2,4,6,7]. Nonetheless, the use of large-scale proteomics in shellfish farming is still in its infancy and the lack of information at the genome level for most of the shellfish cultured species is still a major limiting factor in aquaculture proteomics, making protein identification a challenging task.

With this in mind, this paper will focus on how advanced and throughput proteomic tools have been demonstrated to be very useful, not only for assessing toxicity related to environmental contamination, but also for determining safety and quality of shellfish food. An overview of the different proteomic approaches will be addressed, with reference to the limitations inherent to such methods, new approaches and future challenges.

2. Farming/aquaculture of shellfish

Shellfish aquaculture showed an increasing global production over the last 30 years (from 1 million tonnes in 1984 to 19.2 milion tonnes in 2014), with an actual estimated value of 55 million US dollars [16]. In 2014, ~92% of the shellfish global production came from Asia, whereas Europe and USA produced 4% and 3% of the world's shellfish, respectively (**Fig. 1**). Such global production was much more represented by bivalve molluscs (86%), than crustaceans (14%). Specifically, Asia produced 92% of the total marketed bivalves, followed by Europe (4%) and America (3%); however, this trend dramatically changes for crustacean production, since Asia confirmed to be the main producer (93%), followed by America but not Europe, showing a production near 0%, along with Oceania and Africa (**Fig.1**).

2.1. Main shellfish species used in aquaculture

As previously mentioned, shellfish include primarily molluscs and crustaceans, both of freshwater and marine origin. Bivalve molluscs are the most commonly cultured and widely distributed types of shellfish [16]. Species intended for human consumption are mussels, clams and oysters: they are generally farmed in shallower coastal areas, exploiting bottom and hanging/pole- farming systems. *Mytilus edulis* (blue mussel) and *Mytilus galloprovincialis* (Mediterranean mussel) are the principal cultured mussel species. Blue mussels are widely distributed in European waters, extending from the White Sea, Russia as far as south to the Atlantic coast of Southern France. Due to its abilities to withstand wide fluctuations in salinity, temperature, and oxygen tension, this species occupies a broad variety of microhabitats, expanding its zonational range from the high intertidal to subtidal regions, and its salinity range from estuarine areas to fully oceanic seawaters [17]. *M. galloprovincialis* is mainly cultured in coastal waters from Spanish Galicia to the northern coasts of the Mediterranean Sea. However, in Europe *M. galloprovincialis* is also found in the Black Sea, and in the Atlantic coasts of Portugal and France. The typical habitat for this species is represented by flooded river valley bordered by hills and characterized by muddy bottoms. These sheltered valleys provide an ideal environment for culturing mussels on ropes suspended from floating rafts [18]. The green-lipped mussel (*Perna canaliculus*) has been harvested for human consumption since the beginning of human habitation in New Zealand. Consequently, it is an endemic species of such country, although it is more common in the northern regions. Mussel farming is restricted to areas suitable with respect to its biology (high subtidal) and sea conditions (sheltered in-shore areas). The major growing areas are Coromandel, Marlborough Sounds, and Stewart Island [19].

Veneridae is a cosmopolitan and ubiquitous family of clams, distributed across all oceans, from intertidal flats to deep-sea areas, colonising all types of soft bottoms, including over 680 living species worldwide. In general, venerids burrow in muddy or sandy habitats, but can also colonize mangrove zones, coastal lagoons, estuaries, bays, surf zones and the deep sea. Some venerid species are exploited as a food source for human consumption, reaching high economic value in seafood market. Among them, *Venerups philippinarum* (manila clam), and the carpet shell clam, *Venerupis decussatus*, are mainly farmed in the Mediterranean Sea, Atlantic and Pacific oceans; while the hard shell clam, *Mercenaria mercenaria*, is mainly harvested in both Atlantic and Pacific oceans. *Chamalea gallina*, the common clam, occurs in the Mediterranean Sea and Atlantic Ocean [20].

Among oysters, commonly farmed species include the Eastern oyster (*Crassostrea virginica*), the Pacific oyster (*Crassostrea gigas*), the Belon oyster (*Ostrea edulis*), and the Sydney rock oyster (*Saccostrea glomerata*). In particular, *C. gigas* is native to the Pacific coast of Asia, and it has become the oyster of choice for cultivation in North America, Australia, Europe and New Zealand, because of its rapid growth and tolerance to different environmental conditions. It is an estuarine species, preferring rocky bottoms, but also mud and sand-mud bottoms [21]. Because of its economic importance, evolutionary position and use, *C. gigas* is also considered as an attractive model organism for environmental sciences, in order to investigate population responses to environmental stress and adaptation mechanisms, as well as for elucidating the physiological bases of complex vital process (*e.g.* growth, reproduction, survival) [22,23].

Shellfish aquaculture includes also a wide variety of freshwater and marine crustacean species. Decapods crustaceans, including crayfish, crabs, lobsters, prawns and shrimps, are most commonly farmed. Although fisheries are present in several countries, such as China, Spain and Portugal, crayfish have a major economical and social impact in the southern United States. The most common and widely distributed crayfish is *Procambarus clarkii*. It is found in lentic and lotic freshwater ecosystems, such as sluggish streams, swamps, ditches, sloughs and ponds [24]. *Cherax quadricarinatus* is a tropical crayfish species native to the rivers of northwest Queensland, Australia. Its preferred habitat is in high turbidity, slow moving streams or static water holes (billabongs) characterizing the rivers of the region. Beside the major producer country (Australia), such freshwater crustacean is also farmed in China, Indonesia, Israel, Morocco, Panama, Spain and the southern United States [25].

Scylla serrata, also known as Indo-Pacific swamp and mud crab, is the most widely farmed species among crabs. It is found in the Indo-West Pacific region from East and South Africa to southeast and East Asia, and Northeast

Australia, as well as in the eastern Pacific, around the Marianas, Fiji and the Samoa Islands. Typically associated with estuarine mangroves and sheltered coastal areas, such organisms inhabit muddy bottoms, where they dig deep burrows [26].

Panulirus ornatus and *Palinurus Homarus* are the favourite cultured species among the various tropical lobsters. Lobster production is basically dependent on the collection of naturally settled pueruli or juveniles, which in some areas of Vietnam and Indonesia are particularly abundant and easily caught. Beside Vietnam and Indonesia, Malaysia and Philippines are currently the main producer countries. Although active research and development programmes have focused on the development of lobster fisheries throughout the world, lobster production is to date limited to the mentioned countries. Indeed, the availability of sufficient pueruli and/or newly settled juveniles is potentially a key constraint to initiating farming trials. Nevertheless, lobster farming remains an attractive aquaculture source, since its species are among the most highly valued seafood and, consequently, worldwide traded [27].

Shrimps and prawns constitute most of the crustacean shellfish, and are cultured in two predominant areas, namely Asia and South America. Specifically, the giant freshwater prawn, *Macrobrachium rosenbergii*, is the most widely cultured shrimp in many Asian countries, such as Thailand, Indonesia, India, Vietnam, Sri Lanka, and Malaysia; while the Pacific white shrimp (*Litopenaeus vannamei*) is more common along the eastern coast of the Pacific Ocean, from Mexico to Peru. However, *L. vannamei* is increasingly replacing another important farmed species, the black tiger shrimp farming, *Penaeus monodon*, in many Asian regions, due to higher yields and more convenient production costs [15].

2.2. Use of shellfish as bioindicators of environmental toxicology

The most important bioindicators from the shellfish point of view are bivalve molluscs, being mussels and clams the most represented species [4]. Bivalves are well recognized as valuable bioindicators of environmental contamination, and extensive background information exists on their biological responses (physiological, cellular and molecular) to a wide range of both inorganic and organic compounds, as well as trace metals [28-30]. Mussels *Mytilus spp*. are the bivalve species more used worldwide that filter feeders and accumulate contaminants in their tissues well above that of surrounding waters [31]. Several other features make bivalves extensively used as sentinel organisms: (i) they are sessile, filter and accumulate particles from water, thus, measuring contaminant levels in

their tissues is a good indicator of the contamination of the surrounding environment; (ii) they are relatively resistant to a wide variety of contaminants and environmental stress (*e.g.* salinity, temperature), thus, being able to survive in naturally stressed environments with different degrees of contamination; (iii) they are easily collected and maintained under well-defined laboratory conditions; (iv) they are found in high densities in quite stable populations, allowing repeatedly sampling and time-integrated indication of environmental contamination throughout a sampling area; (v) they are widely distributed worldwide (both in freshwater and marine environments), allowing data comparison from different areas [32-34]. Nonetheless, marine species rather than freshwater species are more commonly investigated in scientific research mainly due to their higher economic value associated to human consumption and aquaculture. For the above reasons, several monitoring programs were developed, including the "Mussel Watch" Project (MWP) initiated in 1986 using *Mytilus edulis*, to assess the current status and the long-term changes in environmental quality in US estuarine and coastal waters. Nowadays, the "Mussel Watch" concept has spread worldwide, where several monitoring programs developed routine sampling using bivalve molluscs [32,33,35]. Although addressed to a lesser extent by the scientific community, many crustacean species are also considered as valuable bioindicators for assessing the impact of environmental pollution. In fact, due to the high abundance and wide distribution, decapods crustaceans represent another ecologically and throphically important component of benthic habitats. Furthermore, the high tolerance to different physico-chemical characteristics of sediment and water, as well as sensitivity to pollutants and toxicants, and the capability of accumulating them, makes these organisms well suitable for marine monitoring programmes and ecotoxicological studies [36]. Marine prawns were investigated for studying the stress response to pollutants, such as pesticides [37], and heavy metals [38]. The crayfish *P. clarkii* was proposed as suitable bioindicator of pesticide exposure [39] and heavy metal and metalloid contamination [40]; while other studies considered crabs for monitoring pollution in freshwater ecosystems [41,42].

3. Proteomics definition and technologies

Proteomics is the study of the proteome, a catalogue of all proteins expressed by a genome within a cell, tissue or organism at a particular time and space. While genome activity is relatively stable, the proteome is very dynamic, as the protein content of cells varies in response to a diverse stimuli (*e.g.* physiological state of the cell, drug administration, health and disease) and environmental factors. Additionally, expression and quantity of proteins

differs between different cell types within an organism, whereas the genome remains relatively unchanged [43,44]. Proteomics technologies have received increased attention since neither genomic or transcriptomic data can provide a complete picture of the dynamic processes inside an organism in response to diverse stimuli [45,46]. Therefore, the identification of a protein function and regulation in the context of its cellular environment is essential to understand the metabolic and structural signals that control growth, development, replication and stress response of cells [46]. When compared to conventional chemical and analytical methods, proteomics offers the possibility to map the entire proteome of an organism, detect toxic effects at significantly lower doses, as well as faster screening for potential adaptive mechanisms by the use of highly sensitive analytical techniques. Furthermore, in combination with already established detection tools, proteomics also provides new insights into molecular mechanisms underlying key cellular processes, as well as the distinction between species-specific effects, thus facilitating more reliable risk assessment in environmental sciences, and safety, quality and traceability in food science, in view of the improvement of human health [47,48].

As a discipline, proteomics includes not only the study of the structure and functional of proteins but also the analysis of their modifications, protein-protein interactions, protein intra-cellular localization and its quantitative expression [4,7]. Proteomics analysis is characterized by high-throughput methodologies that enable high-resolution separations and the display of all proteins in a tissue in a way that allows subsequent analysis and comparison. From the hundreds to thousands of proteins that can be obtained in one experiment, those that are either expressed under a given condition or suppressed can subsequently be identified [43,44,48]. Recent advances concerning proteome analysis methodologies (mass spectrometry detection techniques and bioinformatic treatment of data), sample preparation and genome sequencing has allowed this discipline to evolve and expand from mere protein profiling to accurate and high-throughput protein quantification between two or multiple biological samples [4, 49]. Given the variety of proteome analysis techniques available, information on proteins can be derived either from top-down proteomics or bottom-up proteomics. In bottom-up proteomics, complex peptide mixtures are initially subjected to enzyme cleavage, and the resulting peptide fragments are subsequently analysed by high-speed tandem mass spectrometry instruments and high throughput in silico database searches to relate mass spectra to peptide sequences, then peptides to their parent protein. On the other hand, in top-down proteomics intact proteins are directly subjected to either protein specific separation or fractionation techniques before identification. These two approaches are complementary, and neither can provide the whole information for a protein of interest [47,50,51].

As a large-scale study of the proteome, the field of proteomics ranges from global to "target" protein analysis. While global analysis is focused on large-scale characterization, quantification and identification of all proteins within a particular proteome, the "targeted" mode gives the opportunity to look at alterations within the sub-proteomes (*e.g.* proteins in organelles, nucleus or proteins that are part of signalling pathways) to assess low abundant proteins that otherwise would be overlooked within the high abundant proteins present in the cell [47,52]. Quantitative proteomics (*i.e.* the global analysis of protein expression) has been successfully applied in environmental studies to define protein expression signatures (PESs) of a specific cell or tissue that associate protein expression and post-translational modifications (or PTMs) to the condition and level of environmental stress, thus providing new biomarkers of exposure and effect [53,54]. More recently, this approach has been applied to seafood products, in the search for new potential sensitive biomarkers for evaluation of shellfish quality and safety. Quantitative proteomic approaches can either be classified as gel-based or gel-free, as well as label-free or label-based, of which the latter can be further subdivided into the various types of labelling approaches as chemical and metabolic labelling. Since an in depth explanation and evaluation of the techniques and instrumentation used in proteomics is out of the purpose of this review, a brief and general overview of the available tools used in proteomic studies will be given below, with reference to different methods, new approaches and challenges (for more information see reviews by [47,52,55,56]).

3.1. Overview of proteomic techniques

A typical proteomic workflow includes the following three major steps: a) sample preparation and extraction, b) protein separation and quantification and c) protein identification and characterization. This workflow can either be applied to gel-based or gel-free approaches, depending on the method chosen for protein separation and quantification (bottom-up or top-down) and the accessibility to a mass spectrometer [2,55,57]. The common workflow for gel-based and gel-free proteomic analysis is represented in **Fig. 2**, differentiating between the bottom-up and top-down approaches.

3.1.1. Gel-based proteomics

The success of any proteomic workflow is highly dependent on sample preparation. The first general step for sample preparation starts with protein extraction, where all proteins (or a specific subset) present in the sample of interest

are solubilised in an appropriate solvent (*e.g.* aqueous buffers, organic solvents). For marine organisms, shellfish species included, a precipitation step to eliminate salts is advised in order to avoid interference with gel-based and chromatographic separation methods. Sample fractionation can also be used to improve protein separation and identification, where protein extracts can be simplified and/or the dynamic range of a protein mixture improved. Methods as chromatography, differential solubility, electrophoresis and (ultra) centrifugation are commonly used, aimed at isolation of organelles and sub-cellular compartments (nuclei, mitochondria), enrichment of proteins sub-populations (*e.g.* phosphorylated and membrane proteins) and reduction of highly abundant proteins (*e.g.* albumin and immunoglobulins in blood plasma) [2,51,55].

The most current and common separation technique to study protein and PTMs (including protein synthesis, post-translational modifications, and degradation) is the classical two-dimensional gel electrophoresis (2-DE), particularly the 2-DE SDS-PAGE. This technique allows the separation of several thousands of proteins from protein extract samples (crude or fractionated) first by isoelectric focusing (IEF, first dimension), in which proteins are separated according to their molecular charge (or isoelectric point, p*I*) through an immobilized pH gradient (IPG). Secondly, the focused proteins are separated orthogonally through SDS PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) based on their molecular mass (M*r*), originating a two-dimensional map [43,44,55]. This technique can routinely separate hundred to thousands of proteins in a specific sample (up to 10000 proteins), including their small PMTs and highly homologous isoforms, thus allowing high-resolution proteomic analysis. Consequently, 2-DE has become one of the best experimental tools for reliable separation in protein analysis and has proven suitable for the global analysis of protein expression in shellfish species [6,71]. Nevertheless, 2-DE is known to have some limitations, as it is time consuming and labour intensive, has limited linear dynamic range to display proteins of widely varying concentrations and provides poor solubilisation of proteins at extremes of hydrophobicity and isoelectric point. The limited loading capacity associated with this technique will also affect the sensitivity and dynamic range to detect low abundant proteins resulting in imprecise quantification and misidentification. Another disadvantage of this technique is the lack of reproducibility between gels, as several gels need to be run per treatment leading to large gel-to-gel variations [45,49,50,56,57].

Classically, proteins obtained by 2-DE can be detected and quantified by staining (*e.g.* silver staining, Coomassie brilliant blue) and scanning 2-DE gels in the visible range, giving rise to a visible array of spots with varying intensity [2,56]. However, as different stains present variable sensitivities and different protein ranges, some issues

may also arise in protein visualization especially considering low abundance proteins. Several image analysis systems and software for digitalizing and quantifying protein amounts in spots are commercially available for analysis of 2-DE gels, being the most commonly used PDQuest 2-D Analysis software (Bio-Rad Laboratories), Image Master 2-D Platinum (Amersham Biosciences), Progenesis SameSpots (Non Linear Dynamics) and DeCyder (GE Healthcare). Although several of the mentioned software use simplified, semi-automated analysis to overcome some of the disadvantages associated with 2-DE, some of the results obtained are still ambiguous and therefore require visual inspection and user intervention to manually correct the software and improve the accuracy in spot matching [2,49,51].

Two-dimensional differential gel electrophoresis (2D-DIGE), a modified version of the classical 2-DE, has been recently developed to overcome the 2-DE inherent inter-gel variability. 2D-DIGE involves differential labelling of proteins in various treatment groups with different fluorescent cyanine dyes (CyDye) and separates them on the same 2-D gel, eliminating gel-to-gel variability. This significant reduction in inter-gel variability makes spot matching and identification more sensitive and accurate, increasing the number of spots that can be analysed. Additionally, the use of a pooled internal standard (loaded with the control and treated samples) increases the reliability of quantification and statistical confidence of experimental data. Similarly to 2-DE, 2D-DIGE also provides the ability to detect PTMs, which are generally difficult to detect using other proteomic approaches [45,49,56,58].

Upon protein spot detection and quantitation, gel matching and identification of differences in protein expression between sets of samples using a software-assisted 2-DE gel analysis, sets of differentially expressed proteins can be selected for further identification by mass spectrometry based techniques. These proteins of interest are excised and digested to peptides using site-specific proteases such as trypsin [44,55]. The resultant set of peptide mixtures are then identified and characterized using mass spectrometry (MS). During the first step of MS analysis, the masses of corresponding peptides are measured after a separation on the basis of their mass-to-charge (*m/z*) ratio, giving a unique peptide mass fingerprint (PMF). Proteins can then be identified directly through matching of the PMF with all proteins included in the database or by analysis of the fragmentation spectra of selected peptides, creating a peptide fragment fingerprint (PFF) through tandem MS (MS/MS). The data obtained by MS/MS contains information concerning the peptide amino acid sequence, masses, fragment ion masses, and intensities, which is transformed into a peak list and matched against theoretical spectra fragments obtained from sequences databases

(open-source or proprietary software packages) and the corresponding proteins identified. Three types of databases are available: protein databases (*e.g.* Swiss-Prot, National Centre for Biotechnology Information or NCBI and TrEMBL), genomic databases, and translated expressed sequence tag (EST) databases [2,47,55]. Given the advances and accuracy of current MS techniques and the low number of proteins present in a single 2-DE spot, the MS and MS/MS data obtained are quite straightforward and reliable. However, peptide identification using MS spectra can be challenging and limiting in the case of non-model organisms (as the case of most shellfish species), whose genomes are not yet fully sequenced or available to the public and thus only limited information exists on the available databases. If insufficient information exists on the databases, *de novo* peptide sequencing can be applied to assign an amino acid sequence to the peptide in question and the resulting sequence tag used to search for homology with proteins from other organisms [6,55].

Gel-based proteomics has been so far the main approach used in shellfish proteomics. 2D-DIGE has recently started to be more commonly used, even though the number of studies using this improved technique is still lower compared to classical 2-DE.

3.1.2. Gel-free proteomics

Even though gel proteomics still remains one of the most popular methods for visualizing and processing expression proteomics globally, the technique still presents some drawbacks, mostly related to quantitative reproducibility and ability to study certain classes of proteins. Therefore, with the advances in MS instrumentation, the use of alternative approaches such as gel-free protein isolation techniques is becoming increasingly popular [47,48,50]. The gel free proteomics technology allows higher analytical throughput and larger scale protein expression and characterization than gel-based methods, being suitable for the analysis of proteins with low abundance in complex samples. It profits from the liquid chromatography (LC) system to efficiently separate proteins and peptides in complex samples, assuring more sensitive and more accurate protein quantitation and high quality protein identification. Several new proteomic tools have been derived from LC-based methods and even though their use for seafood research is still in its infancy, they could become an important set of tools for future application in shellfish quality and safety. In shotgun proteomics (bottom-up strategy), crude protein extracts can be digested directly (proteolytic enzymes) without any previous separation and the complex peptide fractions generated can be separated by multi-dimensional separation by LC coupled to MS/MS. Since peptides can be more easily separated by LC than proteins,

this approach offers a faster and cheaper high-throughput analysis of the proteome of a sample of interest and provides a snapshot of the major protein constituents. Given the complexity associated with the digestion of a complex protein mixture, fractionation of samples is commonly associated with gel-free approaches to reduce the number of different peptides present in each fraction. Several methods for multidimensional fractionation are currently available to reduce sample complexity through peptide separation according to size, charge, and hydrophobicity, being the most commonly used linked to liquid-phase chromatography procedures followed by Electrospray Ionization (ESI)-based mass spectrometers. MudPIT (or multidimensional protein identification) is another example, where peptides are separated by charge (Strong Cation-Exchange High Performance Liquid Cromatography, SCX-HPLC) and hydrophobicity (Reversed Phase (RP)-HPLC) preceding MS analysis. Peptide quantification in most gel-free workflows can either rely on label-based (chemical isotope tags or metabolic labelling) or label-free methods, being the applicability of each method defined according to their sensitivity, sample-specific characteristics and experimental set-up. There are several label-based approaches, being the most commonly known the isotope coded affinity tags (ICAT), the isobaric tags for relative and absolute quantification (iTRAQ), the tandem mass tags (TMT), the stable isotope labelling with amino acids in cell culture (SILAC) and dimethyl labelling. Nonetheless, the concept for all approaches is similar, in which tagging results in quantifiable mass shifts of the labelled amino acids [49,51]. Quantitative comparison of protein samples was first demonstrated using ICAT, which derivatizes cysteine-containing peptides with reagents of a different isotopic composition (light or heavy isotopes) that yield a pair of ions with 8 Da mass-shifts. These peptide pairs are detected in MS scans and their ion intensities compared for relative quantitation. However, as cystein do not cover the whole proteome (present in only one of seven proteins), several other methods were developed that allow to globally label peptides, independent of their amino acid composition or PTMs [47,49]. iTRAQ is a widely used chemical labelling technique in which all peptides in a digested protein mixture are labelled using the same amine reactive isotopic tags for derivatization of peptides at the N-terminus and the lysine side chains. After fragmentation using MS/MS, signature ions are produced (separated by small mass shifts) and relative quantitative information estimated upon integration of the peak areas of identical peptides [2,47,48,51,55]. Similarly to iTRAQ, TMT uses coding reagents of equal physicochemical properties and overall mass to guarantee their chromatographic co-elution; however different fragmentation patterns are produced during MS/MS analysis (release of “daughter ions”), which can be used for relative quantification. These techniques are complementary with similar quantitative precision and accuracy, even

though more peptides and proteins can be identified with iTRAQ, while ICAT allows the identification of a higher number of proteins with lower abundance [47,49]. Like the 2-DE approach, information on protein abundance/identity is obtained using computational tools. SILAC is an example of metabolic labelling commonly used that allows protein labelling at the time of protein synthesis. Briefly, essential amino acids are added to amino acid deficient cell culture media and are incorporated into all proteins as they are synthesized. However, this technique was developed towards studies with cell cultures [49,59]. Another labelling method for quantitative proteomics that uses stable isotope incorporation at the peptide level is the dimethyl labelling. This chemical labelling method labels the primary amines (N-terminus of proteins/peptides and lysine residue side chains) through reductive amination after digestion with trypsin. The obtained labelled samples are simultaneously analyzed by LC-MS and the mass shifts of the dimethyl labels are compared with peptide abundance in the different samples. The procedure is very straightforward, reliable and rapid and has the advantage of employing reagents that are commercially available at costs significantly lower than the other labelling methods referred previously. In addition, this procedure can be easily automated and applied in high-throughput proteomics experiments and is applicable to virtually any sample [60,61]. Despite these attractive properties, dimethyl labelling has not been widely used compared to other commercial chemical labelling reagents, especially for shellfish research.

Quantitative proteomics based on labelling approaches present some disadvantages, as they often require expensive labelling reagents, have several sample preparation steps and require high amount of sample, resulting in sample loss and reduced sensitivity in peptide detection. In this context, label-free proteomics is a simpler, cost-effective and reproducible alternative to the traditional labeling techniques, less prone to errors and side reactions related to the labelling process. In this type of approach, quantification can be performed by comparing multiple unprocessed LC-MS datasets (relative peptide peak intensities) using internal standards normalized to the data with commercially available software. Peptides showing differential expression in peak signal intensity are matched against the available databases, for peptide and protein identification. Label-free quantification decreases the time needed to search spectra and at the same time increases throughput, allowing higher proteome coverage and greater dynamic range of quantification, thus being highly advantageous in biomarker discovery [49,51,56]. Even though these techniques are still underrepresented in shellfish proteomics, its applicability for global screening for seafood research has proven to be very convenient and cost-effective [62].

Gel-free proteomics can also use a top-down approach, where intact proteins without previous enzymatic digestion

are identified and characterized by deducing a partial amino acid sequence after fragmentation using MS/MS techniques. However, difficulties related to ionization of whole proteins, complexity of spectra produced (presence multiple charged peptide ions), and infancy in software development make top-down approaches intrinsically more challenging. In comparison with bottom-up approaches, top-down approaches are increasingly practical due to the use higher resolution of MS instruments using Fourier transform ion cyclotron resonance (FT-ICR) or Orbitrap technologies [2,47].

Although the gel-free methods described earlier were initially seen as replacements for gel-based methods, they should instead be used in complement to 2-DE. Several points of comparison and contrast exist between the standard 2-DE and shotgun analyses (*e.g*. sample consumption, depth of proteome coverage, analyses of isoforms and quantitative statistical power), so, the use of several of the mentioned techniques in a complementary way will result in the identification and quantification of non-overlapping groups of proteins, thus more closely reproducing the whole proteome of a sample of interest. Nonetheless, these techniques and the associated procedures still need improvements as they still present limitations due to technical problems associated LC separation and data analysis [63]. Thus, the 2-DE technique still plays an important role in current research of proteomics.

3.1.3. PTMs and redox proteomics

PTMs are modifications to a protein after protein synthesis in response to a wide range of extra- and intra-cellular signals, whose function is to either enhance or repress certain attributes or abilities of a protein. Several cellular processes are regulated by different PTMs including enzymatic activity (activation/inactivation), gene expression, protein/protein interactions, signal transduction, cellular protein localization and protein stability. PTM research is continually growing with specific software, and databases rapidly evolving. More than 300 different PTMs have been described, with protein phosphorylation, glycosylation, acetylation, and methylation receiving most attention [50,64,65]. Identification of PTMs is still suffering from the lack of sensitive methodology and sufficient protein samples for analysis and characterization, often requiring the use of a versatile battery of approaches and methodologies (*e.g.* 2-DE, radiolabeling, western analysis with antibody against specific modification and mutagenesis of modification sites) [47,66]. 2-DE, western blotting and sample preparation techniques useful for the separation, enrichment and purification of peptides and PTMs are available in a number of commercially available kits that can potentially be useful for routine aquatic monitoring using shellfish species [4,56].

Identification of protein PTMs is usually performed at both protein level (top-down proteomics) and peptide level (bottom-up proteomics), facilitated by the use of affinity-purification techniques, thus allowing a specific look at the sub-proteome level. In relation to shellfish species, the majority of the specific protein modifications described so far is due to oxidative stress caused by environmental contamination, thermal stress and exposition to ultraviolet light, and are commonly detected using a combination of gel electrophoresis and Western blot [52,56]. Proteins and their side chains can undergo a range of reversible and irreversible modifications due to oxidative stress and even though some are protective of the protein´s structural integrity, some can lead to inactivation of proteins. More recently, the detection of these oxidative stress-specific protein modifications has been defined as redox proteomics [67-69]. Some of the most commonly reported oxidative modifications in proteins are ubiquitination, the formation of carbonyl groups (carbonylation) and glutathionylation, which can be assessed by 2-DE followed by Western blot immunoassay (oxyblot). Ubiquitin targets the protein-transport machinery to transport short-lived intracellular regulatory proteins or abnormal cytosolic or nuclear proteins for degradation in the proteasome *via* the ubiquitin proteasome pathway (UPP) [70]. The UPP is then responsible to remove damaged proteins from cells by proteolysis, making this process essential for normal cell growth and viability [71]. After protein separation by 2-DE, ubiquitination can be detected by specific staining using anti-ubiquitin antibodies after blotting the proteins to a membrane [72,73]. Ubiquitinylated proteins produce diglycine branched peptides after tryptic digestion, which can be used as markers in MS/MS analysis [74]. Carbonylation is a process by which protein side chains can be irreversibly modified to aldehyde or ketone groups that can lead to protein aggregation, inactivation and degradation [75]. Protein carbonylation can be detected following the chemical derivatization of carbonyl groups with dinitrophenylhydrazine (DNP) prior to electrophoresis to allow identification after transfer to a blot membrane. The proteins spots originated by immunostaining with an anti-DNP antibody can be further analysed and identified by MS/MS [76]. Glutathionylation arises from the reaction between free cysteine groups (-SH) and the reduced form of glutathione, catalyzed by glutaredoxin, and is mostly viewed as a protective strategy to mask these groups until oxidative stress is overcome [76]. Similarly to ubiquitination and carbonylation, protein glutathionylation is readily detectable by Western blotting, using GSH anti-bodies [77].

Modern proteomics technologies evaluating changes in the PTM sub-proteome are slowly gaining weight in environmental research, significantly offering new molecular markers associated with the presence of contaminants in the environment and helping unravel their mechanisms of toxic action [77]. Nonetheless, redox proteomics may

also play an important role in assessing shellfish quality and safety, as the nutritional maintenance of seafood properties can be affected by the oxidative modifications previously mentioned [47,69].

4. Environmental proteomics

Ecotoxicology as a science aims to understand the effects of chemical toxicants (or other biotic and abiotic stressors such as temperature, UV light, predation, *etc.*) on ecologically relevant species [78]. Classically, biological monitoring includes the assessment of the physiological status of bioindicator organisms living in the monitored environment, by determining the values of selected biological parameters that are known to vary in response to the toxic effects of contaminants [79]. Many of these biological responses have been proposed and are used as biomarkers (*e.g.* metallothionein, heat-shock proteins, glutathione transferase), especially those at a molecular level that provide early warnings signals of toxic chemical effects on organisms [80]. However, the use of these conventional biomarkers presents some disadvantages, as they are influenced by confounding factors (*e.g.* abiotic), highly dependent on the route of exposure, bioaccumulation tendency and detoxification mechanisms of chemicals, require a deep knowledge of the toxic mechanisms of contaminants and prevent a more comprehensive view of toxicity by focusing in only few proteins [81]. Therefore, proteomics-based methods can provide a more insightful view on the global changes in protein expression indicative of contaminants exposure and be used not only to i) identify new molecular targets for toxic substances and provide insights into their mechanisms of action, but also to ii) use alterations in specific protein patterns to identify new and unbiased candidate biomarkers for predictive screening of toxic action of compounds [48,82,83]. In this context, a recent discipline has emerged, environmental proteomics or ecotoxicoproteomics, as a tool for the understanding and identification of early molecular events related to an environmental stressor and that are responsible for the adverse effects observed at higher levels of biological organization [52].

The main goal of environmental proteomics is to analyse the proteome of organisms and to identify variations in protein expression induced by contaminants (or other environmental stressors) without the need for thorough detail of their toxicity mechanisms. The comparison of proteomes from a stress condition versus a control has the potential to identify not only single protein markers, but also protein expression patterns specific to the type and degree of stress and, consequently, distinguish between exposure and/or effect to the contaminant. Proteins differential expression can be compared among contaminants, concentrations or complex mixtures in several aquatic

environments, and the resulting up- or down-regulated proteins combined within protein expression patterns that are specific to the condition and the level of environmental stress. These changes are identified as PESs, sets of key proteins that potentially offer greater understanding of underlying toxic mechanisms of stress response, which makes them powerful tools to classify the ecological status of the environment [48,54,56]. Since a PES by itself constitutes a biomarker, protein identification is not indispensable to diagnose adverse environmental effects [54,83-85]. However, without knowing the protein identity, it is not possible to link the key molecules and their functions to provide essential mechanistic information on the underlying toxicity [86]. Upon quantification and identification, these PESs can be used as novel and unbiased biomarkers for exposure and effect of several environmental factors (*e.g.* chemical pollutants, salinity, temperature).

In the case of shellfish species, proteome techniques have been used to study the changes and variety in protein expression in response to environmental shifts, allowing the identification of biomarkers that can be used for tools for quality and safety assessment, production optimization and nutritional assessment of shellfish products [4,7,45,57,86]. However, sequence data from most shellfish species is still scarce in databases, making protein identification more difficult and possible only by homology with sequences from other species [86].

4.1. Proteomics application to environmental contamination

The presence and effects of environmental contaminants in shellfish can affect not only the health and quality of the farmed species but also pose a serious health hazard for the consumers [87]. In the last 10 years, the number of publications dealing with the use of proteomics to study the impact of environmental contaminants and biomarker discovery in shellfish species has rapidly increased (**Fig. 3**), with the majority of the published studies focusing on bivalve species, especially the marine mussel of the genus *Mytilus*. Several PESs have been identified in response to several types of contaminants (conventional and emergent) and other environmental factors (*e.g.* temperature, hypoxia, *etc.*) from field and laboratory studies, thus identifying candidate proteins for further study [88-91]. A compilation of the use of proteomics as a tool for evaluating the responses of shellfish species to environmental contaminants is showed in **Table 1** and described in more detail below.

4.1.1. Conventional contaminants

One of the first environmental toxicology proteomic studies with bivalves was conducted on *M. edulis* after exposure to copper (70 ppb), aroclor 1248 (1 ppb) and lower levels of salinity, using 2-DE and quantitative analysis of protein abundance by gel image analysis [54]. 500 to 600 protein spots were separated and visualized and identified as unique PESs, considered by the authors as key protein changes in the overall pattern for each stressor used. The authors also emphasized the importance of using PESs for the identification of specific stressors and key proteins leading to functional linkages to higher-order effects in biomonitoring [54]. After this first work by Shepard and colleagues [54], several authors have reported the use of proteomic approaches for the establishment of PESs after exposure to a variety of environmental pollutants including polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), crude oil and metals.

Knigge *et al.* [48] analysed the alterations in the proteomic profile of blue mussels *M. edulis* from polluted marine habitats surrounding the island of Karmøy in Norway, using ProteinChip® technology combined with surface-enhanced laser desorption/ionization time-of-flight MS (SELDI-TOF-MS). Two different types of contamination (heavy metals and PAHs) were compared to a clean reference site, after which differentially expressed proteins were found to show a specific induction or a general suppression associated with the field site of origin. A tree-building algorithm was applied to proteomic data to combine sets of protein markers that was able to accurately classify samples from these sites with an accuracy of 90% [48].

The effects of crude oil, oil spiked with alkylphenols or PAHs in protein expression of *M. edulis* hemolymph were investigated employing a gel-free proteomics approach based on weak cation-exchange protein chip arrays combined with SELDI-TOF-MS. Differential mass peaks were detected after mussel exposure to spiked oil or oil respectively, some of which revealed exposure- or gender-specific responses. Multivariate analysis with regression tree based methods distinguished protein expression patterns related with exposure that accurately classified masked samples with 90–95% accuracy [92].

Aiming to enhance detection of low-abundance proteins involved in the detoxification of xenobiotics, several studies first enriched samples from the digestive gland of *Mytilus* spp. for peroxisomes, organelles that use molecular oxygen to produce hydrogen peroxide, which in turn is used to oxidize a number of

substrates, including fatty acids, phenols, and alcohol [82,88,93,94]. Peroxisome proliferation expression patterns associated with exposure to a polluted environment were characterized by Mi and collaborators [93] in *M. galloprovincialis*, in which several protein spots were identified as peroxisomal proteins involved in the degradation of xenobiotics (epoxide hydrolase, peorixisomal antioxidant enzyme and sarcosine oxidase) and oxidation of thiol groups (acyl-CoA oxidase, multifunctional protein sand Cu,Zn-SOD) [94]. Peroxisomal preparations were also used to detect PESs in *M. edulis* exposed to diallylphtalate, polybrominated diphenylether (PBDE-47) and bisphenol A [88]. Digestive gland peroxisome-enriched fractions were investigated using 2D-DIGE, combined with MS/MS analysis. Further statistical analyses (principal component analysis (PCA) and hierarchical cluster analysis) were employed to the proteomic data to distinguish stressor specific PESs. The methodology used proved effective in discriminating exposure groups, and a unique PES of exposure was assigned to each of the stressors. Proteomic analysis provided evidence for alterations in oxidation pathways, xenobiotic and amino acid metabolism, cell signalling, oxyradical metabolism, peroxisomal assembly, respiration, and the cytoskeleton [88]. Using PCA and hierarchical clustering, a related study of *M. edulis* from polluted and control sites near Gothenburg Harbour, Sweden, showed that peroxisomal PESs were sufficient to discriminate polluted and unpolluted areas and to provide a spatial gradient from the polluted source [82]. PESs identification by ESI-MS/MS showed that higher abundance proteins in the polluted sites were involved in protein degradation (cathepsin), β-oxidation (acyl-CoA dehydrogenase), and detoxification (GST), while proteins with lower abundances included constitutive isoforms of HSP70, an actin-binding protein associated with the cytoskeleton (fascin), and an oxidative stress protein (aldehyde dehydrogenase) [82]. A similar approach was used by Mi and colleagues [94] to obtain PESs in *M. edulis* exposed for 3 weeks to crude oil mixtures: crude oil or crude oil spiked with alkylated phenols (AP) and extra PAH. In this study, peroxisome-enriched fractions were analysed by 2D-DIGE and MS, where a minimal PES composed by 13 protein spots and unique PESs of exposure to the two different mixtures were identified. Hierarchical clustering analysis allowed the discrimination of the control and exposed groups on the unique PES. The PESs obtained in this study were consistent with protein patterns obtained in previous field experiments [93], highlighting the usefulness of using peroxisomal proteomics to assess oil exposure in marine pollution assessments [94]. Peroxisomal proteomics was also used to assess the effects of the Prestige's oil spill on

mussels from the Iberian Peninsula [95]. PCA showed differences in protein expression among stations and sampling years, namely the up-regulation of peroxisomal β-oxidation proteins, PMP70 and the antioxidant enzyme catalase. Several proteins were putatively identified by mass spectrometry and immunolocalization, associated with oxidative phosphorylation, signal transduction, cytoskeleton, hydrolases or acyltransferases and arthropod hemocyanins and insect larval storage proteins [95]. All these studies emphasize the relevance of using gel-based sub-proteomics approaches together with multivariate data analysis, to increase the analytical capacity of specific subsets of proteins and thus providing information to interpret the effects of oil spills at cellular level in mussels.

Additionally, proteomics has been used to compare profiles of mussels gills exposed to Cu and Benzo(a)pyrene (BaP), alone and in mixture for a period of 7 days [96]. Different protein expression profiles were detected for each condition, which might reflect different accumulation, metabolism and chemical interactions of BaP, Cu and their mixture. Proteins identified by MS/MS and homology search further indicated that all three conditions are associated with alterations in adhesion and motility, cytoskeleton and cell structure, stress response, transcription regulation and energy metabolism. Moreover, alterations in oxidative stress and digestion, growth and remodelling processes were attributed to Cu exposure alone, while the mixture affected only one protein (major vault protein) possibly related to multi drug resistance. Overall, new candidate biomarkers, namely zinc-finger BED domain-containing protein, chitin synthase and major vault protein, were also identified for BaP, Cu and mixture, respectively [96]. In another study, Wu *et al.* [98] investigated the proteomic response in hepatopancreas and adductor muscle tissue of the green-lipped mussel *Perna viridis* after exposure to Cd and hydrogen peroxide (H_2O_2). Unique sets of tissue-specific PESs were attributed to each stressor, 15 of which were successfully identified by MALDI-TOF/TOF MS analysis. Identified proteins showed alterations in several mechanisms, namely glycolysis, amino acid metabolism, energy homeostasis, oxidative stress response, redox homeostasis and protein folding, heat-shock response, and muscle contraction modulation. Of the tissue-specific mechanisms revealed, the Cd-induced endoplasmic reticulum stress responses were seen by the authors as a new and complementary approach in pollution monitoring using this biomonitor species [97].

Another bivalve species with high commercial importance in shellfish aquaculture is the clam from the genus *Ruditapes* (*R. decussatus*, *R. phillipinarum*, *Tapes semidescussatus*), which in the last decade has

been widely used in environmental toxicology. Proteomics has been employed to characterize the impact of cadmium in the proteome of *R. decussatus* after exposure for 21 days. Tissue-specific protein expression profiles were reported, which might reflect differences in Cd accumulation, protein inhibition and/or autophagy. Proteins identified by LC-MS/MS, homology search and *de novo* sequencing further indicated that cadmium toxicity may imply alterations in cytoskeleton, amino-acid and fatty acid metabolism and regulation of membrane protein traffic [90]. Wu *et al*., [98] combined proteomics and metabolomics to characterize the differential responses of As in clam *R. philippinarum* under different salinities. 25 protein spots were significantly altered in As -exposed clams, with 21 proteins being identified by MALDI-TOF-MS/MS analysis. Disturbance in cytoskeleton, oxidative stress, energy metabolism, osmic regulation, immune system, cell growth, and apoptosis was connected to As toxicity, however differently affected by varying salinities. Metabolomic biomarkers complemented the information given by proteomics, highlighting the usefulness of combining both techniques for elucidating toxicological effects of environmental contaminants [98].

Oysters, another bivalve group of species highly appreciated for human consumption, have also been the targets of several studies aiming the detection of protein expression profiles in response to several contaminants. Sydney oysters (*S. glomerata*) haemolymph was used to assess the effects of environmentally relevant concentration of Cd, Cu, Pb, Zn upon 4-day exposure by 2-DE and tandem mass spectrometry. Unique protein expression profiles were assigned to each metal, and 18 of 25 proteins were affected by only one type of metal, indicating that different metals affect different cellular pathways. Proteins affecting shell properties, cytoskeletal activities and metabolism/stress responses were more drastically altered by all four metals [99]. Another study performed by the same authors with similar experimental set-up revealed distinctive sets of differentially expressed proteins, specific to one metal or affected by more than one metal [100]. The most common biological functions of these proteins were associated with stress response, cytoskeletal activity and protein synthesis. Of the identified proteins, some are commonly used as biomarkers for environmental monitoring (*e.g.* HSP70), while others were suggested as potentially novel biomarkers of exposure to metals (*e.g.* vasa, TIM, arginine kinase, shematrin-7, and insoluble protein) [100]. In both studies, the authors emphasize the inherent difficulty in identifying proteins in species without fully sequenced genomes. Oysters *C. hongkongensis* proteome was also used as

a diagnostic tool for contamination and toxicity of metals/metalloids in a real multiple metal-contaminated estuary, *i.e*., the Jiulongjiang estuary in China [101]. A proteome pattern comprising of 13 commonly altered proteins was identified in oyster gills, which allowed the distinction between contaminated and clean individuals, as well as the differentiation between contamination levels. MALDI-TOF/TOF MS/MS analysis followed by homology search in the NCBInr and Bivalvia EST only allowed the identification of 19 proteins within 173 differentially expressed in contaminated oysters. These identified proteins were involved in a variety of biological processes, such as amino acid metabolism, ATP synthesis, cell structure and motility, glycolysis, fatty acid metabolism, protein biosynthesis, protein degradation, tricarboxylic acid cycle, and superoxide metabolism [101]. More recently, Flores-Nunes *et al.* [102] determined the alterations in PESs of oysters *C. gigas* after transplantation to an area influenced by sanitary sewage in Florianópolis, Brasil. Only 5 differentially expressed proteins were identified in the oysters exposed *in situ* to sanitary sewage discharges, mostly related to the cytoskeleton (CKAP5 and ACT2), ubiquitination pathway conjugation (UBE3C), G protein- coupled receptor and signal transduction (SVEP1), and cell cycle/division (CCNB3). The results obtained by the authors provided initial information on PES of *C. gigas* exposed to sanitary sewage, which can subsequently be useful in the development of more sensitive tools for biomonitoring of oyster farming activities.

Metal toxicity was investigated in *Patinopecten yessoensis* (a commercially valuable scallop species in China and Japan) by exposing the scallops to acute cadmium chloride ($CdCl_2$) over 24h and variations in the proteome of the kidney were analysed by 2-DE, MALDI-TOF mass spectrometry and database searches [103]. Results showed 13 differentially expressed protein spots, mainly involved in many biological processes, such as signalling transduction and transcriptional regulation, indicating various mechanisms involved in Cd toxicity. Guanylate kinase (GK) and C_2H_2-type zinc finger protein were highlighted as being tightly connected with Cd toxicity. In addition, the up-regulated expression levels of GK were confirmed using real-time PCR and this protein was suggested as novel biomarker for monitoring the level of Cd contamination in seawater [103]

Liu and colleagues [104] used a proteomic approach to investigate the effect of Cd on bioaccumulation of other heavy metals in four bivalves: the scallop *Chlamys nobilis*, the clam *R. philippinarum*, the mussel *P. viridis*, and the oyster *S. cucullata*. The authors found that Cd had little effect on the bivalve proteomes,

even though it affected the bioaccumulation of other trace metals, and the identified proteins were insufficient to explain the disruption of trace element metabolism. Nevertheless, PESs allowed the distinction between clams and mussels that had accumulated different Cd levels, while no PESs for oysters and scallops was obtained. Galectin was suggested by the authors a novel biomarker in environmental monitoring due to its strong up-regulation in Cd-exposed oysters [104].

Other less commercialized bivalve species have been used to characterize the effects of several contaminants using proteomics. Romero-Ruiz and co-workers [105] utilized 2-DE, nanospray-ion trap tandem mass spectrometry, *de novo* sequencing and a bioinformatics search to examine proteomic profiles of *Scrobicularia plana* gills (highly commercialized clam in the Iberian Peninsula) residing in the Guadalquivir estuary, Spain. From the 3 sites analysed within the Guadalquivir Estuary, different PESs were found at each site, with the highest number of more intense spots in clams with the highest metal content. From 19 more intense protein spots, only 2 proteins were unambiguously identified, hypoxanthineguanine phosphoribosyltransferase (HPRT) and glyceraldehyde-3 phosphate dehydrogenase (G3PDH) and correlated with highest metal contents. These two proteins, HPRT and G3PDH, were proposed by the authors as novel pollution biomarkers [105]. Another clam species highly appreciated in the Iberian Peninsula, *Chamaelea gallina*, has also been used to screen changes in protein expression upon exposure to arochlor 1254, Cu, tributyltin, and As for 7 days [106]. Altered protein expression was confirmed in the proteome of exposed clams, showing several PESs specific for a particular contaminant. Only 4 of 15 highly altered protein spots were unambiguous identified by PMF and LC-MS/MS followed by homology search in SWISS-PROT and TrEMBL databases, being all of them involved in cytoskeleton and cell structure (tropomyosin, two isoforms of actin and myosin). The authors also highlight the exclusive identification of cytoskeletal proteins to reflect their relative abundance in the proteome of the clam species used, their prevalence in bivalve molluscs' databases or their role as major targets of pollutant-related oxidative stress [106]. In fact, cytoskeletal proteins are frequently detected using 2-DE based approaches with bivalve species, highlighting that this method is most appropriate for the separation and analysis of this group of proteins [86]. Another study also used *C. gallina* to investigate the time course response of heat shock protein (HSP70) isoforms affected by PAH benzo[a]pyrene (B[a]P) using a 2-DE approach. Using 2-DE immunoblotting with specific antibodies against these proteins, the authors detected

changes in constitutive HSC70 and inducible HSP70 forms, which could be related to an adaptation to stress and to a normal protein synthesis capability, respectively [107].

Another 2-DE based proteomic approach increasingly used in bivalves is the study of the PTMs through the use of specific antibodies. One of the first papers published using redox proteomics presented a combination of 2-DE and western blotting for the detection of carbonylation and glutathionylation of proteins in gill and digestive glands of *M. edulis* collected from a polluted and reference site in Cork Harbour, Ireland [77]. Western blotting showed higher levels of specific carbonylation of proteins in tissues from mussels from the polluted site, in contrast to the similar protein patters and expression levels obtained by 2-DE for both sites. Further exposure of mussels from the reference site to H_2O_2 for 24 h revealed more pronounced carbonylation in polluted and exposed animals. Western blot analysis using antibodies to glutathione and actin showed that gill proteins are glutathionylated more strongly than digestive gland proteins, especially in polluted mussels. The authors conclude that carbonylation and glutathionylation occurred in mussels' tissues, with actin as a major target for both PTMs modifications under oxidative stress conditions [77]. Alterations in disulphide bonds (S—S) of proteins were first assessed in *M. edulis* tissues exposed to H_2O_2 to provoke oxidative stress [73]. Diagonal gel electrophoresis was used to detect the proteins displaying S—S modifications, after which western blot analysis revealed the presence of actin among the group of modified proteins. Further carbonylation and glutathionylation of proteins were investigated demonstrating that in addition to S—S modifications, actin may also form glutathione adducts in cells under oxidative stress [73]. Additionally, McDonagh and Sheehan [72] also reported the carbonylation and ubiquitination of model pro-oxidants (H_2O_2, $CdCl_2$ and menadione) in gill, mantle and digestive gland of *M. edulis*. Treatment- and tissue-specific carbonylation and ubiquitination levels were observed in proteomic separations followed by immunoblotting, probably due to subtle differences in the interplay between each pro-oxidant and the detoxification machinery of the tissues investigated. From the results obtained, the authors acknowledge the potential of ubiquitination as a sensitive and specific early marker for oxidative stress in bivalves [72]. The redox proteomics approach has been extended to other bivalve species such as *R. decussatus* to examine the impacts of Cd [108], nonylphenol [109] and p,p'-dichlorodiphenyldichloroethylene (DDE) [110] in clams proteome. High levels of protein ubiquitination and carbonylation were verified by [108] using 2-DE and western blotting with specific antibodies, in

soluble protein fractions from digestive glands and gills of *R. decussatus* exposed to cadmium. Distinct profiles for ubiquitination and carbonylation were found between tissues, with higher levels in the digestive gland compared to the gills [108]. High levels of both PTMs were also showed by 2-DE and immunobloting in *R. decussatus* in response to nonylphenol [109]. Ubiquitination and carbonylation patterns were tissue-dependent, with more ubiquitinated proteins in gills, while the digestive gland showed a significant higher number of carbonylated proteins. Results achieved suggest that protein ubiquitination and carbonylation are independent processes [109]. Protein carbonylation and levels of heat shock proteins were also measured in gill, mantle and digestive gland of *R. decussatus* upon exposure to DDE [110]. Immunoblotting with antibodies specific to heat shock proteins only revealed the presence of HSP90 in DDE-treated mantle. On the other hand, immunoblotting with dinitrophenol-specific antibody showed differences in both extent and number of carbonylated proteins in mantle and digestive gland in response to DDE while the gill was unaffected [110]. Two-dimensional electrophoresis has also been implemented to study glutathione affinity-selected proteins of the clam *Tapes semidescussatus* [111]. Analysis by 2-DE revealed tissue-specific patterns of protein expression with some spots common to gill and digestive gland, which were further identified as glutathione transferases (GSTs) by immunoblotting. Further analysis using reversed phase C-18 HPLC-MS/MSconfirmed that gill and digestive gland share some GST isoenzymes. This study showed complex tissue-specific pattern of GSH-binding proteins in *T. semidecussatus*, which may reflect different redox requirements in each tissue [111]. As shown by all these authors, using redox proteomic approaches, changes in protein structure provide options for affinity selection of sub-proteomes for 2-DE, simplifying the detection of protein biomarkers. Consequently, the use of modern proteomic technologies evaluating PTMs in sub-proteomes will likely expand in the future because of the implied simplification of the subsequent analysis and will increase the understanding of the role of protein PTMs in the toxicity induced by environmental contaminants.

Shellfish species other than bivalve molluscs exposed to various environmental contaminants have also been evaluated. For example, 2-DE followed by MS/MS micro-sequencing was used to characterize differentially expressed proteins of the Chinese mitten crab *Eriocheir sinensis* exposed to Cd [108]. Different responses at the protein expression level were detected by the authors after acute (3 days) and chronic exposure (30 days) to waterborne Cd, with 6 and 31 protein spots differentially expressed after

acute and chronic exposure, respectively. From these spots, 15 protein species were identified. Alpha tubulin, glutathione S-transferase and an isoform, and crustacean calcium-binding protein 23 were connected to acute Cd exposure, while proteins disulfide isomerase, thioredoxin peroxidase, glutathione S-transferase, a proteasome subunit, cathepsin D, ATP synthase beta, alpha tubulin, arginine kinase, glyceraldehyde-3-phosphate dehydrogenase and malate dehydrogenase were differentially expressed after chronic exposure. The authors suggest that Cd mainly exerts its toxicity through oxidative stress induction and sulfhydryl-group binding [112]. ProteinChip technology was used to evaluate the protein profile and the discovery of new and sensitive biomarkers in two species of crabs exposed to various pollutants [113]. In this study, the spider crab *Hyas araneus* was exposed to diallyl phatalate, bisphenol A, and polybrominated diphenyl ether (PDBE-47), while the shore crab *Carcinus maeanas* was exposed to crude oil and oil spiked with alylphenol and 4-nonylphenol. The authors' highlighted gender and species-related protein pattern alterations, with females from both species and shore crabs showing larger number of significantly altered proteins. Major disruption in protein peak expression was also observed in samples exposed to mixtures of pollutants, i.e. oil spiked with APs. The authors conclude that the use of SELDI-TOF technology is a powerful tool to discover PESs for different pollutants and sex dependent responses [113].

Two studies by Vioque-Fernández *et al.* [81] and Fernández-Cisnal *et al.* [114] used different proteomic approaches to assess the environmental quality of the Doñana National Park in Spain using the red crayfish *P. clarkii* as a bioindicator species. In the first study, an integrated approach was used, combining the responses of well-established biomarkers and protein expression alterations. 2-DE resolved >2500 protein spots in the gill tissue, 35 of which showed significant intensity differences between the reference site and the other seven sites studied. Proteomics analysis allowed the discrimination of 4 different protein expression patterns based on the 35 differentially expressed proteins, which correlated with the contamination levels found at the different sites within the Doñana National Park. Furthermore, the authors acknowledge that proteomic approaches are markedly superior to well-established biomarkers in ecotoxicological studies [81]. In the second study by Fernández-Cisnal *et al.* [114], redox proteomics was used as a novel biomarker to assess contamination levels within the Doñana National Park and its surroundings, where agricultural activity is a serious concern. Fluorescence labelling of reversibly oxidized

Cys and 2-DE separation allowed the differentiation of total density of proteins with reversibly oxidized thiols within the studied sites. 35 spots with significant differences in thiol oxidation were revealed by 2-DE analysis, in which several were identified as ferritin, superoxide dismutase, protein disulfide isomerase, actin nucleoside diphosphate kinase, fructose-biphosphate aldolase, fatty acid-binding protein and phosphopyruvate hydratase. Of the identified proteins, evidence of reversible oxidation was found for specific Cys residues, including Cys13 in ferritin as well as Cys76 and Cys108 in nucleoside diphosphate kinase. This study showed that the identified thiol-oxidized proteins provided information about the metabolic pathways and/or physiological processes affected by pollutant-elicited oxidative stress [114].

To complement the lack of information regarding the effects of endocrine disrupting compounds (EDCs) on the proteome of shellfish, Liu and co-workers [115] used a proteomic approach to investigate the effect of nonylphenols and bisphenol A on larval *Haliotis diversicolor supertexta* (a commercially important abalone from Taiwan). Results of proteomic responses revealed alterations in several functional proteins with slight differences between each chemical. Several physiological functions were reported to be affected, such as energy and substance metabolism, cell signalling, formation of cytoskeleton and cilia formation, immune and stress responses at the same time, leading to consequent failure of metamorphosis [115].

4.1.2. Emergent contaminants

As new chemical compounds are constantly being produced and discharged in the aquatic environment, ecotoxicology is continuously improving its understanding of current and past contaminants by the use of new technologies, as proteomics. From a long list of current contaminants, pharmaceuticals and nanoparticles have been recognized in the past years as emerging contaminants (even though pharmaceuticals drugs have been used for much longer than that), due to their increased use and presence in the aquatic environment and consequent toxic impact on shellfish species [116].

4.1.2.1. Pharmaceuticals

One of the first studies to evaluate the effects of pharmaceuticals in the proteome of marine mussels (*Mytilus spp*.) was performed by Schmidt *et al.* [117]. In this study, PESs in the digestive gland of mussels

were obtained by 2-DE after 7, 14, and 21 days of exposure to diclofenac and gemfibrozil. 7 of 12 protein spots significantly altered by both pharmaceuticals were successfully identified by LC-MS/MS analysis. Identified proteins were involved in energy metabolism, oxidative stress response, protein folding, and immune responses. In addition, alterations in PESs over time suggested that mussels were still undergoing oxidative stress for up to seven days post-exposure [117]. Changes in protein thiols were also reported for *M. edulis* exposed to diclofenac using redox-proteomics, where a tissue-specific reduction in total protein thiol groups was detected in gills [118]. Further analysis with 2-DE and PMF showed several proteins oxidized and with altered abundance, namely caspase 3/7-4, heat-shock cognate protein 70, a predicted enolase-like protein, arginine kinase, β-tubulin, actin, isocitrate dehydrogenase, arginine kinase, heavy metal-binding HIP, cytosolic malate dehydrogenase, proteasome subunit alpha type 2, glyceraldehyde-3-phosphate dehydrogenase and superoxide dismutase. Six hypothetical proteins, either oxidized or decreased in abundance, were also identified using bioinformatics analysis, 78 kDa glucose-regulated protein precursor, α-enolase, calreticulin, mitochondrial H^+-ATPase, palmitoyl protein thioesterase 1 and initiation factor 5a. Thus, the authors concluded that diclofenac caused significant oxidative stress in mussel gills that further affected key structural, metabolic and stress-response proteins. More recently, Campos *et al.* [119] used shotgun proteomics to evaluate the individual and combined effects of propranolol and low salinity in the gills of *M. edulis*, and thus elucidate the molecular basis of the action of these two environmental stressors. Analysis of proteome profiling patterns from 587 highly reproductive proteins showed that salinity had a key role in mussel's response to propranolol. Protein identification further indicated that molecular mechanisms associated with cytoskeletal proteins, signalling and intracellular membrane trafficking pathway combined with a response towards the maintenance of transcription and translation were altered upon exposure to low salinity. On the other hand, combined exposure between propranolol and low salinity changed the expression of structural proteins including cilia functions and the expression of membrane protein transporters. The authors emphasize that the results obtained reinforced the concerns of the impact of low salinity in combination with anthropogenic pollutants in the aquatic environment and anticipated the critical physiological conditions for the survival of this mussel species in northern areas [119].

The intensification of shrimp farming has been associated to an increased use of chemotherapeutics as a production management strategy. For this reason, 2D-DIGE has been used to assess the stress levels of the giant tiger shrimp, *P. monodon* in response to the antibiotics enrofloxacin or furazolidone in both laboratory and field conditions [120]. No alterations in protein expression profiles were induced upon laboratory exposure to both antibiotics. On the other hand, 9 protein spots displayed different abundance in the field experiment (extensive ponds versus intensive ponds), and were identified as several forms of hemocyanin and sarcoplasmic calcium-binding protein. The data obtained from this study demonstrated that the effect of both antibiotics on patterns of haemolymph protein expression was overwhelmed by the effects of external farming conditions, such as different oxygen and nitric concentrations [120].

4.1.2.2. Nanoparticles (NPs)

Tedesco and co-authors published the first study using a proteomic approach (redox proteomics) in *M. edulis* tissues exposed to Au NPs-citrate (13 nm) [121]. After protein separation by 1 dimensional electrophoresis (1-DE), higher levels of protein carbonyls were detected in the gills in contrast to the digestive gland, where higher protein ubiquitination occurred. Subsequent analysis with 1-DE and 2-DE showed a decrease in protein thiol oxidation in the digestive gland of *M. edulis* exposed to the same NPs (~15 nm) as a response to reactive oxygen species (ROS) formation and targeting of protein thiols [121]. The same authors also reported alterations in proteins patterns in the sub-proteome of thiol-containing proteins after exposure to smaller Au NPs (5.3 ± 1 nm), consistent with higher protein thiol oxidation in response to a decrease in NP size. Nonetheless, no protein identification was performed in this study [122]. Alterations in carbonyl and protein thiols were also detected in *M. edulis* gill extracts upon exposure to CuO NPs (50 nm), where an increase in protein carbonyls and a decrease in reduced protein thiols were revealed [123]. PMF in combination with MS analysis allowed the identification of six proteins: α- and β-tubulin, actin, tropomyosin, triosephosphate isomerase and Cu–Zn superoxide dismutase, revealing significant protein oxidation of cytoskeleton and key enzymes in response to CuO NPs [123]. According to the authors, the use of redox proteomics helps to unravel more specific effects in tissues than traditional biomarkers of oxidative stress in sentinel species, as bivalves. The effects of CuO NPs (31 ± 10 nm) were also investigated in *M. galloprovincialis* tissues using 2-DE and compared to that of Cu^{2+} [124]. Several

copper and tissue-specific PESs were found for CuO NPs and Cu^{2+} and linked to tissue-specific redox requirements associated with different toxic mechanisms of CuO NPs and Cu^{2+}. PMF and MS analysis enabled the identification of 15 of 40 differentially expressed proteins with higher expression changes. Modifications of proteins involved in cytoskeleton and cell structure, transcription regulation, stress response, oxidative stress, energy metabolism, apoptosis, proteolysis and adhesion and mobility were detected in response to both forms of copper. Of the identified proteins, some are traditional molecular targets of CuO NPs exposure in mussel tissues (*e.g.* HSPs, GST, ATP synthase), while others were suggested by the authors as putative new biomarkers for CuO NPs exposure (caspase 3/7-1, cathepsin L, Zn-finger protein and precollagen-D) [124]. Proteomes obtained by 2-DE were also investigated in *M. galloprovincialis* gill and digestive gland to characterize the effects of Ag NPs (42 ± 10 nm), also in comparison to its ionic form [91]. Tissue-specific PESs were reported for both Ag NPs and Ag^{+}, reflecting differences in uptake, tissue-specific functions, redox requirements and modes of action. 15 proteins with higher expression change were subsequently identified by MALDI–TOF–TOF and database search: catchin, myosin heavy chain, HSP70, GST, nuclear receptor sub- family 1G, precollagen-P, ATP synthase F0 subunit 6, NADH dehydrogenase subunit 2, putative C1q domain containing protein, actin, a-tubulin, major vault protein, paramyosin and ras, partial. The differential expression patterns obtained for Ag NPs and Ag^{+} exposures helped to identify new putative candidate molecular biomarkers to assess Ag NPs (major vault protein, ras partial and precollagen-P) and Ag^{+} toxicity (MgC1q60 and precollagen- P) [91].

4.1.3. Other environmental stressors

Apart from exposure to different type of contaminants, shellfish farming habitats are also subjected to numerous other variables that include different temperatures, salinities, amounts of dissolved oxygen, and changes in pH, which can negatively impact shellfish species and lead to decreased physiological status. Due to the close relationship between environmental conditions and the success of aquaculture production, the wide impacts of climate change are becoming a major concern. Carbon dioxide emissions are directly responsible for ocean acidification (OA) and increased water temperatures, which in turn, leads to oxygen limitation [125].

To test the impact of OA in protein expression and PTMs of the barnacle *Balanus amphitrite* (an economically and ecologically important barnacle species), Wong and co-workers [126] used current and projected CO_2 concentrations for the year 2100 as test conditions. Alterations at the total proteome level of exposed larva were detected in combination with two PTMs (phosphorylation and glycosylation) using a 2-DE based proteomic approach. Several proteins were differentially expressed in response to altered CO_2 levels and attributed to three major functional groups: energy-metabolism, respiration, and molecular chaperones, possibly reflecting the strategy employed by the barnacle larvae to tolerate OA stress. Proteomic data allowed the identification of OA-responsive PESs for an OA scenario in 2100, which include 2 hemoglobin-like proteins, 1 elongation factor 2-like protein, 1 fatty acid oxidation-associated enzyme, 1 heat-shock-like protein, 1 protease, and 1 endocytosis-related protein [126]. In a study investigating the effect of OA on *C. virginica*, the proteomes of oysters' mantle tissue were analysed for changes induced by altered CO_2 conditions [127]. Proteomic analysis (2-DE in combination with MS/MS) revealed 54 of 456 proteins (12%) differentially expressed in response to high CO_2 conditions, 17 of which were identified and found to either be associated with the cytoskeleton (*e.g.* several actin isoforms) or oxidative stress (e.g. superoxide dismutase, several peroxiredoxins, thioredoxin-related nucleoredoxin). The results obtained in this study suggested that the cytoskeleton is a major target of oxidative stress under high CO_2 levels either caused by direct and/or indirect production of ROS. The authors highlight that even though several species are already exposed to higher and more variable levels of CO_2, climate change may further increase the extremes and thereby cause greater levels of oxidative stress [127].

Another direct consequence of climate change is the alteration of distribution patterns of shellfish species as a function of increased water temperatures, which can lead, for example, to the colonization of new habitats by more adapted invading species [4,86]. An example is the Mediterranean blue mussel *M. galloprovincialis*, an invasive species along the southern Californian coast, which is currently displacing the more heat-sensitive native species *M. trossulus* possibly due to climate change [128]. Accordingly, in order to gather new insights on the molecular mechanisms that account for the different temperature tolerance in the two mussel species and that could explain the invasion capability of *M. galloprovincialis*, the gill proteomes of both congeners were investigated in response to acute heat stress [128]. Protein expression was assessed quantitatively using high-resolution 2-DE and statistical analysis accompanied

with cluster and PCA to help unravel which proteins contributed most to heat tolerance. The identification of proteins involved in protein folding, proteolysis, energy metabolism, oxidative damage, cytoskeleton and deacetylation revealed a common heat stress response in both mussels. A lower sensitivity to high-temperature damage in the warm-adapted Mediterranean mussel was also shown in this study, explaining its increased colonization range in warmer waters. On the other hand, the cold-adapted *M. trossulus* showed increasing levels of a number of molecular chaperones, (acidic) HSP70 and some HSPs among the most abundant, at lower temperatures in comparison with *M. galloprovincialis*, showing interspecific differences in protein thermal stability. Both mussel congeners responded to heat stress by increasing the abundance of NADPH-producing proteins, possibly facilitating the scavenging of ROS. However, *M. trossulus* seemed to be incapable of responding to heat-induced oxidative stress beyond 28°C, after which resorts to the down-regulation of NADH-producing pathways to lower the production of ROS by the electron transport chain. Overall, the authors conclude that the systems-level differences in the proteome's response to acute thermal stress may represent some of the molecular factors responsible for the differences in thermal tolerance seen for both mussel species. In a follow-up study that investigated temperature acclimation in both mussel species, warm acclimation was found to affect proteins that comprise the cytoskeleton and function in energy metabolism, which is consistent with increased filtration and respiration rates associated with increased ciliary activity [129]. Increased levels of molecular chaperones were detected in *M. trossulus* at the highest temperature tested (19°C) that were not evident in *M. galloprovincialis*, suggesting differences in the long-term upper thermal limits of the congeners. On the other hand, a stronger response to cold acclimation was seen for *M. galloprovincialis*, including alterations in abundance in more proteins and different protein expression profiles between the lower tested temperatures (7 and 13°C), a pattern not detected in *M. trossulus*. Although the results obtained in this study indicated that *M. trossulus* might have a competitive advantage at colder temperatures, the authors suggest that data also showed why *M. galloprovincialis* displaced *M. trossulus* in southern California and indicated that anthropogenic global warming could increase this competitive advantage [129].

The capacity of marine species to survive chronic heat stress was also investigated by Artigaud and co-authors [130] in the king scallop *Pecten maximus*. In this study 2-DE proteomics in combination with RNAseq were employed to decipher the molecular response of this bivalve to elevated temperatures.

Proteomic analysis identified 24 differentially expressed proteins, with five identified from ESTs sequenced in the RNAseq experiments described in the study, and the other four proteins from the scallop hemocytes ESTs used to build the authors database. Network analysis of the results obtained revealed a pivotal role for GAPDH and AP-1 signalling pathways, in addition to cell structure remodelling and a diversion of energetic metabolism towards the mobilization of lipid energy reserves to fuel the increased metabolic rate at higher temperatures. The authors emphasize that the preliminary insights into the response of *P. maximus* to chronic heat stress obtained with this work can provide a basis for future work on the tipping points and energetic trade-offs of scallop cultures in warming oceans [130]. Proteomics has also been employed to characterize the changes induced by hypoxia in the same scallop species subjected to three different temperatures [131]. At the lowest temperature tested no significant changes were induced by hypoxia, suggesting that the low energy demand due to hypoxia at this temperature did not require extra proteins adjustment. Differently expressed proteins were only revealed upon exposure at the highest temperatures tested of 18ºC and 25ºC. 11 proteins were identified by MS, with known functions in protein modifications and signalling (*e.g.*, casein kinase 2 alpha catalytic subunit, Serine/threonine-protein kinase), energy metabolism (*e.g.*, ENO3) or cytoskeleton (GSN), giving insights into the thermal-dependent response of scallops to hypoxia. Overall, the proteomic data suggested a decrease of several mechanisms included in the energetic metabolism of scallops, and a role for apoptosis in the hypoxia response following thermal acclimation [131].

The effects of hypoxia were also investigated in the hepatopancreas of the Chinese shrimp *Fenneropenaeus chinensis* using 2-DE in combination with LC-ESI-MS/MS. 67 protein spots showed changes in expression as a result to stress exposure by hypoxia, with 33 being identified as proteins involved in energy metabolism, metabolism-related proteins, immune response, antioxidant processes, chaperoning and cytoskeleton [132]. As one of the first studies focusing on the differential expression in the hepatopancreas of shrimp after hypoxia, this study provided new knowledge that can serve as basis for further studies.

5. Proteomic applications to shellfish safety and quality

The issue of food safety and quality is of great concern to the consumers of both importing and exporting countries, and particularly important for the shellfish industry, which needs to maintain consumer

confidence in its products. The safety and quality hazards associated with shellfish farming generally vary according to the species and the type of culture system. Since it is expected that commercial aquaculture, along with the risks related to shellfish consumption, will continue to grow in coming years, effective and preventive measures are mandatory for assessing shellfish safety.

Consequently, this section will provide information on proteomic techniques exploited for detecting hazards that may occur during farming, processing and storage stages, and may affect the safety and quality of shellfish food.

5.1. Shellfish safety proteomic applications

Most health risks associated with seafood safety are related to the anthropogenic contamination of the environment where the organisms come from. Indeed, infectious disease outbreaks (*e.g.* typhoid fever, hepatitis and salmonellosis) [133] and poisoning by methilmercury [134], heavy metals [135] and organochlorine compounds [136], are notoriously related to the unsafe consumption of shellfish, acting as vectors of chemicals, toxins and pathogens derived from human activities. Additionally, an intensive or super-intensive use of aquaculture systems, involving factors such as heavy stocking densities and use of certain feed, chemicals and drugs, can be further responsible for a potentially unsafe shellfish production and for a negative impact on the surrounding environment. Proteomics is an affordable tool not only to the environmental research but also to food science, giving a snapshot of the organism's state and map the entirety of its adaptive potential and mechanisms. In fact, it may provide valuable insight into the health status of shellfish; the stress or contamination levels at the breeding place [137].

5.1.1. Environmental contaminants

Since the most important sentinel species represent at the same time some of the most common shellfish, great effort is being made to develop strategies to predict and quantify the effects of environmental contamination on seafood from an ecotoxicological and productive perspective. As seen in the previous section, most of the reported studies mainly focused on the employment of proteomic techniques to investigate the impact of metals, pharmaceuticals, PAHs, and other contaminants/stressors on shellfish proteome. In general, such a tool provided conclusions about different stress responses induced by specific

proteins, deriving, at the same time, valuable insight about the level of contamination and, therefore, safety of shellfish.

5.1.2. Algal toxins

Beside environmental contaminants, other environmental stress conditions, such as climate change and global warming, could affect shellfish safety by inducing an increase of dinoflagellate and cyanobacterial blooms [4]. The harmful aspect of these algal blooms includes not only considerable damages to the aquatic ecosystems (eutrophication), but also the production of toxic secondary metabolites which, accumulating in shellfish, can impact human health through severe respiratory, hepatic, digestive, and nervous diseases (*e.g.* paralytic shellfish poisoning toxins, or PSTs) [137]. In view of the potential risk for human consumers, Current European regulation [138] has established that the total PST content in the edible parts of molluscs must not exceed 80 μg/100 g of flesh, providing, consequently, a minimum margin for safety.

Many studies have already characterized the proteome of typical cyanobacteria and dinoflagellates to identify several biomarkers of toxicity. In a study conducted by Chan and colleagues [139], an approach based on 2-DE followed by MALDI-TOF was exploited to successfully discriminate the protein patterns of toxic and non-toxic strains of *Alexandrium minutum*. Once proteome reference maps were established for toxic and non-toxic strain, 4 proteins were found only in the toxic strain; while 2 proteins were specific to toxic strain of *A. minutum*. Between them, only one protein successfully matched with protein database, and, consequently, was identified as the chain M, peridinin-chlorophyll protein. Overall, the proteins characterizing non-toxic strains could be considered as potential taxonomic biomarkers for strain differentiation; while the two proteins found in the toxic strain were likely correlated to toxin biosynthesis and could be used as potential toxin biomarkers. In a subsequent study, a combined 2-DE and MALDI-TOF approach helped to develop biomarkers of toxicity in toxic and nontoxic strains of another toxin producer, *A. tamarense* [140]. Results of this study clearly showed that 2 proteins were specific to toxic strains of *A. tamarense* and may serve as biomarkers of toxicity. An exhaustive overview of proteomic studies conducted on toxin-producer cyanobacteria was reported by D'Agostino and colleagues [141]. A first attempt to elucidate both the origin of contamination (*e.g.* the toxin producer), and the proteomic responses of the target organism was recently developed. In this study, the proteome of the digestive gland

of *M. galloprovincialis* contaminated by dinoflagellate toxins was mapped and specific proteins indicating toxin contamination were identified. Such protein biomarkers were expressed not only by algal organisms, but also by mussels' tissues, which activated adaptive mechanisms in response to the bioactive stimuli [142]. In another work, the protein interaction of a group of dynoflagellate toxins, azaspiracids, was investigated in the digestive gland of blue mussels through SDS-PAGE followed by MALDI-TOF/FOF and nanoLC-ESI-MS/MS. Four proteins were detected only in the digestive gland of challenged mussels. They were characterized by a high homology with cathepsin D, superoxide dismutase and glutathione S-transferase. Furthermore an unexpected bacterial flagellar protein was identified. Such toxicity biomarker was probably involved in the azaspiracid infection process, since previous studies have already highlighted the involvement of bacteria in the production of shellfish toxins by dinoflagellates [143]. Other proteomic works focused on the characterization of toxin producer proteome and protein interactions between algal toxin-shellfish are summarized in **Table 2.** Taken together, these findings provide relevant implications for the consumers of shellfish contaminated by algal toxins.

5.1.3. Pathogenic agents

Shellfish can also uptake and accumulate pathogenic microorganisms naturally present in harvest waters, compromising their safety. Protozoa, such as *Cryptosporidium spp.*, *Giardia duodenalis* and *Toxoplasma gondii* [148], bacteria, such as *Escherichia coli, Salmonella spp*., *Staphylococcus aureu*s, *Shigella spp.* and *Vibrio cholera*, [149] as well as virus, such as Rotavirus, Astrovirus and Hepatitis A [150], are among the most common pathogens able to develop disease outbreaks in human consumers**.** Furthermore, such pathogens may be also responsible for massive losses of shellfish, leading to significant damage of aquaculture production and economy.

MALDI-TOF MS proved to be a technique with high potential for foodborne microorganism identification due to its rapidness, reduced cost and minimal sample preparation compared to conventional techniques [151]. Thanks to MALDI-TOF MS, intact bacterial cells can be analyzed in a rapid way, obtaining high specific protein fingerprints. Bacterial cells are generally pre-treated with trypsin and lysozyme, so that the obtained spectral patterns are generally attributed to intracellular proteins [152]. However, only few works focused on the employment of MALDI-TOF for the identification of common shellfish pathogens. These

works include the classification and identification of some shellfish pathogens, such as *Listeria spp.*, *Salmonella spp*., *Campylobacter spp., V. parahaemolyticus* and *E. coli* [154-157]. However, proteomics revealed to be a useful tool not only for characterizing food borne microorganisms, but also for deepening pathogenesis mechanisms, host-microorganism protein interaction, as well as potential targets for therapeutic and preventative strategies. An approach based on 2-DE and MS/MS analysis by MALDI-TOF/TOF was exploited to characterize and compare the proteome gill tissue of *M. galloprovincialis* infected with *V. anguillarum* and *M. luteus*. *M. luteus* infection was confirmed by specific protein biomarkers, such as matrilin, ependymin-related protein and peptidylprolyl-cis-trans-isomerases, responsible for immune stress, disturbance in signalling pathways and protein synthesis. On the other hand, oxidative stress and alteration of energy metabolism caused by *V. anguillarum* in mussel gills were highlighted by altered procollagen-proline dioxygenase, protein disulfide isomerase, nucleoside diphosphate kinases, electron transfer flavoprotein and glutathione S-transferase [153]. An interesting work conducted by Sangsuriya and collaborators [158], focused on the building of a protein interaction map of the White Spot Syndrome Virus, a microorganism representing an impendent threat for shrimp aquaculture. A series of viral proteins with a large number of interactions (hub proteins) were successfully characterized. Such proteins seem to be more essential than non-hub proteins, since are probably involved in many biological processes, such as nucleotide metabolism, DNA replication and virion assembly, and, consequently, may be candidate targets for the antiviral strategy. In another study, 2-DE electrophoresis was applied to study the tolerance/resistance mechanisms of haemocytes of *C. gigas* (resistant) *and O. edulis* (susceptible) challenged by the protozoan *Bonamia ostreae*. The haemolymph protein profiles of the two oysters were compared and differences in the number of protein spots, as well as in expression levels were found. Nevertheless, a further MS characterization will be necessary for identifying the proteins responsible for such proteome changes, and elucidating their role in the infection process [159]. Further proteomic works focused on the proteomic response of different shellfish species to pathogen microrganisms are summarized in **Table 2.**

5.1.4. Allergens

It is well accepted that the consumption of seafood can induce allergy in susceptible individuals, and shellfish are among the major causes of Immunoglobulin E (IgE)-mediated allergic reactions [150]. The European directive 2007/68/EC [167] sets the list of all the allergenic foods, including seafood and products thereof that must be labelled when allergens have been intentionally introduced in such foodstuffs. However, some products could unintentionally contain traces of allergens, due to cross-contaminations during food manufacturing processes. Consequently, accurate, sensitive and fast detection methods allowing the detection of allergens in food samples are highly recommendable. In the last decades, proteomics has particularly been devoted to investigate shellfish allergens. Yadzer and coworkers [168], for instance, investigated the IgE reactivity pattern of the freshwater prawn *Macrobrachium rosenbergii* by western blot, performed with serum of positive patients. The set of IgE reactive proteins were further characterized by 2-DE electrophoresis and MALDI-TOF, and two major allergens, namely tropomyosin and arginine kinase, were identified. Other allergens, such as phosphagen kinase, creatine kinase and sarcoplasmic calcium-binding protein, were identified by means of different proteomic techniques in several molluscan and crustacean species [150,169]. Proteomics applied to seafood allergies presents a so wide potential that it would be more convenient defining it as "allergenomics". Although only a few major allergens in seafood have been characterized, and the molecular basis of the interaction food allergens-immune system is still unclear, allergenomic applications have revolutionized the food-allergy field, since they have positively contributed to the diagnosis/prognosis of allergies, providing tools for an accurate characterization and quantification of protein allergens [170]. Further studies on detection of allergens in shellfish by proteomic tools are summarized in **Table 2.**

5.2. Shellfish quality proteomic applications

Seafood quality is a complex concept, since it relies on different characteristics that may be grouped in appearance, odour, flavour and texture. In most cases, seafood quality is determined by its appearance. However, a more objective evaluation requires that the product meets certain criteria set by suppliers, before it can be deemed to be of quality. In particular, freshness requirements must be maintained to maximize the value and quality of seafood, and, therefore, great attention must be paid to the employed handling and storage processes. Among omic tools, proteomics can provide relevant conclusions about the

deterioration of product quality before, during and after processing or storage, as well as guarantee from all potential hazards, which can impact consumer health [177].

5.2.1. Handling and storage processes responsible for post-mortem changes

A serious threat to the seafood industry is the degradation of meat, occurring during post-mortem handling and storage, and affecting quality and shelf-life of such food products. This phenomenon, also known as "softening or mushiness", is mainly due to the autolysis of muscle proteins, which has deleterious effects on flesh texture [178]. Although the molecular mechanisms underlying the post-mortem softening are still not fully understood, it is well established that muscle proteins are hydrolysed by endogenous or microbial (exogenous) proteases [179,180], in a manner dependent on time and conditions of shellfish storage [181,182]. Seafood myofibrillar and cytoskeletal proteins seem to be the proteins most affected by such processes [7]. Within this context, sensitive and accurate diagnostic techniques, such as proteomics, are mandatory to understand the molecular mechanisms of post-mortem changes, and develop biomarkers for determining freshness of species, or groups of related species, in relation to specific genetic and environmental factors. Although post-mortem changes were addressed more in fish than in shellfish [7], many studies aimed to map the proteomic changes occurring in the meat of different prawn species after ice storage. In a study of Martinez and coworkers [183], the muscle proteome of three different prawn species (*Pandalus borealis*, *P. japonicus* and *P. monodon*) stored in ice for different hours (0-92h), was analysed by 2-DE and immunological techniques. The muscle of *P. borealis* showed a significant decrease in the relative amount of myosin heavy chain (MHC). Other relevant results were the disappearance of protein bands of about 67 and 50 kDa after 2h of ice-storage, and the appearance of a band >50 kDa, after 24h and 5h of ice-storage, respectively. Two bands of ~100 and ~96 kDa showed a positive reaction with the α-actinin antibody in the zero-time extract of *P. borealis*, but after 24 h of ice-storage only one faint 96 kDa band was detected. On the other side, the extracts of *P. japonicus* and *P. monodon* did not suffer significant alterations during the entire examined period. Although many of these protein bands were not identified, they could be considered as potential freshness biomarkers in prawn [183]. The freshness of Pacific white shrimp (*L. vannamei*) stored for 0, 2, 4, 6, 8, and 10 days under vacuum-packing at 4°C was also evaluated by 2-DE coupled to MALDI-TOF/TOF. Three proteins, namely, arginine kinase, phosphopyruvate

hydratase, and actin T^2, were down-regulated with the increase of storage time, and could be involved in the molecular mechanisms of post-mortem changes [184]. Differently from crustaceans, the proteomic assessment of freshness in bivalve molluscs has not yet been addressed. Therefore, it could be an appealing opportunity to develop a proteomic approach to study the post-mortem changes occurring in the adductor muscle of most common bivalves, after ice storage.

Additionally, the post-mortem integrity of muscle proteins can also be altered by typical handling practices, such as slaughter [7]. However, despite few studies investigated the impact of such practice on fish quality [185], no work has been focused on shellfish, which are commonly slaughtered by asphyxia in air or ice. One of the future trends may be to compare the effects of asphyxia in air and ice on the muscle proteome of shellfish, in order to determine which slaughter method causes less severe post-mortem protein changes.

Handling and storage techniques may be responsible not only for meat tenderizing but also for the loss of precious sensorial and nutritional properties of fishery products, primary due to non-enzymatic PTMs (nePTMs). Such nePTMs are mainly caused by oxidation and by the non-enzymatic reaction of sugars with amino acid side chains (Maillard glycation). In addition, other nePTMs, such as condensation, hydrolysis of side chains or break down of the peptide backbone, were described [186]. In general, the conformational modifications are responsible for changes in key protein features, such as hydrophobicity, protein aggregation and protein solubility, and induce relevant consequences on the nutritional and toxicological properties of seafood. Although nePTMs induced by oxidative stress have been widely investigated for assessing the effect of environmental pollutions in bivalves (see section 3.1.3), no work has still dealt with the characterization of nePTMs in shellfish induced by aquaculture practices. Such aspect of proteome analysis may be of potential interest for assessing shellfish quality and, therefore, need to be further investigated.

5.2.2. Species authentication

One of the most relevant issues concerning seafood quality is species authentication. In fact, seafood may be adulterated by substituting, partially or entirely, valuable species with similar but cheaper ones (food fraud) [187]. Although without a strict definition for food fraud, the European Union has provided guidance in the European Directive No. 178/2002/EC [188], affirming that food law shall prevent: (a) fraudulent or

deceptive practices, (b) food adulteration and (c) any other practice which may mislead the consumer. Additionally, the European Directive No. 104/2000/EC [189] on the common organization of the markets in fishery and aquaculture products, has declared that seafood products should be labelled indicating the commercial designation of the species, the production method (wild or farmed), and the geographical origin. Within this context, it is mandatory to adopt measures aimed at ensuring the identification of fraudulent seafood, and at guaranteeing that it is not placed on the market, in order to protect human health. Faster, sensitive and high-throughput proteomics tools have recently been proposed for the assessment of the authenticity and traceability of marine species in seafood. In fact, the presence/absence of specie-specific peptide biomarker in marine organisms may evidence species authenticity in a short analysis time. Recent works employed proteomics for authentication purposes in shellfish. To identify and sequence species-specific peptides from commercial mussel species, namely *M. trossulus*, *M. edulis* and *M. galloprovincialis*, Lopez and co-workers [190] developed a straightforward proteomic approach based on the combination of different MS techniques, taking advantage of the capacity of MALDI-TOF to produce accurate peptide maps, and the ability of ion trap (IT) to sequence peptides, both off-line and coupled to a micro-HPLC peptide separation system. Considering 51 peptide mass maps, coming from 6 major proteins characterizing the three mussel species, it was found that the sequence of a specific peptide (YDTAASKLEEASKAADESER) allowed a reliable identification of individuals belonging to the *M. trossulus* taxonomic form, when compared to those belonging to *M. edulis* and *M. galloprovincialis*. This peptide sequence could be exploited for the production of monoclonal antipeptide antibodies, useful to develop highly accurate species-specific profiling assays. In another study, two phylogenetically close prawn species, namely *P. monodon* and *Fenneropenaeus indicus*, were characterized by 2-DE and MALDI-TOF to compare the respective peptide mass fingerprints (PMF). Although these two species showed very similar PMF, the sarcoplasmic protein arginine kinase resulted to be species-specific, and, therefore, useful to commercially differentiate them [191]. Many other works confirmed the potential use of arginine kinase as authentication biomarker, due to the inter-specific variability and its high concentration in shrimp muscle [192,193]. Furthermore, as mentioned in section 4.2.1.5, arginine kinase has been recognized as one of the most relevant allergens in shrimp and, consequently, its detection could be useful, not only for authentication purposes, but also for confirming the presence of shrimp allergens in foodstuff.

6. Challenges and way forward

From all the information gathered and discussed in this review, it is possible to acknowledge the potential of proteomic approaches for the evaluation and assessment of quality and safety in shellfish products, not only from the detection of PESs indicative of environmental stress exposure (as contaminants, climate change, algal toxins, etc.), identification and detection of pathogenic bacteria and allergens but also from effects caused by handling, processing and storage of products. Nevertheless, the use of proteomics in shellfish aquaculture has been limited until recently, even though some of the biological questions have been well addressed in some cases to ensure quality and safety controls, especially in terms of consumer protection.

To date, even though the studies carried out have clearly demonstrated the potential of proteomics to identify important proteins and unravel potential biomarkers and mechanisms related to shellfish farming, there are still major drawbacks for shellfish products due to their limited available genomes and proteomes in databases. This results in the identification of only a limited number of proteins, which affects not only aquaculture related research, but also all the other areas using proteomics with these non-model organisms. As a consequence, 2-DE based proteomics still remains the most widely used approach in shellfish proteomic research, even with all the inherent limitations. Meanwhile, this major gap in shellfish related proteomics could be overcome in the mid-short term with the use of alternative approaches, as de novo sequencing of transcriptomes using next generation sequencing platforms (see Suárez-Ulloa *et al.*, [194] for more information on the current transcriptomic data bases already available for bivalve species). Additionally, the recent publication of the draft and complete genome sequences for two oyster species, the pearl oyster *Pinctada fucata* [195-196] and the Pacific oyster *C. gigas* [197] will also greatly improve proteomics application using these species.

With the continuous development of more cost-effective and sensitive technologies (*e.g.* multi-dimensional liquid chromatography, MALDI imaging or protein array/protein chip approaches), the use of MS-based proteomics approaches will greatly contribute to a better assessment of quality and safety of shellfish products and become indispensable tools in the field of aquaculture (and other aquatic monitoring areas in general). Furthermore, the integration of this technology with others as transcriptomics, metabolomics and

bioinformatics is currently the direction of future research in the area, which will definitely provide a wider high-tech tool in large-scale screening for control, authentication, safety and quality identification of shellfish products.

List of abbreviations

ACT 2 – Actin 2

Acyl-CoA – Acyl-CoA dehydrogenase

ADH – Alcohol dehydrogenase

AK – Arginine kinase

Aki – Adenylate kinase isoenzyme

ALDH – Aldehyde dehydrogenase

CaM – Calcium-binding messenger calmodulin

CAT – Catalase

CCNB3 – Protein homologue to cyclin B3

CKAP5 – Cytoskeleton-associated protein 5

CRT – Calreticulin

Cyt. – Cytochrome

ECH – Enoyl-CoA hydratase

EF2 – Elongation factor 2

eIF – Eukaryotic translation initiation factor

ENO – Phosphopyruvate hydratase or enolase

FDH – Formyltetrahydrofolate dehydrogenase

FGH – Formylglutathione hydrolase

GALE - UDP-glucose 4-epimerase-like

GFAT – Glucosamine–fructose-6-phosphate aminotransferase

GNB – Guanine nucleotide-binding protein subunit beta

GPDH – Glyceraldehyde-3-phosphate dehydrogenase

GPx – Glutathione peroxidase

GRP – Glucose-regulated protein

GST – Glutathione s-transferase

HSP – Heat shock protein

ICDH – Isocitrate dehydrogenase

MCAD - Medium chain-CoA dehydrogenase

MD – Malate dehydrogenase

Mit – Mitochondrial

MVP – Major vault protein

PDI – Protein disulfide isomerase

PPI – Peptidyl -prolyl cis-trans isomerase

PPIA – Peptidyl -prolyl cis-trans isomerase A

PPIB – Peptidyl -prolyl cis-trans isomerase A

PPT – Palmitoyl protein thioesterase

Precol – Precollagen

Rab GDP – Rab GDP dissociation inhibitor

RDH – Retinal dehydrogenase

Rib – Ribosomal

SILAC- Stable isotope labeling by amino acids in cell culture

SLC25A3 – Mitochondrial phosphate carrier protein

SLC5A25 – Calcium-binding mitochondrial carrier proteins

snRNP – Small nuclear ribonucleoprotein

SOD – Superoxide dismutase

SVEP1 – Sushi, von Willebrand factor type A, EGF and pentraxin domain- containing protein 1

TIM – Triosephosphate isomerase

TK – Tyrosine kinase

TPx – Thioredoxin peroxidase

UBE3C – Ubiquitin-protein ligase E3C

Vtg – Vitellogenin

Conflict of interest

The authors declare the inexistence of any conflict of interest.

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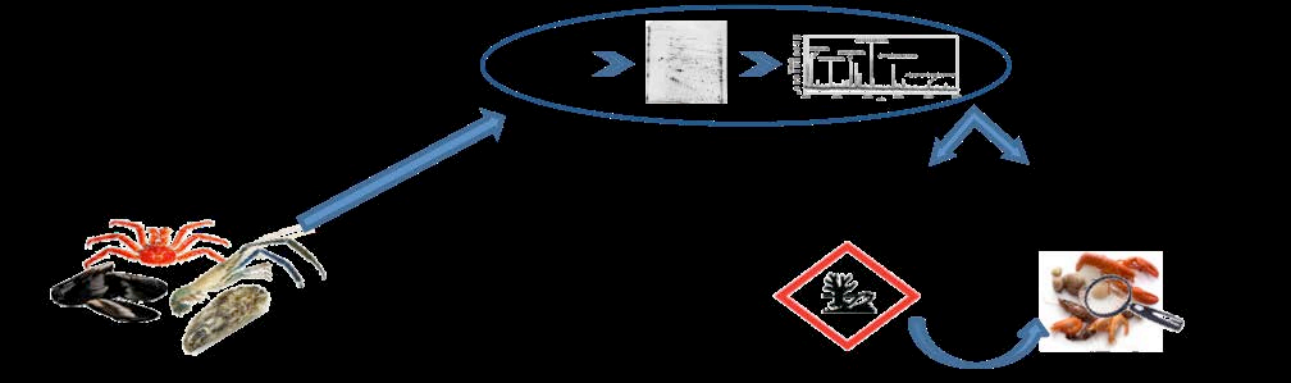


Table 1 – Summary of proteomic-based studies in ecotoxicological research using shellfish species. AM – adductor muscle; DG – Digestive gland, G- Gills, H – Haemolymph, HP – Hepatopancreas, K – kidney, L – Larvae, M – mantle, W – Whole soft body. See abbreviation section for proteins full name.

Species	Environmental stressor	Tissue	Proteomic technique	Proteins of interest	Reference
Mytilus edulis	Sediments from Gothenburg Harbour, Sweden	DG	2-DE, ESI-MS/MS	Cathepsin B, GST, acyl-CoA, fascin-like protein, ALDH, isoforms Hsp70	[82]
Mytilus edulis	Diallylphtalate, PBDE-47, bisphenol A	DG	2D-DIGE, MS/MS	β –tubulin, ADH, CAT, GST, ECH, phospholipase A2, peroxin 10, cyt. c oxidase subunit II	[88]
Mytilus galloprovincialis	Contaminated area in the Bay of Biscay, Spain	DG	2-DE, ESI MS/MS	Epoxide hydrolase, peorixisomal antioxidant enzyme, sarcosine oxidase, acyl-CoA oxidase, multifunctional protein sand, Cu,Zn-SOD	[93]
Mytilus galloprovincialis	Prestige´s oil spill in the Iberian Peninsula	DG	2-DE, MALDI-TOF MS	Peroxisomal β-oxidation proteins, PMP70, CAT	[95]
Mytilus galloprovincialis	Cu, BaP and their mixture	G	2-DE, MS/MS	Zinc-finger BED domain-containing protein, chitin synthase, MVP	[96]
Perna viridis	Cd, H_2O_2	H, AM	2-DE, MALDI-TOF/TOF MS	Cu/Zn SOD, ENO isoforms, DJ-1/ThiJ/PfpI family proteins, DI and CRT (ER chaperones), PDI, ATP synthase beta subunit, CRT	[97]
Ruditapes decussatus	Cd	G, DG	2-DE, LC-MS/MS	Actin, β –tubulin, Rab GDP, Rab GDI alpha, actin, ALDH, MCAD	[90]
Ruditapes philippinarum	As and salinity	G	2-DE, MALTI-TOF/TOF MS	ATP synthase, succinyl-CoA synthetase, nucleoside diphosphate kinase	[98]
Saccostrea glomerata	Cd, Cu, Pb, Zn	H	2-DE, LC-MS/MS	Non-gradient byssal precursor, Ca^{2+} ATPase, myosin, tropomyosin, precol D, ATP-synthase, tubulins, vasa, laminin receptor 1, actin, shematrin-7, TIM, vtg, omega-crystallin	[99]
Saccostrea glomerata	Cd, Cu, Pb, Zn	H	2-DE, LC-MS/MS	Vasa, TIM, AK, shematrin-7, insoluble protein	[100]
Crassostrea hongkongensis	Jiulongjiang estuary, China	G	2-DE, MALDI-TOF/TOF	Acyl-CoA, 4-hydroxyphenylpyruvate dioxygenase, methionine adenosyltransferase, mitochondrial H^+ ATPase	[101]
Crassostrea gigas	Area influenced by sanitary sewage in Florianópolis, Brasil	DG	2-DE, MALDI-TOF-TOF	ACT2, UBE3C, SVEP1, CCNB3, CKAP5	[102]
Patinopecten yessoensis	Cd	K	2-DE, MALDI-TOF MS	Guanylate kinase, C_2H_2-type zinc finger protein	[103]
Saccrostea cucullata	Cd	W	2-DE, MALDI-	Galectin	[104]

			TOF/TOF MS		
Scrobicularia plana	Contaminated sediments Guadalquivir Estuary in Spain	G	2-DE, MALTI-TOF-MS	Hypoxanthineguanine phosphoribosyl-transferase, GPDH	[105]
Chamalea gallina	Aroclor, Cu, TBT, As	W	2-DE, MALDI-TOF-MS	Tropomyosin (Aroclor, Cu), light chain myosin (Aroclor, Cu), actin (all)	[106]
Eriocheir sinensis	Cd	G	2-DE, MS/MS	GST, α-tubulin, AK, crustacean calcium-binding protein 23, PDI, TPx, proteasome subunit, cathepsin D, ATP synthase beta, GPDH, MD	[112]
Procambarus clarkii	Doñana National Park in Spain	DG	2-DE, MALDI-TOF/TOF	Ferritin, SOD, PDI, actin nucleoside diphosphate kinase, fructose-biphosphate aldolase, fatty acid-binding protein, ENO	[114]
Haliotis diversicolor supertexta	Nonylphenols and bisphenol A	L	2-DE, MALDI-TOF/TOF MS	ATP-dependent protease, Hsp60, chaperone containing t-complex protein, actin, β-tubulin, fascin, TK-like protein, annexin A11, ADH	[115]
Mytilus spp.	Diclofenac and gemfibrozil, 7, 14, 21	DG	2-DE, LC-MS/MS	GPDH, class 1 ADH, beta subunit, BRAFLDRAFT_282392, aconitase 1, PPI 5 precursor, PPIB, IDGF precursor	[117]
Mytilus edulis	Diclofenac	G	2-DE, MALDI-TOF/TOF	Caspase 3/7-4, hsp 70, ENO-like protein, AK, β-tubulin, actin, ICDH, AK, heavy metal-binding HIP, MD, proteasome, GPDH, SOD, 78 kDa GRP precursor, α-ENO, CRT, mit H^+-ATPase, PPT 1, initiation factor 5a	[118]
Mytilus edulis	Propranolol, salinity	G	Shotgun proteomics, Nano-LC–MS/MS	Dynein proteins, CaM, Ras-related protein Rab-1A, eIF 3 subunit M, snRNP, PPIA, rootletin, vinculin, severin, rib protein L11, AKi 5, GFAT, ATP synthase, RDH, citrate synthase, transaldolase, GALE, ADP, ADP/ATP carrier protein, GNB, GST, SLC25A25, SLC24A3	[119]
Penaeus monodon	Enrofloxacin, furazolidone	H	2D-DIGE, nano-LC–ESI–MS/MS	Several forms of hemocyanin and sarcoplasmic calcium-binding protein	[120]
Mytilus edulis	CuO NPs	G	2-DE, MALDI-TOF/TOF	A-tubulin, actin, tropomyosin, triosephosphate isomerase, Cu–Zn SOD	[123]
Mytilus galloprovincialis	CuO NPs, Cu2+	G, DG	2-DE, MALDI–TOF–TOF	Caspase 3/7-1, cathepsin L, Zn-finger protein, precol-D	[124]
Mytilus galloprovincialis	Ag NPs, Ag^+	G, DG	2-DE, MALDI–TOF–TOF	MVP, ras partial, precol-P, putative C1q domain containing protein	[91]
Balanus amphitrite	CO_2	L	2-DE, MALDI-TOF/TOF MS	Hemoglobin-like proteins, EF2-like protein, fatty acid oxidation-associated enzyme, hsp-like protein, protease, endocytosis-related protein	[126]
Crassostrea virginica	CO_2	M	2-DE, caMALDI-TOF/TOF	Several actin isoforms, SOD, peroxiredoxins, thioredoxin-related nucleoredoxin	[127]

Mytilus trossulus, Mytilus galloprovincialis	Temperature	G	2-DE, MALDI-TOF/TOF	NADPH-producing proteins, Hsp70, proteasome isoforms	[128]
M. trossulus and M. galloprovincialis	Temperature	G	2-DE, MALDI-TOF/TOF	Ubiquinol cyt. c reductase, ATP synthase, tubulin isoforms, ubiquinol cyt. c reductase, hsp isoforms	[129]
Pecten maximus	Temperature	M	2-DE, MALDI-TOF/TOF	GPDH	[130]
Pecten maximus	Hypoxia and Temperature	G	2-DE, MALDI-TOF/TOF	Casein Kinase 2 alpha catalytic subunit, TANK binding kinase 1, enolase 3, GSN	[131]
Fenneropenaeus chinensis	Hypoxia	HP	2-DE, LC-ESI-MS/MS	Preamylase, AK, ENO, citrate synthase, ATP synthase, chymotrypsin BI, chitinase, ferritin, C-type lectin receptors, transketolase, FGH, FDH, ALDH, GPx, Mn-SOD, PDI, actin, oncoprotein nm23, crustacyanin-C1	[132]

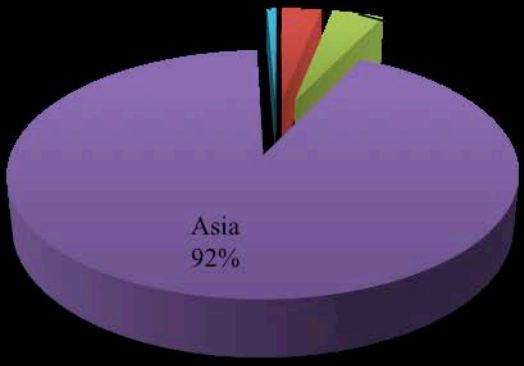
Table 2 – Shellfish safety proteomic applications.

Agent affecting shellfish safety	Topic	Reference
Algal toxin	Comparative proteomics of toxic and non-toxic *M. aeruginosa* strains	[144]
	Comparative proteomics of toxic and non-toxic *A. catenella* strains	[145]
	Proteomic effects of the exposure of *M. galloprovincialis* and *C. fluminea*, to toxic and non-toxic *C. raciborskii* cells	[146]
	Overview of the analytical techniques exploited for the detection of poisoning shellfish toxins in shellfish	[147]
Pathogen organism	Proteomic analysis of *E. sinensis* hemocytes to elucidate the crustacean immune responses at the translational level upon spiroplasma challenge	[160]
	Analysis of the protein profile involved in the anti-Vibrio immune response of the Zhikong scallop	[161]
	Proteomic analysis of shrimp hemocytes to identify differentially expressed proteins in response to a *V. harveyi*	[162]
	A comparative proteomic approach was performed to investigate the differentially expressed proteins in healthy and challenged by *V. Anguillarum* lymphoid organ of *F. chinensis*	[163]
	Proteomic study of the haemolimph of *S. serrata* challenged by *V. parahaemolyticus*	[164]
	Proteomic response of *F. chinensis* (hepatopancreas) to white spot syndrome virus	[165]
	A comparative proteomic analysis was performed to characterize altered proteins in the yellow head virus infected lymphoid organ of *P. monodon*	[166]
Allergenic protein	Evaluation of the effect of different cooking methods on the allergenicity of cockle and identification of proteins most frequently bound by IgE antibodies, using a proteomic approach	[171]
	IgE-binding proteins in various organs of *F. merguiensis* were identified by immunoblotting and tandem mass spectrometry	[172]
	The muscle protein extract of snow crab was profiled on SDS-PAGE and screened against patients' sera to characterize allergenic proteins	[173]
	Characterization of allergenic proteins of *P. monodon* using bottom up tandem mass spectrometry	[174]
	The major allergens of greasy-back shrimp were investigated by MALDI-TOF-MS	[175]
	2-D immunoblotting and MALDI-TOF were employed for the identification of a novel allergen from *P. monodon*, designated as Pen m2	[176]

Figure 1 – Percentage of shellfish global production worldwide and the percentage attributed to mollusc versus crustacean aquaculture.

Figure 2 – Overview of a typical proteomic workflow applied to either gel-based or gel-free approaches, differentiating between bottom-up and top-down strategies. Abbreviations: 2-DE – two dimensional gel electrophoresis, 2D-DIGE – two dimensional differential gel electrophoresis, IEF – isoelectric focusing, SDS-PAGE – sodium dodecyl sulphate-polyacrylamide gel electrophoresis, PMF – peptide mass fingerprint, PFF – peptide fragment fingerprint, PTMs – post-translational modifications, MALDI-TOF/TOF – matrix-assisted laser desorption/ionisation time of flight, HPLC-ESI-IT – high performance liquid chromatography electrospray-ion trap, SILAC – stable isotope labelling with amino acids, ICAT – isotope-coded affinity tag, TMT – tandem mass tags, iTRAQ – isobaric tags for relative and absolute quantification, MS – mass spectrometry, MS/MS – tandem mass spectrometry, SELDI-TOF: surface-enhanced laser desorption/ionisation time of flight, FT-ICR – Fourier transform ion cyclotron resonance mass spectrometry (adapted from [2]).

Figure 3 – Number of published manuscripts with proteomics in shellfish species from 2000 to 2015. Search was performed on Web of Science Scopus using the terms “Proteomics” and “Bivalves” or “Shellfish” or “Crustaceans” in Title, Abstract and Keywords. Books and Reviews were not considered in the search criteria.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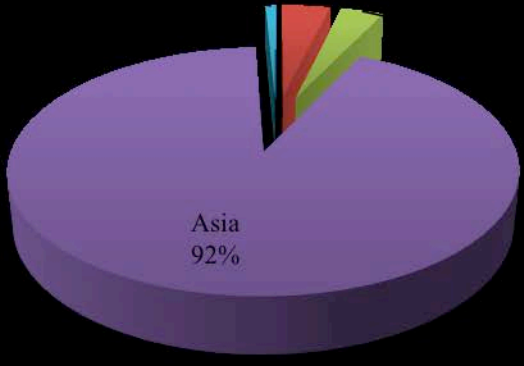
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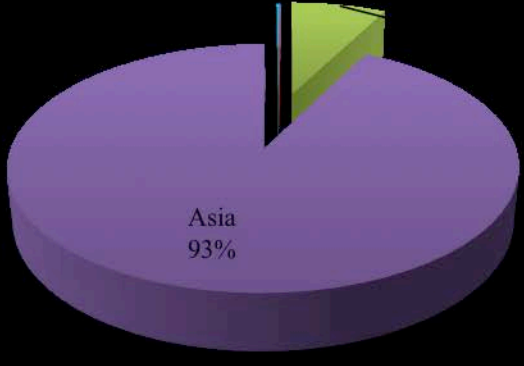
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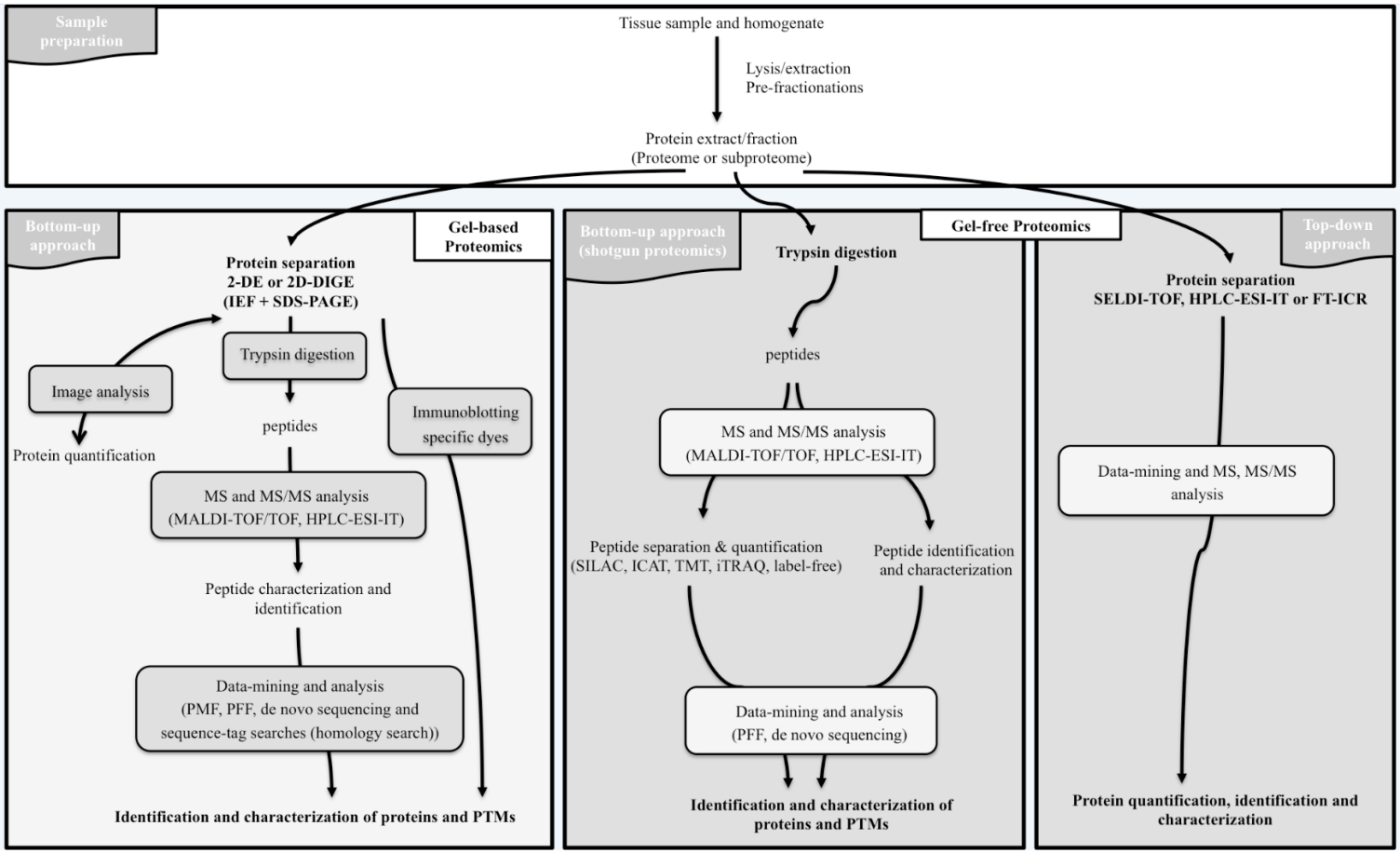
Sample preparation
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Lysis/extraction
Pre-fractionations
Protein extract/fraction
(Proteome or subproteome)
Bottom-up approach
Gel-based Proteomics
Protein separation
2-DE or 2D-DIGE
(IEF + SDS-PAGE)
Image analysis
Protein quantification
Trypsin digestion
peptides
Immunoblotting
specific dyes
MS and MS/MS analysis
(MALDI-TOF/TOF, HPLC-ESI-IT)
Peptide characterization and identification
Data-mining and analysis
(PMF, PFF, de novo sequencing and sequence-tag searches (homology search))
Identification and characterization of proteins and PTMs
Bottom-up approach (shotgun proteomics)
Gel-free Proteomics
Trypsin digestion
peptides
MS and MS/MS analysis
(MALDI-TOF/TOF, HPLC-ESI-IT)
Peptide separation & quantification
(SILAC, ICAT, TMT, iTRAQ, label-free)
Peptide identification and characterization
Data-mining and analysis
(PFF, de novo sequencing)
Identification and characterization of proteins and PTMs
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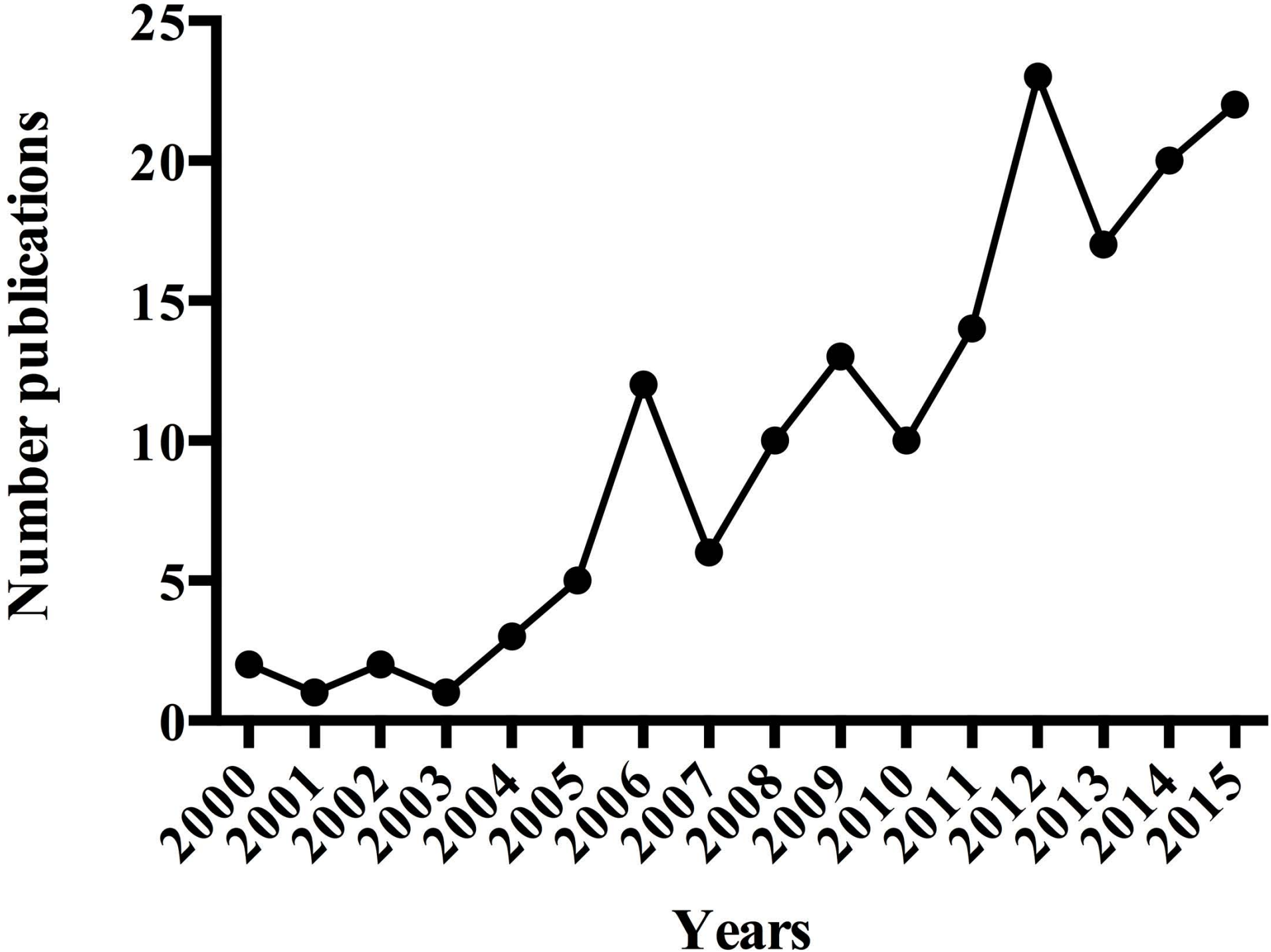
Protein quantification, identification and characterization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