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Ecotoxicoproteomics: A decade of progress in our understanding of anthropogenic impact on the environment

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ABSTRACT

Anthropogenic pollutants are found worldwide. Their fate and effects on human and ecosystem health must be appropriately monitored. Today, ecotoxicology is focused on the development of new methods to assess the impact of pollutant toxicity on living organisms and ecosystems. *In situ* biomonitoring often uses sentinel animals for which, ideally, molecular biomarkers have been defined thanks to which environmental quality can be assessed. In this context, high-throughput proteomics methods offer an attractive approach to study the early molecular responses of organisms to environmental stressors. This approach can be used to identify toxicity pathways, to quantify more precisely novel biomarkers, and to draw the possible adverse outcome pathways. In this review, we discuss the major advances in ecotoxicoproteomics made over the last decade and present the current state of knowledge, emphasizing the technological and conceptual advancements that allowed major breakthroughs in this field, which aims to "make our planet great again".

Significance: Ecotoxicoproteomics is a protein-centric methodology that is useful for ecotoxicology and could have future applications as part of chemical risk assessment and environmental monitoring. Ecotoxicology employing non-model sentinel organisms with highly divergent phylogenetic backgrounds aims to preserve the functioning of ecosystems and the overall range of biological species supporting them. The classical proteomics workflow involves protein identification, functional annotation, and extrapolation of toxicity across species. Thus, it is essential to develop multi-omics approaches in order to unravel molecular information and construct the most suitable databases for protein identification and pathway analysis in non-model species. Current instrumentation and available software allow relevant combined transcriptomic/proteomic studies to be performed for almost any species. This review summarizes these approaches and illustrates how they can be implemented in ecotoxicology for routine biomonitoring.

1. The importance of ecotoxicology for environmental health assessment

Concerns relating to anthropogenic pollution have increased worldwide in recent decades. Due to this persistent menace, increasing efforts have been made to improve environmental quality. An estimated 80,000 chemicals are available on the market [1], and only a small fraction has been subjected to rigorous safety or toxicity testing. The chemical characteristics and intensive use of toxic chemicals, such as the historically well-known dichlorodiphenyltrichloroethane, polychlorinated biphenyls (PCBs), or chlordecone, to cite some, has resulted in high persistence of these pollutants in soil and water, despite their banning decades ago because of their known toxicity to humans and wildlife. New synthetic chemicals are currently being produced in large

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quantities, and unfortunately released into the environment. Thus, the list of emergent pollutants – which includes pharmaceuticals, personal care products, artificial sweeteners, flame retardants, *etc.* – continues to grow [2,3]. Moreover, environmental pollutants often occur as mixtures which may have synergistic/antagonistic effects that are often poorly characterized. Dissecting the contribution of each component is a daunting task. In the case of natural exposure scenarios when studying field samples, the effects and impact of the mixture are observed alongside confounding effects. These combined effects make it much more difficult to understand the toxicity of the individual chemicals contained in the mixture. As a result, the fate and behaviour of emergent pollutants in the environment is largely under-documented and poorly understood.

The "One Health" initiative is a holistic and transdisciplinary approach aiming to improve conditions for people, animals, and the environment as a single, global, highly interconnected ecosystem [4]. The rationale behind this concept is strongly related to the early definition of ecotoxicology, which is the branch of toxicology assessing the toxic effects of pollutants on the constituents of ecosystems in an integrated context [5]. Indeed, ecotoxicology is essential for human and ecosystem health and conservation in the so-called "Anthropocene", the present era of extensive anthropogenic influence on the biosphere [6]. Ecotoxicology deals with the fate of contaminants in air-water-soil-sediment systems, food chains, and how they affect Life in the short-term and long-term. One of the main challenges in ecotoxicology (Fig. 1) is that it involves disciplines ranging from molecular biology to ecology in its attempts to predict the impact of pollutant on the broad natural diversity of biological species.

In ecotoxicology, the health status of organisms and assessment of the environmental risks linked to bioavailable chemicals are determined through laboratory-based toxicity testing and biomonitoring performed *in situ* in the field. The toxicity of a particular contaminant or class of contaminants is often tested in the laboratory on individual surrogate organisms to examine adverse effects on important biological functions such as growth or reproduction. Surrogate organisms are model animals used as representatives of or substitutes for other species from a specific environmental compartment and/or taxonomic group. Results from toxicity testing are then used to determine the predicted no-effect concentration [7] or environmental quality standard, which is used, for example, by Member States of the European Union to establish safe levels and monitor concentrations of a list of priority substances in all water bodies, as required by the Water Framework Directive.

Untargeted *in situ* biomonitoring is used to assess overall environmental toxicity resulting from interactions between numerous contaminants and additional abiotic factors. In this approach, living organisms are selected as sentinels of the quality of a given environment under contaminant pressure. The rationale behind biomonitoring is that sentinel organisms give quantifiable biochemical, physiological, and/or organism-level responses (*i.e.*, biomarkers) which reflect the state of pollution in their environment. Birds, plants, fishes, bees, mussels, earthworms, and other aquatic and terrestrial invertebrates have been used over the years as sentinels to monitor for air, soil, and water pollution. The classical example of sentinels from the early 1900s were the caged canaries used by miners operating in coal mines to provide early warning of the presence of lethal carbon monoxide. Precursor signs of stress from these sensitive birds indicated an unsafe environment. Today, the Mussel Watch program monitors concentrations of more than a hundred contaminants present in mussels in US coastal waters to give a toxicology picture of the marine environment [8].

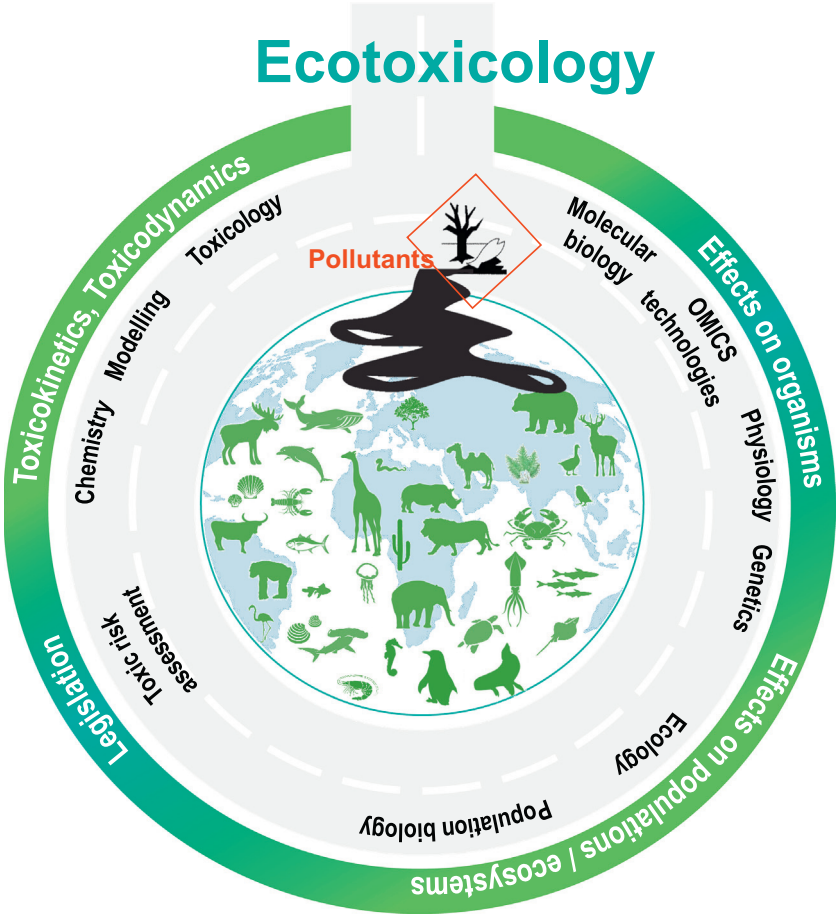

Fig. 1. Ecotoxicology: the study of the effects of toxic chemicals on biological organisms and ecosystems, at the crossroads linking several disciplines.

The goals of ecotoxicology are to understand and diagnose toxic effects in living organisms and rapidly predict adverse effects on ecosystem services. The development of holistic biomarkers could improve assessment of these effects. Interestingly, the latest “omics” methodologies provide suitable tools to highlight the most valuable molecular biomarkers. Indeed, large-scale molecular information can be quickly obtained through RNAseq or proteomics, even when working with organisms for which genome sequences are not currently available. This information can be used to elucidate the molecular modes of actions of contaminants, and/or to develop sensitive methods for biomarker quantification [9]. Proteomics is of particular interest for ecotoxicology as it involves the study of proteins, which are the agents of biological change creating an organism's response to toxic pressure. The term “ecotoxicoproteomics” was introduced a decade ago in a study assessing the proteome dynamics of filter-feeding mussels following oil exposure [10]. Applications of proteomics in ecotoxicology were highlighted in several interesting review articles during the onset of the last decade [11–17]. These reviews provide deep literature searches and examples performed mainly in aquatic species, setting the foundations for new-generation proteomics in ecotoxicology. Outcomes were found quite systematically limited by i) the lack of protein sequences in databases for most of the sentinel species routinely used, ii) the low consistency of quantitative proteomics, and iii) the difficulty of applying proteomics-based approaches in field studies.

In this review, we describe the dawn of ecotoxicoproteomics, and how the field developed during the last decade in order to overcome some of the limitations previously described in the literature. We give a special emphasis to the technological and conceptual developments, and propose a proteomics-based framework for species-specific protein identification in non-model species and biomarker quantification for routine ecotoxicological assays. This is illustrated through two case studies performed on aquatic animal species. The current limitations and potential, possible applications, and future directions of exotoxicoproteomics are then thoroughly discussed. The outstanding contribution of the *Journal of Proteomics* to the field of exotoxicoproteomics over the last decade is also highlighted and commented upon.

2. The potential of proteomics for ecotoxicology: significant progress from the ecotoxicologist's point of view over the last decade

Ecotoxicologists often aim to integrate several levels of information provided by a sentinel chosen because it is known to be sensitive to toxicants and it has a key position on the food-chain or in the ecosystem. Alternatively, studies can be performed on several organisms in parallel. These studies may provide sufficient information about the impact of the potential pollutant to extrapolate to other species in the ecosystem. Different objectives may be achieved: i) establish and document the pollutant's toxic effects on environmental species, ii) understand their mechanisms of toxicity, iii) predict the environmental impact of exposure to anthropogenic pollutants; and iv) develop methods that could provide this integrated information.

Classically, the effects of chemical stressors on living organisms have been studied at the individual level based on physiological [18], behavioural [19], and biochemical responses [20]. At community level, species richness, abundance, diversity and similarity indices can be assessed [21]. Several studies have described morphological abnormalities - such as deformed eyes, mouthparts, spinal cord, and haemorrhaging - in fish exposed to endocrine disruptors and other synthetic chemicals, *e.g.* [22,23]. Passerine birds from PCBs-contaminated sites were found to present a variety of contaminant-induced external heart deformities [24]. In the freshwater macrophyte *Juncus effusus* L., growth inhibition is used as an indicator of atrazine exposure, and in the amphipod *Gammarus fossarum,* feeding inhibition is used as an indicator of chemical pressure [25]. To assess exposure to pollutants, several other endpoints can be used, including survival [26], reproductive parameters [26], feminization/masculinization events [27], or biochemical responses as determined based on the enzymatic activity of some key proteins [28]. Among these endpoints, those assessed at the molecular level present specific advantages as they may provide earlier warning of a toxic effect, before it becomes detectable at higher physiological levels. However, conventional individual biomarkers are not sufficiently representative of the entire set of modes of action and specific health effects of contaminants present in ecosystems. Moreover, in most cases, the biomarkers available for animals are derived by analogy from human and/or vertebrate biology. Their transfer to other species may not result in equivalent response specificities because of the evolutionary distance between lineages [17]. In recent years, ecotoxicologists have sought to obtain more precise assessments by using high-throughput molecular screening – made possible thanks to the “omics” revolution, and reviewed in [29,30] – and multi-biomarker approaches [31]. These strategies aim to integrate the responses and specificities of several biomarkers in order to correlate phenotypic and molecular data, and document the toxicity mechanisms of pollutants. Progress in our understanding of the mechanistic basis for toxicological responses identified by “omics” techniques is also deeply motivated by the potential of these methods to provide a sound framework for extrapolation to other species as part of ecological risk assessment [32]. The identification of critical molecular pathways and regulatory networks involved in the most sensitive species should help with this cross-species transfer thanks to the functional similarities detected by comparative genomics.

Ecotoxicoproteomics has always been led by the impact that proteomics has had on human health management, offering a myriad of diagnostic and prognostic tools. When seeking to determine the environmental impact of pollutants, ecotoxicoproteomics has focused on analyzing the dynamics of proteins from environmental species by mass spectrometry (MS)-based high-throughput analytical methods. The resulting protein fingerprints can be compared across several conditions to understand the modes of action of chemicals, decipher the adaptive mechanisms adopted by organisms, and identify pollutant- and species-specific biomarkers. Large-scale discovery-led proteomics does not require *a priori* hypotheses. As it is not hypothesis-led, it may reveal novel mechanisms of pollutant action. From an ecotoxicological point of view, this is highly significant because of the wide range of chemicals that exist and their strongly contrasting effects on the different species present in an ecosystem. One of the best-studied examples is endocrine disrupting chemicals (EDCs), which have a well-known mechanism of action in restricted phylogenetic groups (*e.g.* synthetic oestrogens provoke dysfunctions in male vertebrates [33], and juvenoid-mimicking insecticides are massively used to kill insects [34]). However, in non-target species their effects differ from those predicted, either because the mode of action has changed, or because we know little about the basic endocrinology of non-model species, even those routinely used in ecotoxiocology studies. Unlike single-biomarker approaches, high-throughput proteomics can not only decipher changes occurring in a few target proteins, but can also reveal the cascade of biochemical events associated with the up- or down-regulation of the proteome. Therefore, it facilitates understanding of the potential toxic effects of chemicals on different organisms, and the establishment of links between molecular and physiological/organismal variation. For example, Martyniuk and co-workers [35] used proteomics to assess the effects of 17α-ethinylestradiol, an oestrogen present in birth control pills, on the telencephalon of male fathead minnows. Their results highlighted a set of proteins modulated by exposure to this pollutant. These proteins were involved in cellular and endocrine pathways (cell differentiation and proliferation, neuron network morphology, long-term synaptic potentiation). This type of study can provide early evidence of toxic effects on organisms that may affect the sustainability of their wild populations. A well-known example is the collapse of fish populations due to the xenoestrogen-related male feminization events reported by Kidd and co-workers [36].

High-throughput mechanistic proteomics studies can not only determine the modes of action of pollutants, but also resistance mechanisms developed by organisms in response to chronic contamination of their environment with toxic pollutants. Comparisons between reference and contamination-adapted populations often reveal cellular processes that are critical for adaptation to environmental stressors. For example, proteomics has been applied to determine insect resistance to insecticides [37], plant adaptation to soil contamination with heavy metals [38], and fish adaptation to highly toxic aquatic environments [39].

Proteomics data could be useful in the future to help regulatory agencies make decisions relating to toxic risk assessment and monitoring. These are just some of the numerous examples of studies demonstrating that ecotoxicoproteomics has significant potential for deciphering the varied modes of action of contaminants and discovering new biomarkers of toxicity in representative species from an ecosystem. Biomarkers have been proposed as reliable indicators of the toxic exposure of several sentinel species to a wide range of pollutants, and modern omics-based diagnostic tools will soon revolutionize the routine assessment of the health status of sentinel organisms.

3. Technologies and concepts for tackling some of the complex problems presented by ecotoxicology

As shown in Fig. 2, bidimensional gel (2D-PAGE)-based identification and quantification of proteins that are under- or over-represented in samples from environmentally-challenged organisms was relatively common a decade ago. The toxicity of environmental substances to organisms was assessed simply based on the patterns and intensities of protein spots observed on the gel [40]. Later, with the advent of MS-based proteomics, differentially-expressed protein spots could be analysed by MALDI-TOF-MS (matrix-assisted laser desorption/ionization coupled to a time-of-flight analyser) or tandem mass spectrometry (using electrospray ionization –ESI-MS/MS). The advantage of the latter is that it allows identification of peptide sequences and is compatible with error-tolerant searches that retrieve related proteins from other organisms (homology-based identification), even when the target protein is not itself present in the database. One of the first studies to apply 2D-PAGE-MS examined the quantitative differences in protein expression profiles in peroxisomal proteins from *Mytilus galloprovincialis* sampled from different control and polluted sites [41]. ESI-MS/MS analysis of 100 protein spots highlighted 55 proteins that were differentially-expressed between the two conditions. This pioneering work proposed a methodological strategy to circumvent the bottleneck of 2D-PAGE: that only the most highly abundant proteins are visible on the gel. By delving into a subproteome, the peroxisome, low-abundance proteins that proved to be highly sensitive to contamination could be identified. Further improvements based on the introduction of orthogonal fractionation by LC coupled to 2D-PAGE, led to an application of this approach in marine pollution monitoring programs [42]. These and many others pioneering methodologies have been the premise for ecotoxicoproteomics studies in plants [43–46] and animals [41,47–52] throughout the initial years of the field. Nevertheless, these approaches were unfortunately restricted to the proteins visible on the stained gel, which were the most abundant in the sample and that overlap other less-abundant but more relevant proteins. The poor representation of gene/protein sequences from sentinel organisms in generalist databases also hindered peptide/protein matching, resulting in very low percentages of protein spot identifications.

More recently, shotgun proteomics, *i.e.*, direct analysis of the whole-proteome *via* liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) [53], surpassed gel-based approaches as the gold standard for whole-proteome analysis in ecotoxicology. The application of specific enzymes (*e.g.* trypsin, the most widely used) to the whole pool of proteins to generate peptides which are then subjected to reverse phase separation prior to mass measurements provides a much more comprehensive and faster approach than 2D-PAGE. Recent publications describe the identification of thousands of proteins using shotgun proteomics. Using high-resolution mass spectrometers, 1075 proteins were detected in the reproductive testes of the freshwater fathead minnow (*Pimephales promelas*) [54], and 4000 proteins were identified in the whole-body of the crustacean sentinel *Daphnia pulex* (for which the genome has been sequenced and annotated) [55]. While protein identification is straightforward for genome-sequenced organisms, it remains challenging for sentinel species used in ecotoxicology for which genome-sequencing has yet to be completed. A homology-driven identification strategy, although useful, is inappropriate in most cases due to the high molecular divergence acquired by species during evolution. A recent paper that compared the proteome of the ovaries from five different amphipod species illustrates these difficulties [56]. Indeed, the interpretation of the MS/MS spectra using RNA-seq derived databases of other phylogenetically closely-related species diminished greatly spectra attribution rates. Furthermore, proteins identified based on sequence similarity often correspond to highly conserved, ubiquitous proteins with housekeeping functions that are of relatively low interest in ecotoxicology, since they are not representative of key

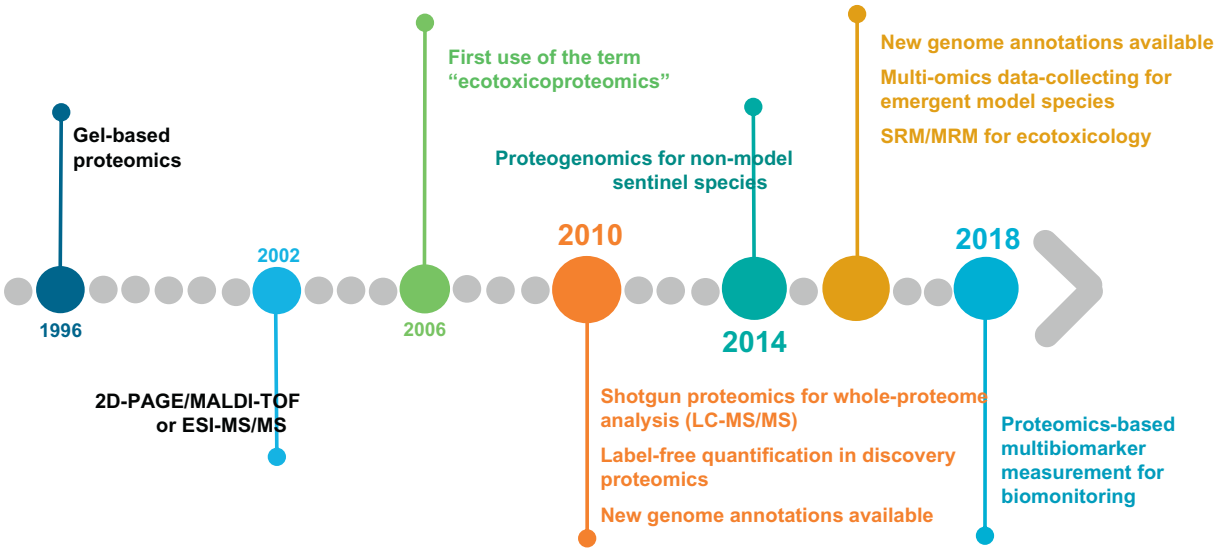

Fig. 2. The contribution of proteomics to ecotoxicology over the last two decades.

functions involved in the organisms' response to pollution. As exemplified earlier, minimizing the complexity and dynamic range of the proteome, *i.e.* working with a sub-proteome, is an effective solution for identifying less-abundant proteins, which in an ecotoxicological context are believed to play important roles in these key functions. Moreover, the completion of sequencing of several genomes in the last decade and numerous ongoing genome-sequencing projects using next-generation sequencing (NGS) should offer many opportunities for transcriptome and proteome characterization of different species. According to data from the National Center for Biotechnology Information, 463 eukaryotic genomes were newly annotated in the last decade (19 had been annotated in 2008 compared to 482 in September 2018). Nevertheless, correct annotation of complex eukaryotic genomes remains challenging, often requiring manual curation to improve the quality produced by automated genome annotation pipelines [57], which would otherwise result in sub-optimal protein-sequence databases.

In this context, proteogenomics has been proposed as a rapid and effective alternative to genome-sequencing, combining RNA-sequencing of poly-A RNAs (mostly protein-coding) and high-throughput shotgun proteomics to discover species-specific protein sequences [9]. Proteogenomics modified the molecular vision of non-sequenced species. Nowadays, thanks to advances in RNAseq technology, cost-effective deep sequencing of the transcriptome can be used to rapidly identify protein-coding genes, and consequently transcriptomics-based studies took off over the decade. Proteogenomics has been applied in ecotoxicoproteomics by using transcriptomic data to create a custom protein database. As shown in Fig. 3, systematic three- or six-base reading frame translation of whole-transcriptome sequences from a particular species can be used to create a theoretical species-specific protein database which can then be searched to interpret MS/MS spectra. The translated contigs can be further optimized using bioinformatic tools to select only the protein-coding portions, and thus reduce the size of the databases [58]. These databases contain true protein sequences mixed with erroneous polypeptide sequences, but the proteomics data will separate the wheat from the chaff. This “proteomics informed by transcriptomics” approach has been successfully applied in several research areas using non-model species, and discovered, for example, protein sequences specific to the nematode *Heligosomoides polygyrus* [59], the domesticated tomato *Solanum lycopersicum* [60], the crustacean amphipod *G. fossarum* [61], the apogamous fern *Dryopteirs affinis* [62], or the bivalve *Mytilus edulis* [63]. Proteogenomics offers a valid alternative to the long-lasting problem of the lack of protein-sequence databases for non-sequenced species. However, as highlighted by Trapp et al. [17], inferring functions for the proteins identified is another challenge. Current approaches to inference are based on sequence similarity or physiology-guided functional correlations in species where more physiological and biological data are available [61,64].

As discussed above, comparative shotgun experiments necessarily require the abundance of proteins in the samples to be determined. Due to rapid technological developments and the shift from gel to shotgun proteomics, label-free approaches (not requiring fluorescent or isotopic labels) have gained relevance in quantitative proteomics. Indeed, a high correlation was observed between protein abundance and chromatographic peak areas [65] or number of MS/MS spectra [66]. Despite its simplicity, this robust approach not only produces large amounts of data that require rigorous statistical treatment, but also identifies unique expression patterns for single or multiple proteins in the study conditions. This large scale quantification method of entire proteomes is the technique of choice in clinical proteomics for the initial biomarker discovery phase, since it allows the robust identification of expression patterns of modulated proteins in response to a stimulus. In environmental sciences, it has been applied in shotgun analysis for obtaining relative quantification of differentially expressed proteins after toxicant exposures [53,63,67].

Because they allow precise quantification, targeted proteomic approaches based on Selected Reaction Monitoring (SRM) is also widely used in clinical proteomics as part of biomarker validation pipelines, after the discovery phase [68]. Over the years, SRM-based protein quantification has been establishing itself as a valid alternative to classical immunoassays (such as ELISA - enzyme-linked immunosorbent assay), having already demonstrated comparable or superior performances [69]. By adding isotope-labelled peptides to the samples, precise, fast and reproducible quantification can be obtained [70]. In contrast to shotgun discovery proteomics, which analyses the whole proteome, SRM uses a quadrupole analyser as an ultra-precise filter to exclusively target pre-selected peptides identified as surrogates for the proteins of interest and then focuses on specific fragments generated by fragmentation of these peptides. This technique is highly sensitive and selective and presents large dynamic quantification ranges spanning 4

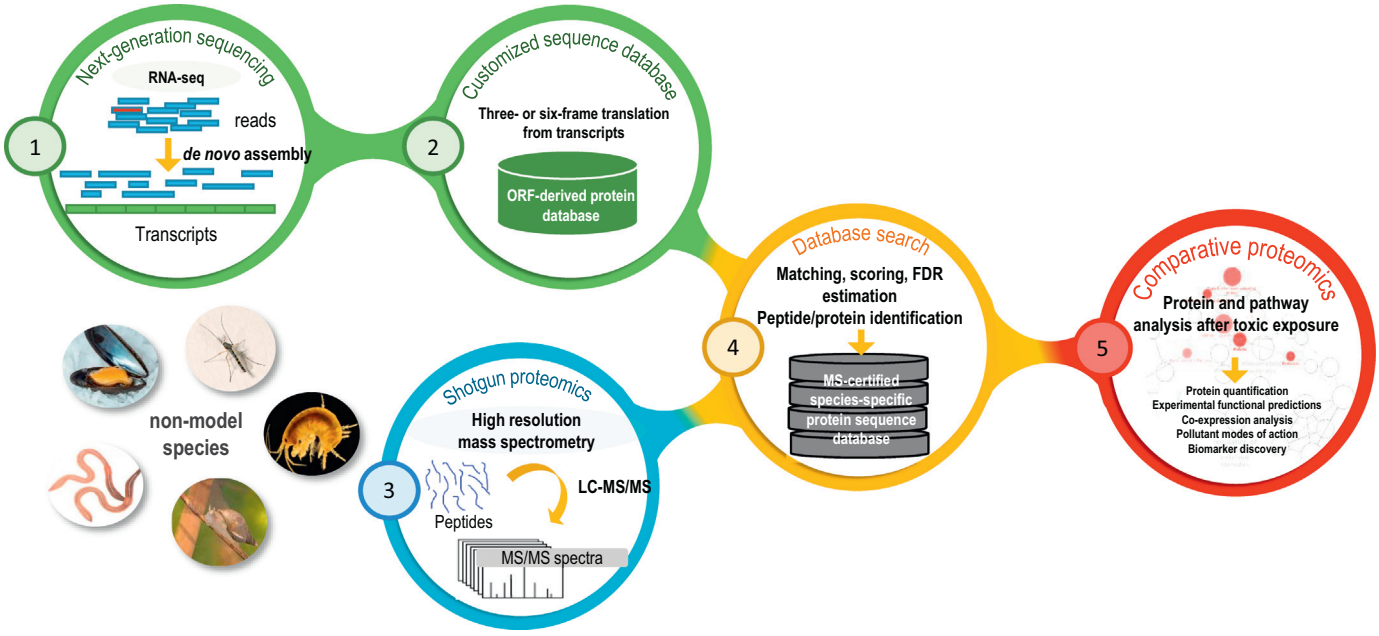

Fig. 3. Proteogenomics strategy to deal with emergent non-sequenced model species.

to 5 orders of magnitude [71]. Using both chromatography retention times and *m/z* values for specific ions, unequivocal identification/quantification of target proteins is possible even in highly complex samples. SRM uses protein-specific surrogate peptides to determine the concentration of the corresponding proteins. Therefore, some *a priori* knowledge must be available - such as the protein sequences and some chromatographic/MS properties of the surrogate peptides (normally derived from the shotgun discovery/proteogenomics stage). This strategy has been proposed as a diagnostic tool to assess the health status of sentinel organisms in ecotoxicology using single- or multi-biomarker approaches. SRM has been used to quantify the vitellogenin protein in the sentinel shrimp *G. fossarum* [72,73] and, more recently, in different fish species [74]. Also in *G. fossarum*, a multiplexed SRM approach (known as MRM – multiple reaction monitoring) has been developed and used to validate multiple species-specific biomarkers and assess their relevance for biomonitoring in field studies conducted in a regional river monitoring program [64,75–77].

However, SRM is still limited by the power of selection of appropriate peptides and the detection of their corresponding transitions after peptide fragmentation. For each project, a dedicated "SRM assay" should be developed, requiring the optimal selection of several peptides representative of each of the proteins of interest and their transitions, and their experimental validation. This choice is limited to a given number of transitions per nanoLC-MS/MS run (up to 1250 depending on instrumentation), which limits the multiplexing capabilities of the classical SRM approach [78]. In order to select the most intense transitions uniquely describing the target peptide, one can use empirical data obtained from public data repositories (*eg.* PRIDE or SRMatlas) or from experimental testing with eventually *in vitro* synthesized peptides/proteins. There are also bioinformatic tools to predict *in silico* the best transitions for a given peptide based on its sequence [79]. SRM holds great potential for a validation or a routine diagnostic tool to detect and accurately quantify a large set of proteins in large cohorts of samples with a high degree of reproducibility [71]. Because the approach is robust and standardized and can be easily transferred across platforms, SRM is a valuable tool that meets one of the main gaps for biomarker development and application in ecotoxicology as recently shown [64,75].

4. Environmental biomonitoring with ecotoxicology-relevant species

Biomonitoring has become an essential part of assessing the health status of the environment. This monitoring requires the use of several species of animal and plant indicators covering the largest possible spectrum of ecosystem biodiversity so as to better determine the impact of pollutants on different environments. Model species are good laboratory models, but one of the major challenges for ecotoxicologists is the transfer of results from proteomics studies performed in the laboratory as part of a proof-of-concept to field studies. Successful transfer requires the study of ecologically relevant wildlife species that are mostly "non-model" organisms in the sense that they cannot be easily shared in the scientific community to compare results on the same basis. When applying a species-sensitivity distribution approach in risk assessment studies, a minimum number of species, taxonomic groups and endpoints must be used to comply with regulatory guidelines, as a result, standard and non-standard tests and organisms are required [80]. Despite the significant growth of ecotoxicoproteomics over the last decade, only a few organisms have been studied, even though additional genomic data are now available. Moreover, in many cases little or no functional information is available about a significant number of protein sequences directly affected by pollutants. This lack of information is most likely due to the molecular divergence acquired by organisms during evolution and the importance of species-specific proteins in the response to environmental stimuli.

Addressing standardization of procedures used in field studies is of utmost importance in order to provide data that could correlate with the requirements that regulatory agencies would consider and/or use as additional information to corroborate reports or international guidelines. This may appear as a conundrum for ecotoxicology-relevant species. Next-generation shotgun proteomics allows for unprecedented data generation. Although standardized methods and first-class quality control environment are conventional in most proteomic platforms, reproducibility and variability are still difficult challenges. This is due to the defaults inherent to instrumentation and sample preparation methodology, to the high dynamic ranges of proteomes from sentinel species, and to the low number of biological replicates usually analysed. Another important challenge is data interpretation as numerous sentinel organisms should be analysed while their genotype may be slightly differing. This genomic variability may be important for specific biomarkers and thus, it is important to assess the sequence conservation of any molecular biomarker among the whole species or subspecies to be considered. Based on of the known evolutionary variability of biomarker sequences, two alternative strategies can be adopted to make operational the biomarker assay. First, only strictly conserved peptide sequences across the species are considered. Alternatively, a series of peptide sequences accounting for the known polymorphism of sequences within the phylogenetic group of interest (population, species, genus) can be monitored (*e.g.* [81]).

Last, when defining relevant biomarkers the study design should be carefully considered. The diversity of sentinel species needed for representative ecotoxicological testing and the many external confounding factors such as, season, water temperature, salinity, or pH should be taken into consideration. Calibrated organisms from reference populations, with synchronized reproductive status should be used, deployed and exposed in study sites in order to reduce bias related to some individual-related variables, as recently shown in some examples of active biomonitoring [75,82,83]. Moreover, fundamental studies on organism's physiology that assess the influence of individual (*e.g.* sex, age, reproductive status) or environmental variables (*e.g.* temperature, pH, salinity, feeding) should also be preferably conducted beforehand in order to know the variability of the proteome of reference organisms [25,84–86].

5. The ecotoxicoproteomic odysseys of *Mytilus edulis* and *Gammarus fossarum*, two aquatic sentinels

From our point of view, among the wide panel of sentinels used in aquatic ecotoxicology, two animal species have attracted the most interest in the last decade for omics-based biomonitoring purposes: the marine bivalve *Mytilus edulis* and the freshwater amphipod *G. fossarum*. These two organisms are well established sentinel species used to monitor aquatic pollution, and results from studies involving them illustrate the remarkable advances that have been made in the application of proteomics in ecotoxicology.

5.1. *Mytilus edulis*

Due to their suitable size, wide distribution, and filtering activity, mussels are ideal species for biomonitoring in marine environments, and they have been used in several monitoring programs all around the world, such as the Mussel Watch program [8]. The blue mussel *M. edulis* is one of the species most common to ecotoxicological studies, and has been used in a large number of proteomics studies over the last fifteen years.

Apraiz et al. [87] used a gel-based approach to identify proteomic signatures of exposure to several marine pollutants (diallyl phthalate, PBDE-47, bisphenol-A). Their results highlight unique protein modulation patterns associated with each of the contaminants. The effects of menadione-induced oxidative stress in thiol-containing proteins were investigated [88], and key protein targets for oxidative processes were identified, which underlie the response of the organism to the pro-

oxidant pollutant. Chip-based SELDI-TOF-MS (surface-enhanced laser desorption/ionization-time of flight-mass spectrometry) approaches were used to discover biomarkers associated with exposure to oil, heavy metals, and polyaromatic hydrocarbons in laboratory-controlled [10] and field experiments [89]. Campos et al. [90] reviewed all applications of gel- and chip-based approaches in ecotoxicoproteomics involving bivalves in 2012. In their interesting review, they predicted the advantages that state of the art technology, shotgun and targeted MS approaches could provide to the field, while also highlighting the limitations imposed by the lack of genomic information for invertebrate sentinel species. In 2015, the same authors published the first gel-free shotgun proteomic approaches with *M. edulis* as model species [91], describing the proteome of the hemolymph and revealing a group of molecular functions which contribute to the mussel's immune defences. Using modern sample preparation tools and instrumentation, and adding a transcriptomic database from *M. galloprovincialis* for the database search stage, they identified 1121 hemolymph proteins, of which 595 were successfully functionally annotated by sequence similarity. The use of a transcriptomic-inferred database increased protein identification three-fold compared to the UniProt KB database alone. Another proteogenomics approach identified 2071 proteins from mussel gills [63]. Using spectral count quantitative data to perform multivariate analysis, the authors managed to decipher the molecular mechanisms involved in mussel adaptation to low salinity stress, and documented how salinity modulates the effects of exposure to propranolol.

5.2. Gammarus fossarum

Detritivore gammarids are essential animals in aquatic systems. They are sensitive to a wide range of pollutants and are thus highly suited for ecotoxicological studies. *G. fossarum* and *Gammarus pulex* have been the most frequently used in ecotoxicology in recent decades [92]. Similar to the blue mussel, the studies using *G. fossarum* published in the last decade have allowed invaluable advances in the field of ecotoxicoproteomics. The application of proteogenomics to decipher the reproductive proteome of *G. fossarum* provided the necessary tools for a series of proteomics-based studies using this species (summarized in Fig. 4). These data were used to create a database of coding gene sequences consisting of 1873 MS-certified proteins, and to classify proteins with high sexual dimorphism that are implicated in key reproductive processes [61,93]. Molecular responses of male gammarids to several EDCs were also investigated through a comparative shotgun approach [67,94], resulting in the proposal of several proteins as biomarkers of reproductive disorders and endocrine disruption in males. The responses of *G. fossarum* to metal exposure over 10 weeks were recently reported, identifying protein deregulation profiles specific to cadmium, lead, and copper [95]. The high-throughput studies conducted in our laboratories yielded specific protein sequences related to key physiological processes along with dozens of biomarker candidates. We subsequently developed an MS-based precise multibiomarker quantification strategy to verify these biomarkers. With this strategy, up to 40 protein biomarkers could be simultaneously quantified in a sample from a single animal by SRM [76,77]. The method was used in physiological and ecotoxicological laboratory studies to validate the relevance of each candidate biomarker [64]. Importantly, the method was also successfully applied with animals sampled in the framework of a regional river biomonitoring program [75], thus validating its robustness and applicability in field conditions. By comparing organisms collected from contaminated *versus* reference (non-polluted) sites, clear contamination-related responses could be quantified and delineated. These proof-of-concept studies demonstrate that SRM is an interesting tool to quickly and quantitatively assess the health status of organisms by simultaneously measuring a wide panel of biomarkers. Furthermore, we have shown that SRM data can contribute to the construction of multibiomarker indices, providing a clear, visual integration of multiple responses, such as those illustrated in Fig. 4. Recently, a similar integration of proteomics data for an index integrating 34 candidate biomarkers was proposed to discriminate between pollutant types based on the eel proteomic response [96].

In addition to biomarker development, molecular physiology studies have been performed in *G. fossarum*. Shotgun proteomics led to the discovery of an unexpected range of proteins with yolk function in this species [93]. Proteogenomics analysis of five different species of crustacean amphipods shed new light on amphipod biodiversity, and was used to construct a database containing the core-proteome of amphipod female reproductive systems for future studies [56].

6. Challenges and perspectives for the coming decade

Developments in NGS technologies will facilitate transcriptome- and genome-sequencing, and have already improved MS-based protein identification over the last decade. In the future, continuously increasing power and more affordable costs will make sequencing platforms even more accessible to quickly read complex mixtures of RNA and DNA samples. Recent long-read sequencing methods such as PacBio [97] or Nanopore sequencing [98] provide improved perspectives for more accurate *de novo* genome-sequencing and genome assembly. Moreover, continuous improvements in proteogenomics-based genome annotations are being made, allowing discovery of all the coding parts of the genome. Despite the greater depth of RNAseq and the valuable knowledge obtained from transcriptome data, proteomics provides unique information - identifying functional transcripts that will produce mature proteins-, while also corresponding to a cheaper and faster time-to-results pipeline.

Nevertheless, there remain some hurdles associated with proteogenomics and proteomics studies, such as the limited coverage and dynamic range of MS-based analysis. Improvements in MS technology over the last decade have been remarkable, and will continue apace in the coming years, so these issues should be gradually overcome. Meanwhile, methodological breakthroughs make the best use possible of the available technology and have produced outstanding results. Innovative approaches such as data-independent acquisition (DIA) [99] or BoxCar acquisition [100] provide a broader picture of the complexity of proteomes by dramatically increasing proteome coverage. The unbiased DIA method aims to fully exploit the capabilities of mass spectrometers, by fragmenting and analyzing all ions within a given *m/z* range. In parallel, BoxCar acquisition is focused on improving the detection of intact precursor ions (at the MS level) to increase the dynamic range of detection, and has already been reported to detect 10,000 proteins from complex tissues in just 100 min [100].

In addition to the advances in discovery-led proteomics, new targeted approaches for highly multiplexed accurate protein quantification are also being developed. SCOUT-MRM, uses scout peptides to trigger complex transition lists, thus bypassing tedious chromatographic time scheduling. It has been successfully used for highly multiplexed targeted quantification of 445 proteins in a phytopathogen species [101]. In the same vein, the development of a targeted DIA approach - sequential window acquisition of all theoretical fragment ion spectra or “SWATH” - holds great promise for label-free protein quantification [102]. Through library-assisted mining of the complete fragment ion maps produced by DIA, SWATH can combine the best of shotgun and targeted approaches, allowing deep proteome analysis, and accurate and reproducible label-free quantification of entire proteomes. Another emerging field is elemental analysis based on Inductively Coupled Plasma Mass Spectrometry (ICP-MS) for the absolute quantification of peptides and proteins [103]. For now, the technique has only been applied to low-complexity samples, and it is not yet compatible with complex proteomes, but it shows great potential.

These technological and methodological advances will allow the main questions surrounding ecotoxicoproteomics to be addressed, as illustrated in Fig. 5. First, the molecular mechanisms involved in the

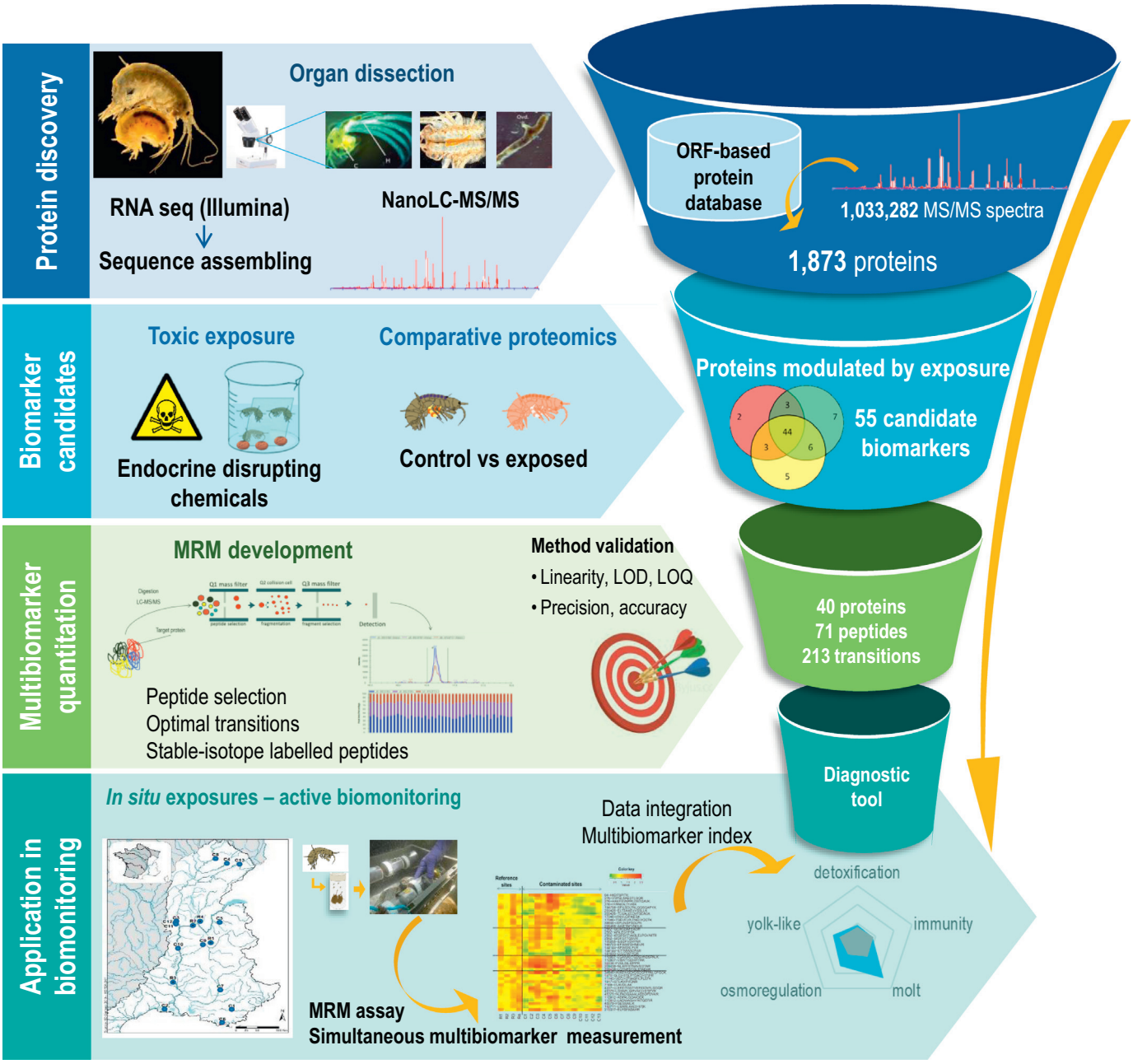

Fig. 4. The whole proteomics pipeline developed for *G. fossarum*.

mode of action of pollutants, leading to the development of biomarkers of toxicity. Moreover, this new knowledge of factors driving the sensitivity to contaminants could help to assess and predict the vulnerability of organisms, populations or species. The study of biodiversity is essential to ecotoxicology since it allows differences in toxic sensitivity of sentinels (and the species that they represent) to be assessed. Given the decreasing costs of sequencing technologies, proteogenomics could be used to study inter-population heterogeneity, intra-species diversity, and even inter-species variability. Indeed, selection of protein biomarkers for a given sentinel species should obviously take into account the possible polymorphism that may exist in the sample cohorts. For this, a good knowledge of the biodiversity of the sentinel species is required. Inter-individual or population genetic polymorphism, and proteoforms diverging between organs or development stages (alternative splicing, polypeptide cleavage, post-translational modification) should be explored. These studies will be crucial if we are to propose peptide and protein biomarkers that will be applicable to groups of phylogenetically similar populations or species [81]. As nicely exemplified by the multiplexed SRM biomonitoring assay developed for gammarids [75], targeted proteomics will play a key role in ecotoxicoproteomics. More recent approaches such as SCOUT-MRM or SWATH could be used in the near future to develop rapid high-throughput assays to accurately quantify multiple biomarkers for biomonitoring purposes. Alternatively, if monitoring of massive cohorts of sentinels is planned, the most relevant biomarkers highlighted by discovery proteomics could be assayed by rapid antibody-based biomarker detection kits. Proteomics data can also be integrated with Adverse Outcome Pathways (AOPs) to establish the links between sub-individual biomarker responses and potential effects at higher levels of biological organization. AOPs are an emerging concept in risk assessment, and omics data can provide essential weight of evidence information for molecular initiating events and key events in AOPs, as well as filling all the knowledge gaps between molecular markers and individual effects [104]. Like AOPs proposed for human health,

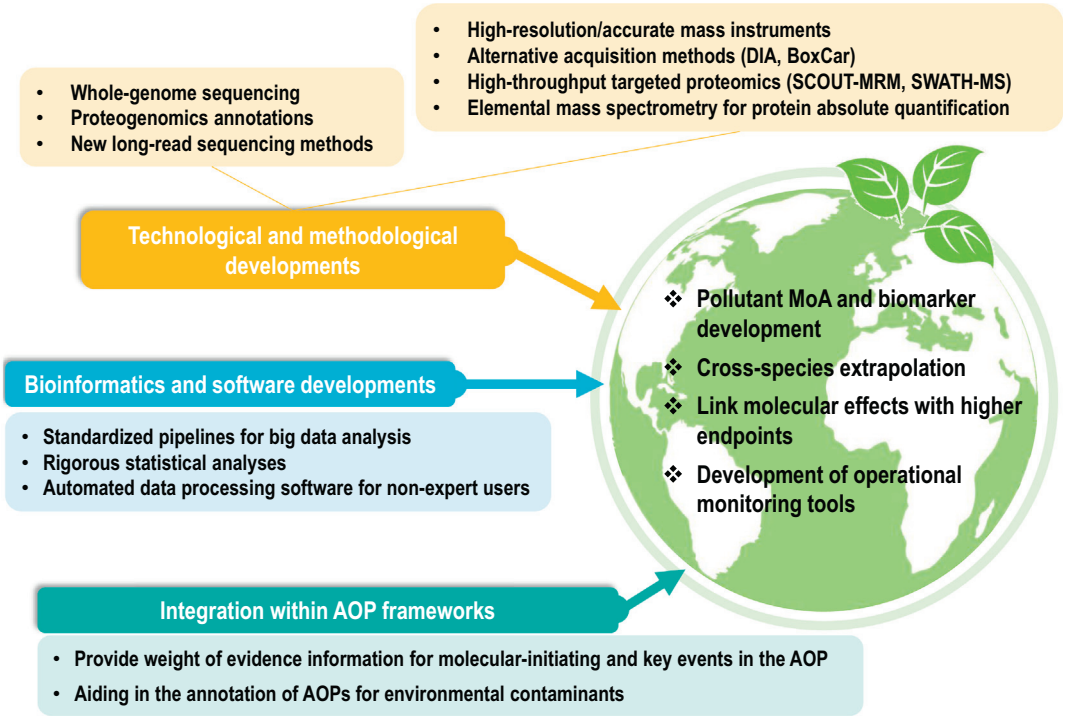

Fig. 5. The future of Ecotoxicoproteomics: how the expected developments can help determine the toxicity of pollutants.

biological events leading to detrimental effects on global environmental health could be documented. We propose to name this concept "Adverse Outcome Ecosystem Services (AOES)". Because animals and their microbiota should be considered as a whole, the holobiont principle - a more integrated view - will be necessary to better understand the response of sentinel organisms. More generally, as the Earth's microbiome is a key item for ecosystem services, further exploration and integration of microbiological data with data for sentinel species will be an important target for future research. Other proteomics-based methods such as metaproteomics could help to reach this long-term holistic goal.

6.1. Concluding remarks

This review was written in the context of the celebration of the tenth anniversary of the *Journal of Proteomics*. Under the decisive influence of its Editor in Chief, Dr. Juan Calvete, over this past decade *Journal of Proteomics* has provided a platform for numerous papers describing methodological advances in proteomics, while also giving audience to a wealth of studies on biomarker discovery, non-conventional and non-model organism proteomics, proteogenomics methodological developments, and pioneering ecotoxicoproteomics studies. Indeed, the influence of *Journal of Proteomics* in ecotoxicoproteomics is highlighted by the numerous citations in the present review to articles published in this journal. No doubt the next decade will be even more exciting for ecotoxicoproteomics with more biology, multi-omics integration, revolutionizing systems biology approaches, and reinforced interactions with other methods and scientific fields. We gratefully acknowledge the support of the *Journal of Proteomics* over this last decade and look forward the next decade of the journal with enthusiasm.

Important advances in ecotoxicoproteomics have been made in the last decade, especially for the monitoring of marine and freshwater environments, but there is still a long way to go to determine how these tools can be integrated into risk assessment frameworks and environmental policies. Large numbers of biomarkers are being proposed as a result of the comparison of "control versus exposed" proteomes, but very few are being subjected to further validation. However, the recent use of high-throughput shotgun approaches, proteogenomics, and targeted proteomics reinforces the potential of next-generation proteomics tools for the discovery of new toxic responses or adaptive paths, discovery and validation of species-specific biomarkers, and providing tools for reliable prediction and diagnosis of the health status of organisms. In this context, the pipeline proposed in Fig. 5 constitutes a relevant approach that provides an answer on how to integrate proteomics in routine environmental monitoring studies for regulatory purposes. These tools need to be properly implemented by determining their inter- and intra-laboratory performance through appropriate quality control procedures. For biomarker development, bioinformatics tools and statistical analysis to ensure the elimination of false negatives/positives from datasets need to be improved. This must be done alongside studies on the influence of other biotic/abiotic influences, introduced as confounding factors when measuring biomarker responses to ensure that the biological response is effectively due to the pollutants. Ecotoxicoproteomists must integrate all of the new proteomics developments into their research and go beyond the classic protein expression signatures that have dominated the field for so long. By developing structured "omics" studies using appropriate technical, methodological, and bioinformatics tools, it will be possible to dig deeper into large volumes of molecular data, whatever the species chosen to answer our research objectives. For the next decade, ecotoxicoproteomics should become an essential pillar of the plan to "Make our planet safe again".

Conflict of interest

No conflict to declare.

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