

Review

Can Proteomics Be Considered as a Valuable Tool to Assess the Toxicity of Nanoparticles in Marine Bivalves?

Beatrice De Felice and Marco Parolini * 

Department of Environmental Science and Policy, University of Milan, via Celoria 26, I-20133 Milan, Italy; beatrice.defelice@unimi.it

* Correspondence: marco.parolini@unimi.it

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Abstract: Exposure to nanoparticles (NPs) has been identified as a major concern for marine ecosystems. Because of their peculiar physico-chemical features, NPs are accumulated in marine organisms, which suffer a variety of adverse effects. In particular, bivalve mollusks represent a unique target for NPs, mainly because they are suspension-feeders with highly developed processes for cellular internalization of nano- and micrometric particles. Several studies have demonstrated that the uptake and the accumulation of NPs can induce sub-lethal effects towards marine bivalves. However, to understand the real risk of NP exposures the application of the so-called “omics” techniques (e.g., proteomics, genomics, metabolomics, lipidomics) has been suggested. In particular, proteomics has been used to study the effects of NPs and their mechanism(s) of action in marine bivalves, but to date its application is still limited. The present review aims at summarizing the state of the art concerning the application of proteomics as a tool to investigate the effects of nanoparticles on the proteome of marine bivalves, and to critically discuss the advantages and limitations of proteomics in this field of research. Relying on results obtained by studies that applied proteomics on bivalve tissues, proteomics application needs to be considered cautiously as a promising and valuable tool to shed light on toxicity and mechanism(s) of action of NPs. Although on one hand, the analysis of the current literature demonstrated undeniable strengths, potentiality and reliability of proteomics, on the other hand a number of limitations suggest that some gaps of knowledge need to be bridged, and methodological and technical improvements are necessary before proteomics can be readily and routinely applied to nanotoxicology studies.

Keywords: nanoparticles; proteomics; marine invertebrates; mussels; clams

1. Nanoparticles as Contaminants of Marine Ecosystems

Nanomaterials (NMs) are defined as natural or engineered items having at least one dimension <100 nm, whereas nanoparticles (NPs) are defined as items having all their dimensions <100 nm [1]. NPs can originate from both natural and anthropogenic processes; the former include volcanic eruptions, forest fires, photochemical reactions in the atmosphere, degradation and oxidation of minerals and organic matter, while the latter include unintentionally created (i.e., diesel engines exhaust, ashes and other combustion by-products such as PM_{2.5} and PM₁₀) and intentionally designed NPs by humans to be used in different applications in medicine, industry and everyday life (i.e., engineered NPs) [2]. Because of their nanometric size, which is comparable to the size of biomolecules, and their large surface-to-volume ratio, NPs can interact with biological systems [1]. Several studies have suggested that the emission of both natural and anthropogenic NPs in the atmosphere, soil and water can result in their accumulation in natural ecosystems, representing a risk for living organisms [1,3]. In particular, marine ecosystems have been identified as a major sink of diverse anthropogenic contaminants and they are prone to receive notable amounts of NPs, whose fate, exposure and biological effects represent

one of the main challenges that ecotoxicology has to face [4]. The importance of assessing the impacts due to NP exposure towards marine organisms has been pointed out by a variety of ecotoxicological studies performed on diverse marine organisms belonging to different trophic levels, from planktonic to fish species [3,5–10]. All these studies have been focused on the identification of potential biological targets and/or suitable models for risk assessment [7,8,11], demonstrating the uptake, accumulation and toxicity of diverse NPs. In particular, studies of marine invertebrates returned a pivotal contribution to the understanding of accumulation pathways, toxicity and mechanism(s) of action (MoA) of NPs [12]. These studies confirmed the capability of marine invertebrates to ingest and to accumulate NPs that can interact with the immune system and cause different adverse effects mediated by the onset of oxidative stress and cell injury in proteins, membrane and DNA damage, which have been identified as the main MoA of NPs in marine invertebrates [10].

Among marine invertebrates, bivalve mollusks have been identified as a unique target group for nanoparticle toxicology because of their particular ecological and physiological features [13]. Bivalves are suspension-feeders and can filter large volumes of water, ingesting microalgae, bacteria, sediments, particulate matter, natural and anthropogenic nanoparticles, as well as accumulating different chemicals in their soft tissues [6]. Bivalves have long been considered as valuable indicators of pollution and vast background information on their biological responses to both inorganic and organic chemicals is currently available [13,14]. Several experimental research studies have highlighted the usefulness of marine bivalves, including mussel, clam and oyster species, to shed light on the toxicity of different typologies of NPs. Such studies mainly focused on the investigation of NP-induced developmental or sub-lethal effects, which have been explored by the application of ecotoxicity tests on early life stages or opportune batteries of biomarkers, respectively [12]. Ecotoxicity tests returned contrasting results, which differed according to the bivalve species used as model organisms and/or to the typology and concentrations of NPs used during experiments. For instance, the exposure C₆₀ fullerene induced developmental changes in the oyster *Crassostrea virginica* [15], but no effects were noted in embryos of the Mediterranean mussel (*Mytilus galloprovincialis*) exposed to n-TiO₂ [16,17] or Fe₂O₃ NPs [18]. Also, the application of batteries of biomarkers on different organs from marine bivalves (i.e., hemocytes, gills and digestive gland) have demonstrated that NP exposure can induce notable adverse effects at different levels of the biological organization (i.e., from the molecular to tissue level) [19–24]. These studies, which were performed according to both in vitro and in vivo approaches, revealed that in marine mussels, and generally in marine invertebrates, the immune system represents a major target for the effects of NPs [12]. For instance, in vitro studies of *M. galloprovincialis* showed that the quick uptake of metal-oxides and carbon-based NPs (i.e., carbon black and fullerenes) in hemocytes affected lysosomal function, phagocytic activity and oxyradical production, as well as the induction of pro-apoptotic processes [6]. Similarly, exposure to different types of NPs returned similar effects on phagocytic activity in hemocytes collected by the oyster *Crassostrea gigas* [25] and the clam *Ruditapes philippinarum* [26]. In vivo experiments exposing mussels to different NP types confirmed the results on the immune system suggested by in vitro tests [27]. Other studies have demonstrated that one of the main MoA of NPs is directly or indirectly mediated by reactive oxygen species (ROS) and free radicals, and the consequent onset of oxidative stress and cell injury to proteins, membranes and DNA [10]. NP-induced ROS overproduction can originate from different pathways, depending on the typology of the NP. For instance, metal-based NPs can induce adverse effects, including oxidative stress, both per se or after the dissolution into ions [28], while the toxicity of carbon-based NPs can be exacerbated by the presence on their surface of other toxic compounds according to a sort of “Trojan horse” effect [29].

Focusing on oxidative stress-related effects in marine invertebrates, many findings showed that it depends on different factors, including particle size and composition (e.g., metal- or carbon-based NPs), experimental approach (e.g., concentration, pathway and time of exposure) as well as target organ [12]. Thus, the application of a battery of biomarkers can be suggested as a tool for the screening of the effects and the MoA of different types of NPs in marine bivalves. However, although biomarkers

can provide an early signal of a potential NP-induced biological effect, especially at sub-organism level (e.g., investigating responses at the molecular, biochemical and physiological levels) [30], their use is biased by some limitations. The application of biomarkers relied on an a priori knowledge of the toxicity mechanisms of a specific contaminant and it is essentially hypothesis driven [31]. Biomarkers currently available for animals often derive by analogy from human and/or vertebrate biology, so the transposition to invertebrate species might not result in equivalent response specificities because of the evolutionary distance between lineages [32]. The use of conventional individual biomarkers could also be biased because they investigate only a specific target or relatively few proteins, excluding many others that might also be altered by exposure to a contaminant but that cannot be measured because of the lack of specific assays or because no a priori hypotheses on their involvement in the MoA of the contaminants have been suggested [33], being not sufficiently representative of the whole set of MoA and the specific adverse effects induced by contaminants. Thus, the urgent need to understand the real risk of NP exposure towards marine organisms pushed researchers to the application of diverse global screening strategies to integrate the information obtained by ecotoxicological approaches based on acute or chronic tests or biomarker assays. In recent years, some studies have relied on the so-called “omics” techniques (e.g., proteomics, genomics, metabolomics, lipidomics), to explore the effects and to shed light on the MoA of NPs towards marine bivalves. Among the “omics”, proteomics is one of the most used technique in ecotoxicology. Proteomics is a useful tool that can help to provide an overview of both structural and functional cellular information, as well as to explore the MoA of diverse contaminants. Moreover, compared to ecotoxicity tests and the application of biomarkers, the use of proteomics can allow the display of protein networks and the identification of alterations of specific proteins or protein patterns that could be used to identify biomarker candidates for particular contaminants [32], as well as to integrate existing biomarkers in a multi-biomarker approach or to identify different isoforms of existing biomarkers in order to obtain a better overview of a specific biomarker response. Lastly, the identification of critical molecular pathways and regulatory networks involved in the most sensitive species should allow a cross-species comparison due to the functional similarities detected by comparative genomics [34].

In recent years, ecotoxicoproteomics, i.e., the application of proteomics in ecotoxicology, has been increasingly used in environmental hazard identification, through the monitoring of protein expression in sentinel organisms exposed to environmental pollutants under both laboratory and field conditions [33,34]. Changes in the molecular machinery of an organism that experience a pollutant-induced stress represent the starting points of its physiological response [35–37] and can provide an early diagnostic of the onset of potential adverse effects at higher-levels of biological and ecological organization. Proteomics has been conducted on ex vivo, in vitro and in vivo experimental models to assess the toxicity and MoA of different NPs. A wide array of proteomics studies have been applied on cell cultures through ex vivo or in vitro approaches, showing that a general stress response of cells exposed to different typologies of NPs, both metal- and carbon-based NPs, altered the expression of diverse proteins generally associated with oxidative stress, energy metabolism, cytoskeleton organization and apoptosis [1]. Similarly, in vivo proteomics studies returned the evidence that a general stress response raised in NP-exposed animals, pointed out by altered expression of proteins associated with metabolism, cellular response to oxidative stress and immune response [1]. Despite these findings, ecotoxicoproteomics studies exploring the toxicity of NPs towards invertebrates or environmentally related biological indicators remain under-represented and focused only on a limited number of species [1]. Focusing on marine invertebrates, to date, only few studies have been applied to assess the toxicity and the MoA of different typologies of NPs. These studies have been focused only on three different taxa: a study on the diatom species *Phaeodactylum tricorutum* exposed to quantum dots [38], one on the sea urchin (*Paracentrotus lividus*) exposed to polystyrene nanoparticles [39] and 12 on bivalves exposed to different typologies of NPs (reviewed in the paragraphs below).

Considering that most proteomics studies that investigated the toxicity of NPs towards marine invertebrates have been focused on bivalves, the present review aimed at (1) outlining the current state of the art concerning the ecotoxicological investigation of NP toxicity on marine bivalves, pointing out

the benefits of proteomics application compared to traditional approaches (i.e., standard ecotoxicity test and biomarkers); (2) summarizing the main changes on the protein profile of marine bivalves induced by exposure to different typologies of NPs; (3) identifying possible advantages (pros) and limitations (cons) regarding the use of proteomics in NPs ecotoxicology using marine bivalves as model organisms; and (4) suggesting implementations and new perspectives on the application of proteomics to assess NP toxicity.

A systematic literature research review was carried out using Google Scholar, Scopus and Web of Science databases, focusing on papers published in the years 2005–2020. Different combinations of keywords, including nanoparticles, proteomics, invertebrates, mussels, clams and toxicity were used to search for published papers on the focal topic.

2. NPs-Induced Changes in Bivalve Proteome

To the best of our knowledge, to date only 12 studies have used proteomics to investigate the possible changes in protein profiles induced by NPs exposure on marine bivalves (Table 1). According to studies of uptake, accumulation and sub-lethal toxicity [6,9,10], the *Mytilus* spp. represent the most used bivalve species to assess the toxicity of NPs through the application of proteomics. *Mytilus galloprovincialis* was the selected model species in 50% of the studies while 33% of them analyzed the effects on *Mytilus edulis*. Only two studies (17%) investigated the effects of NPs on the clam species *Ruditapes philippinarum*. Different proteomic techniques have been applied on diverse tissues of bivalves. Gel-based proteomics represent the most used approach, whereby in detail the 75% of studies relied on a non-redox proteomics, while only 25% of the studies used redox proteomics (among them 66% used two-dimensional (2-DE)-based and 33% mono-dimensional (1-DE)-based; Figure 1a). Electrophoresis gel separation was the most commonly used technique for proteins separation, whereby 42% of the studies used two-dimensional (2-DE), 17% used mono-dimensional (1-DE) and 17% used both mono- and two-dimensional gel electrophoresis. The so-called “gel-free” proteomics were less used in studies assessing the toxicity of NPs towards marine bivalves. Only one study (8%) used LC-MS (liquid chromatography-mass spectrometry), while two studies (16%) used a 2nd-generation isobaric Tags for Relative and Absolute Quantitation (iTRAQ-8plex) to analyze changes in protein profile due to NP exposure.

Regarding the typology of NPs analyzed in studies on marine bivalves, although in the environment, countless different typologies of NPs occur, metal NPs were the most studied (Figure 1b). In detail, 42% of the studies have investigated the changes to proteome of marine bivalves induced by gold nanoparticles (AuNPs) [40–44], while 33% of the studies have focused on silver nanoparticles (AgNPs), uncoated or coated with Poly N-vinyl-2-pyrrolidone/Polyethyleneimine (PVP/PEI) [45–48]. In addition, AgNPs-induced changes on mussel proteome were investigated through functional [45,49] and redox proteomics [46,47]. Seventeen percent of the proteomic studies of NPs have been focused on copper oxide nanoparticles (CuONPs) [49,50], while only one study (8%) has used non-metal NPs focusing on the changes of bivalve proteome induced by fullerene (C₆₀) [51].

2.1. NP-Induced Changes of Protein Profile in Mussels

The first studies aimed at evaluating the effects of NP exposure to the proteome of marine bivalves was performed on *Mytilus edulis* [40]. Mussels were exposed for 24 h to 750 µg/L of AuNPs (13 nm in size) and changes in protein profile of digestive gland and gills was assessed through one dimensional gel electrophoresis. Higher levels of ubiquitination were observed in the digestive gland compared to the gills, which conversely showed higher levels of protein carbonylation. A companion study performed by the same research group investigated the effects of 24-h exposure to 750 µg/L of AuNPs (15 nm in size) on the proteome of *Mytilus edulis* through 1-DE and 2-DE-based proteomics [41]. The 1-DE highlighted that exposure to AuNPs decreased the levels of protein thiols suggesting a moderate pro-oxidant activity of these NPs, confirming the results obtained by a previous study [40]. In addition, the exposure to a smaller AuNPs (5 nm in size) caused a decrease in thiol-containing proteins [42]. The overall amount

of thiol-containing proteins was lower in treated mussel within respect to individuals from the control group. Moreover, the 2-DE-based analyses allowed us to identify a total of 24 spots (i.e., proteins) that occurred in tissues isolated from the control mussels but not in the AuNPs-treated ones. These results suggested that the exposure to AuNPs reduced the amount of thiols proteins and caused a notable protein thiol oxidation. The variation of *Mytilus galloprovincialis* proteome after the exposure to AgNPs was investigated by [45]. Mussels were exposed to 10 µg/L of AgNPs (<100 nm in size) for 15 days and, at the end of the exposure, the proteome of the gills and digestive gland was analyzed by 2-DE-based proteomics. The 2-DE-image pattern analyses showed that AgNPs modulated the expression of 347 and 248 proteins for the gills and the digestive gland, respectively. In detail, focusing on proteins showing a twofold or higher variation compared to the controls, 129 (104 were up-regulated and 25 were down-regulated) and 83 (60 were up-regulated and 23 were down-regulated) proteins were differentially expressed in the gills and the digestive gland isolated from the treated mussels compared to the control individuals, respectively. Further analyses of peptide mass fingerprints (PMS) and mass spectra were performed by using matrix-assisted laser desorption/ionization (MALDI) coupled with a TOF/TOF (time-of-flight) analyzer allowing the identification of 15 proteins belonging to 4 different functional classes. In detail, identified proteins belong to structural proteins (i.e., actin, paramyosin, catchin protein, α -tubulin, precollagen P and myosin heavy chain), metabolic proteins (i.e., ATP synthase F0 subunit 6, NADH dehydrogenase subunit 2), stress response protein (i.e., major vault protein, glutathione s-transferase, putative C1q domain containing protein, heat shock protein 70 and ras, partial) and transcription proteins (i.e., nuclear receptor family 1G). These results showed a tissue-specific protein expression; only ras, partial, NADH dehydrogenase subunit 2 and myosin heavy chain were differentially expressed in the digestive gland, while the other proteins were differentially expressed in the gills.

A similar study was performed on the gills and digestive gland isolated by *Mytilus galloprovincialis* specimens exposed for 15-days to 10 µg/L of CuONPs (<50 nm in size) [49]. 2-DE-based proteomics showed a tissue-specific change in mussel proteome, whereby most of the proteins were up-regulated in the gills and down-regulated in the digestive gland of treated organisms compared to the control specimens. In detail, 103 gill proteins and 119 digestive gland proteins of the treated specimens showed a twofold or higher change in their volume compared to the controls. Eighty-six gill proteins showed an up-regulation, while a down regulation occurred for 17 proteins, while for the digestive gland 68 proteins were up-regulated and 41 proteins were down-regulated. The identification by MALDI TOF/TOF showed that CuONPs altered the expression of protein involved in cytoskeleton and cell structure, oxidative stress, energy metabolism, apoptosis and proteolysis. In detail, in the gills, an up-regulation of protein related to oxidative stress (i.e., glutathione), energy metabolism (i.e., ATP synthase F0 subunit 6) and stress response (i.e., heat shock cognate 71) was noted, while down-regulation concerned proteins related to cytoskeleton and cell structure (i.e., actin), transcription regulation (i.e., zinc-finger BED domain-containing protein 1 and nuclear receptor subfamily 1G) and stress response (i.e., putative C1q domain containing protein). Conversely, in the digestive gland, up-regulated proteins were related to proteolysis (i.e., cathepsin L) and stress response (i.e., heat shock cognate 71), while down-regulated proteins were related to apoptosis (i.e., caspase 3/7-1), cytoskeleton and cell structure (i.e., paramyosin and actin). Interestingly, this study suggested the consideration of caspase 3/7-1, cathepsin L, Zn-finger protein and precollagen-D as new target proteins for the study of CuONPs exposure [49]. Interestingly, protein identification clearly showed that the toxicity of CuONPs is not solely due to the Cu²⁺ ion release [49].

Table 1. List of proteomic studies performed on marine bivalves, mussels and clams, to investigate changes in protein profile due to the exposure to different nanoparticles (NPs).

| NPs | Concentration | NPs Size | Exposure Time | Model Species | Tissue | Technique | Reference |
|-----------------|-------------------|---------------|------------------|---|--------------------------|---|-----------|
| Mussels | | | | | | | |
| Au | 750 µg/L | 13 nm | 24 h | <i>Mytilus edulis</i> | Gills Digestive gland | 1-DE SDS-PAGE | [40] |
| Au | 750 µg/L | ~5 nm | 24 h | <i>Mytilus edulis</i> | Digestive gland | 1-DE SDS-PAGE, 2-DE (IEF/SDS-PAGE) | [41] |
| Au | 750 µg/L | ~15 nm | 24 h | <i>Mytilus edulis</i> | Digestive gland | 1-DE SDS-PAGE, 2-DE (IEF/SDS-PAGE) | [42] |
| Ag | 10 µg/L | <100 nm | 15 days | <i>Mytilus galloprovincialis</i> | Gills Digestive gland | 2-DE (IEF/SDS-PAGE); MALDI-TOF/TOF MS/MS | [45] |
| Ag | 100 µg/L | <50 nm | 3–6–12 h 12 h | <i>Mytilus galloprovincialis</i> | Gills Digestive gland | 1-DE | [46] |
| Ag | 100 µg/L | <100 nm | 3–6–12 h 12 h | <i>Mytilus galloprovincialis</i> | Gills Digestive gland | 1-DE | [46] |
| Ag | 100 µg/L | <50 nm | 12 h | <i>Mytilus galloprovincialis</i> | Gills Digestive gland | 2-DE (IEF/SDS-PAGE); MALDI-TOF/TOF MS/MS | [47] |
| Ag + PVP/PEI | 10 µg/L | 5 nm | 2 days | <i>Mytilus galloprovincialis</i> Female only | Digestive gland | 2-DE (IEF/SDS-PAGE); MALDI-TOF | [48] |
| CuO | 10 µg/L | <50 nm | 15 days | <i>Mytilus galloprovincialis</i> | Gills Digestive gland | 2-DE (IEF/SDS-PAGE); MALDI-TOF/TOF MS/MS | [49] |
| CuO | 400–700–1000 µg/L | ~100 nm | 1 h | <i>Mytilus edulis</i> | Gills | 2-DE (IEF/SDS-PAGE); MALDI-TOF/TOF MS/MS | [50] |
| C ₆₀ | 0.01–0.1–1 mg/L | 653 ± 87 nm | 3 days | <i>Mytilus galloprovincialis</i> | Digestive gland | LC-HRMS | [51] |
| Clams | | | | | | | |
| Au | 0.75 µg/L | 21.5 ± 2.9 nm | 1 days 7 days | <i>Ruditapes philippinarum</i> | Digestive gland | iTRAQ-8plex | [43] |
| Au | 0.75 µg/L | ~20 nm | not available | <i>Ruditapes philippinarum</i> | not declared | iTRAQ-8plex MS/MS | [44] |

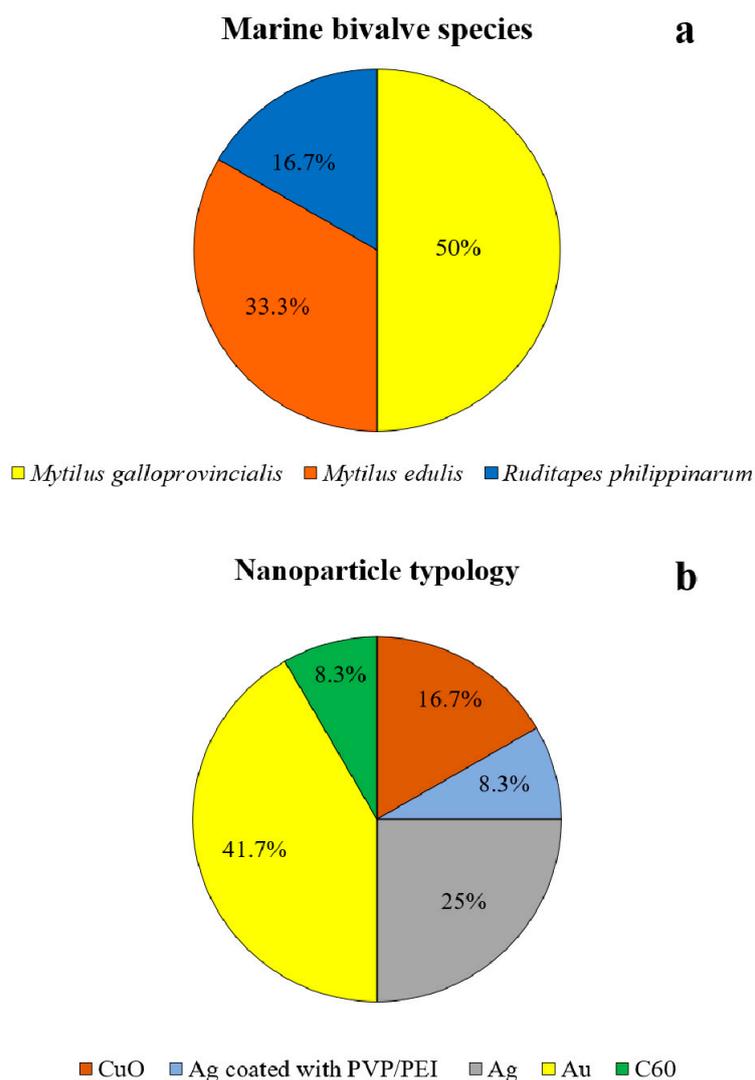


Figure 1. Percentage of proteomic studies performed on different bivalve species (a) and different nanoparticles (b) to investigate changes in protein profile. Au = gold nanoparticles; Ag = silver nanoparticles; PVP/PEI = Poly N-vinyl-2-pyrrolidone/Polyethyleneimine; CuO = copper oxide nanoparticles; C₆₀ = fullerene nanoparticles.

In most proteomic studies the individual sex was not considered, although metabolic and enzymatic pathways of mussels could vary according to several factors, including the gender and the season [52]. For example, some studies of mussels suggested a gender-specific response to contaminants [53], and for this reason used in the experiments only females and not males [37,38]. Accordingly, [48] used female individuals of *Mytilus galloprovincialis* to investigate the changes in protein profile after 21 days' exposure to 10 µg/L of PVP/PEI-coated AgNPs (5 nm in size). Moreover, the experiments were performed in autumn and in spring in order to take into account the seasonal variability of mussel physiology. The 2-DE-based proteomics showed that in autumn, 36 proteins, from digestive gland tissue, were present only in the treated mussels, while 22 proteins were differentially expressed between the treated and control specimens, whereby 9 proteins were up-regulated and 13 proteins were down-regulated. In contrast, 2-DE on mussels exposed in the spring showed that 83 proteins were significantly expressed in the treated mussels, while 33 proteins showed a twofold change or higher between the treated and control mussels, with 11 proteins up-regulated and 22 down-regulated. Further analyses showed that the exposure to AgNPs led to the alteration of diverse metabolic pathways (i.e., amino sugar

and nucleotide sugar, carbon metabolism, glycolysis/gluconeogenesis and the biosynthesis of amino acids). In detail, the MALDI-TOF analyses showed that differentially expressed proteins in mussels exposed to AgNPs in autumn were mostly related to metabolic process, cell adhesion and transcription proteins. Glyceraldehyde-3-phosphate dehydrogenase showed a different expression between spring (down-regulated) and autumn (up-regulated); partial was under-expressed in spring while the same protein was over-expressed in autumn. The nuclear receptor subfamily 1DEF, NR1DEF was expressed only in mussels exposed to AgNPs in autumn, while the putative C1q domain containing the protein MgC1q52 was only expressed in spring. Similarly, AgNPs exposure altered the transcriptional regulation and protein related to cysteine, methionine, pyruvate and citrate metabolism in autumn, while proteins involved in the formation of phagosomes and hydrogen peroxide metabolism were influenced only in spring. Lastly, AgNPs exposure modulated the expression of proteins involved in the organization of the cytoskeleton (i.e., actin and paramyosin), independent of the season. These results suggest that in natural environments the effects of AgNPs on the proteome of mussels can be affected by seasonality, which can influence the physiology of the organisms.

Several studies have shown that the onset of oxidative stress represents one of the main MoAs of NPs in marine bivalves [10]. To perform an in-depth analysis of the role of NPs in inducing oxidative stress, redox proteomics was suggested as a valuable approach. Redox proteomics analyzed the components of the proteome that undergo reversible redox reactions and those modified irreversibly by reactive species during oxidative stress [53]. Redox proteomics investigation was used to explore the changes in protein profile induced by 1-h exposure to 400, 700 and 1000 $\mu\text{g/L}$ of CuONPs (~100 nm in size) on *Mytilus edulis* [50]. The 2-DE-based proteomics suggested that CuONPs exposure induced oxidative damage to proteins. In detail, an overall, dose-dependent decrease in protein thiols and an increase in carbonylated proteins was noted in the gills and the digestive gland of treated mussels compared to the controls. Further MALDI-TOF/TOF MS analyses allowed the identification of six proteins (i.e., α -tubulin, β -tubulin, actin, tropomyosin, triosephosphate isomerase and Cu-Zn superoxide dismutase), whose expression was significantly changed between the treated and control mussels. Among these proteins, two were also targets for thiol oxidation (i.e., actin and triosephosphate isomerase) and three were targets for carbonylation (i.e., α -tubulin, tropomyosin and Cu-Zn superoxide dismutase). Interestingly, four of the CuONPs-modulated proteins were cytoskeletal components. Overall, these results confirmed that exposure to CuONPs can cause an oxidative stress situation in mussels and induce oxidation to gills and digestive gland proteins. Redox proteomics was applied to gills isolated from *Mytilus galloprovincialis* exposed for 3, 6 and 12 h to 100 $\mu\text{g/L}$ of AgNPs (<50 nm and <100 nm in size) [46]. Both sizes of AgNPs induced the oxidation of protein thiol and/or protein carbonylation. The 1-DE approach showed that 3-h exposure to 100 nm of AgNPs led to an increase in the carbonyl content of gills, while in the digestive gland after exposure to 50 nm AgNPs. After 6 h of exposure an increase in thiol content after exposure to 50 nm AgNPs was noted in the gills, while an increase in thiol content was noted in the digestive gland after exposure to AgNPs of both sizes. Lastly, 12 h of exposure to 50 nm AgNPs caused a decrease in thiol content, while an increase in carbonyl content was noted after exposure to both sizes of AgNPs in the gills. No effects were noted in the digestive gland. Lastly, a redox proteomics study was performed to evaluate the redox-based changes in the proteome of *Mytilus galloprovincialis* after 12 h of exposure to AgNPs (50 nm in size) [47]. The 2-DE analysis showed a significant volume change of at least 1.5-fold either in carbonyl content, thiol content and/or protein expression level. Overall, changes in thiol content were mainly found in proteins from the digestive gland, while changes in carbonyl content were found only in the gills. All the proteins detected in the digestive gland showed a significant change in thiols when exposed to AgNPs. Fourteen of the proteins with changed volume between the treated and control mussels were identified and belonged to different functional classes, including cytoskeleton and cell structure, energy and metabolism, general and oxidative stress as well as transcription and regulation. In detail, in the digestive gland no significant changes were shown in carbonyl content protein extract from the digestive gland of treated mussels, while thiol content (i.e., HSP 70, trypsin, shell myostracum collagen-like

protein, collagen like protein, actin, cationic trypsin, predicted peptidyl-prolyl cis-trans isomerase) and protein content (i.e., chitinase-like protein 3, HSP 70, trypsin, shell myostracum collagen-like protein, collagen like protein, actin, cationic trypsin, predicted peptidyl-prolyl cis-trans isomerase) showed at least a 1.5-fold change of volume in both directions (up- or down-regulated). In the gills a significant increase in carbonyl content (i.e., trypsin) and in thiol content (i.e., procollagen-proline dioxygenase β subunit) was noted, as well as a decrease in protein content volume for trypsin and procollagen-proline dioxygenase β subunit.

To date, only one study used liquid chromatography-high resolution mass spectrometry (LC-HRMS) to analyze modulations in the protein profile of marine bivalves [50]. In this work, authors investigated the ecotoxicological effect induced by a 3-day exposure to 0.01, 0.1, 1 mg/L of fullerene (C₆₀) on *Mytilus galloprovincialis*. At the end of the exposure, the digestive gland was processed in order to evaluate the differentially expressed proteins. Surprisingly, none of the selected concentrations led to significant changes in the protein profile of the digestive gland isolated from C₆₀-treated and control mussels.

2.2. NP-Induced Changes of Protein Profile in Clams

To the best of our knowledge, to date only two investigations have been aimed at assessing the NP-induced changes of protein profiles in marine clams. A first study investigated the alteration of *Ruditapes philippinarum* proteome after 1 and 7 days of exposure to 0.75 μ g/L of AuNPs (21.5 \pm 2.9 nm in size) [43]. A 2nd-generation isobaric tags for relative and absolute quantitation (iTRAQ-8plex) proteomic approach was used to quantify and to identify differentially expressed proteins between control and treated clams. The iTRAQ-8plex analysis showed that AuNPs could affect the proteome of the digestive gland isolated by treated clams. In detail, over 2500 proteins involved in several metabolic pathways and/or fundamental physiological process were modulated. A further investigation on the potential effect of 0.75 μ g/L of AuNPs (~20 nm in size) on *Ruditapes philippinarum* proteome was performed [44]. Proteins were quantified and identified by a bottom-up iTRAQ-8plex proteomic approach followed by a tandem mass spectroscopy. Moreover, deep investigation with Lassoland Elastic-Net allowed for better information and the identification of 105 proteins that showed differential expression between the control and treated organisms. The identified proteins were involved in oxidative stress, inflammatory response, cytoskeleton and cell structure.

3. Pros, Cons and Future Perspectives

By critically analyzing the current literature, it is difficult to univocally answer the title question concerning the valuable role of proteomics in the assessment of NP toxicity for marine bivalves. Based on the findings obtained by the 12 studies reporting the effects of NPs on the proteome of marine bivalves, proteomics application can be considered cautiously as a promising and valuable tool to shed light on the toxicity and MoA of NPs. In fact, regardless of the undeniable strengths, potentiality and reliability of proteomics, a number of limitations suggest that some knowledge gaps need to be bridged, and methodological and/or technical improvements are necessary before applying proteomics routinely in nanotoxicology studies.

The studies of proteomics that to date have investigated the changes of protein profile induced by exposure to NPs were performed on different tissues isolated from three species of marine bivalves (*Mytilus edulis*, *Mytilus galloprovincialis* and *Ruditapes philippinarum*), namely the hemocytes, the gills and the digestive gland. Such studies revealed a species-specific and tissue-specific response to NPs exposure, precluding the comparison of results between organisms and the identification of a unique MoA of specific NPs. In addition, bivalves suffer an additional limitation in proteomics that is related to the size and/or the amount of total proteins characterizing each specific tissue. Compared to other aquatic organisms, such as fish, the total protein content is often relatively low and generally shows a decreasing amount as follows: digestive gland, mantle and gills [54]. For this reason, in order to obtain an appropriate amount of protein it is often necessary to pool the tissues isolated from different

individuals, leading to a potential disappearance of specific proteins that can be expressed only in the individual gels [55].

Proteomics were applied to assess the changes in protein profile induced by a limited number of NPs (mainly metallic ones), which were administered to bivalves at different concentrations. Considering the amount of different natural and anthropogenic NPs entering marine ecosystems, further research should be necessary to explore the potential alterations of protein profile of bivalves induced by exposure to other typologies of NPs (e.g., carbon-based or plastic NPs), both alone and in combination. In fact, proteomics can help to disentangle whether the adverse effects and MoA of NPs can originate from the particle per se or by the dissolution of ions from metal-based NPs or to other contaminants adsorbed on carbon-based NPs. All these studies could help to obtain useful information concerning the hazard of NPs towards marine organisms and, consequently, to improve further screening methods and risk assessment strategies [56]. For instance, the development of proteomics, and “omics” in general, could support the development of new biomarkers to be used as indicators of both chemical exposure and related biological effects. The application of proteomics has been demonstrated as a useful tool to obtain complex information of modulations in different biochemical pathways in tissues from model organisms exposed to xenobiotics under laboratory or field conditions (see the review by [54]), as well as to predict the MoA of specific contaminants [57]. Thus, proteomics could help to shed light on the MoA of NPs in marine bivalves, as the identification of proteins that varied their expression after NP exposure might support the understanding of molecular pathways or processes involved in NP toxicity. However, this information is likely to be incomplete for non-model organisms whose genome is not fully sequenced, because not all the proteins that suffer a change as a consequence of NP exposure can be separated or identified due to methodological or technical limitations, respectively. For these reasons, proteomics applied on marine bivalves is still far from being a tool to be used in developing risk assessment strategies for marine ecosystems. To bridge this gap of knowledge, a novel approach, named proteogenomics, has emerged as a straightforward strategy for discovering proteins in non-model organisms [58,59]. This approach relies on fast genome or transcriptome sequencing, six-reading frames translation of coding nucleic acids, and the creation of a molecular database comprising protein sequences validated proteomics data obtained by next-generation proteomics [60–62]. To date, this approach has not yet been applied on marine bivalves but it has shown all its potential in other non-target aquatic species [61].

Different methodological approaches and analytical techniques have been used to apply proteomics on bivalve tissues to assess NP toxicity. Most studies explored the alterations of protein profile by applying 1-DE or 2-DE-based proteomics, which are the prevalent techniques used in proteomics on bivalves. Interestingly, as biomarker studies have shown that one of the MoA of NPs is related to oxidative stress [6,12,16,17], one of the pertinent gel-based proteomics approaches is represented by redox proteomics, which explore protein modification due to oxidative stress. Although carbonylation and glutathionylation of diverse proteins induced by NP exposure were shown by gel-based proteomics, the coupling of these techniques with mass spectrometry analysis is suggested to contribute to the identification of modified proteins and their residues [54]. Because of some strengths (i.e., robustness, parallelism and ability to analyze complete proteins at high resolution) and limitations (i.e., time-consuming, costly, insensitive to low-abundance proteins and unable to separate properly the whole proteome), 2-DE-based proteomics remain a valuable top-down proteomics approach [63]. For instance, a typical 2-DE can visualize only 30–50% of the entire proteome, depending on the type of tissue [64], as proteins that are present in low concentrations or cannot be separated because of their physicochemical properties (e.g., pI, hydrophobicity, molecular weight) are not visualized on the gel. To overcome 2-DE limitations, “gel-free” proteomics, using, for example, multi-dimensional LC-MS/MS, have been implemented but scarcely applied on bivalves to assess NPs proteotoxicity. This approach can allow the separation and identification of peptides obtained from the enzymatic digestion of a protein extract. However, in spite of a shorter time for the analyses compared to 2-DE, the main limitation of this approach consists in the identification of a specific protein based on the sequence of a single (or a few) tryptic peptide(s) derived from this specific

protein, precluding to discriminate between isoforms or protein posttranslational modifications [64]. Another “gel-free” approach used in bivalve proteomics concerns the isobaric tag for relative and absolute quantitation (iTRAQ). The use of iTRAQ is currently in its infancy in bivalve proteomics and it seems far from application in studies on NP toxicity on bivalves. Although this technique allows the display of many proteins, it attenuates the differential display between the proteins. In fact, severe statistical problems are related to iTRAQ, which render it difficult to confirm statistically significant up- or down-regulation of proteins. Overall, despite the accuracy and sensitivity of “gel-free” mass-spectrometry-based methods (i.e., LC-MS/MS or iTRAQ), a high-quality spectral library based on fragmentations and retention time of proteins is necessary for protein identification but to date is not exhaustive for non-model organisms [64]. Interestingly, in order to improve reproducibility and the quantitative power of traditional techniques, in recent years multiplexed liquid chromatography mass spectrometry–selected reaction monitoring (SRM)-based assay and targeted acquisition methods, such as multiple reaction monitoring (MRM) or parallel reaction monitoring (PRM), were proposed as a promising tool for specific multi-biomarker measurements in non-target aquatic species, especially in environmental biomonitoring [61,62]. To date, these techniques have not been applied on marine invertebrates, but their application to other non-target species suggests that they can return a valuable contribution to ecotoxicoproteomics.

Despite some differences in the proteomics methods and experimental settings applied in NPs toxicity studies, it is interesting to note that certain groups of proteins associated to diverse biological functions modulated their expression recurrently (e.g., cytoskeleton, oxidative stress, energy metabolism and apoptosis-related proteins). Specifically, the groups of proteins that often changed in response to NPs included cytoskeleton proteins (e.g., actin, paramyosin, α -tubulin, myosin heavy chain) or stress response proteins, (e.g., HSP70-family of proteins or proteins related to the antioxidant system). This is not true only in marine bivalves but also in other invertebrate and vertebrate model organisms, suggesting a general cross-taxa MoA of NPs. Interestingly, the vast majority of proteomic studies performed on different biological models (both vertebrates and invertebrates) according to different experimental approaches (ex vitro, in vitro and in vivo) have revealed that, regardless of the typology of NP whose toxicity was assessed, modulation of oxidative-stress related proteins, such as antioxidant ones, occurred. Similarly, all the redox proteomics studies showed that oxidation of different proteins occurred as a consequence of NP exposure. These findings support those obtained by the application of biomarkers, and clearly confirmed the role of oxidative stress in the MoA of NPs towards marine bivalves and, in general, living organisms. However, proteomics studies of marine bivalves have shed light on the modulation of proteins specifically induced by certain typologies of NPs that were not related to oxidative stress and/or cannot be investigated through biomarkers, suggesting other potential pathways involved in NP toxicity. However, it is important to consider that proteins whose expression commonly varied in response to NPs can be also be modulated as a consequence of exposure to other environmental stressors. Although the modulation of these proteins can point out a response of bivalves to counteract stressful situations due to contaminant exposure or environmental stressors, they are commonly identified because they are well-described, well-conserved and represented in databanks. One of the main limitations in proteomics of bivalves concerns the lack of sequenced genomes, also for the species that are commonly used as model organisms in laboratory and field ecotoxicological studies. In spite of recent efforts in sequencing, as in the case of *Mytilus galloprovincialis* [65], bivalves remain a not well-annotated species and many proteins can either not be identified or their function in these species remains uncertain.

Another interesting point that deserves to be discussed concerns the number of proteins that appear to be differentially displayed in the studies mentioned above. Although some studies did not report the number of proteins that were differentially displayed as a consequence of exposure to NPs, a huge variability occurred in other investigations, whereby tens to thousands of proteins were differentially displayed. This variability could be due to different reasons, including experimental design (e.g., concentration, pathway and time of exposure), differences in sensitivity or protein content among tissues as well as methodological and technical concerns. Overall, these results are in

agreement with those obtained by the application of proteomics in other model organisms, whereby approximately 500 to 1000 proteins were revealed in 2-DE-based proteomics or 2000–3000 proteins can be obtained in MS-based proteomics. Although, statistically, the oftentimes low numbers of proteins differentially displayed fall in the range of what can be obtained by chance, the fact that most of them consistently appear in proteomics studies might provide an argument against their appearance by chance, as proteins that appear by chance would change from study to study. In spite of this consideration, there is a great degree of uncertainty concerning the number of proteins that can be modulated by exposure to NPs; this is also exacerbated by the fact that most of these proteins cannot be appropriately identified because of the limitations mentioned above (i.e., lack of completeness of the non-model organism's genome). Thus, further pathway analyses and enrichment tools should be useful to elucidate more comprehensively the MoA of specific NPs and to furnish a more detailed information on their toxicity towards marine bivalves.

Another gap in proteomic research on marine bivalves concerns the lack of data on field-exposed organisms. To date proteomics were applied only in bivalves exposed to NPs under controlled laboratory conditions, while no study investigated how NP exposure might affect the proteome of free-living bivalves, likely because a series of limitations (e.g., estimation of exposure concentrations, changes in physiology of the organisms due to gender or seasonality, co-exposure with other contaminants) greatly complicate the interpretation of results, limiting the use of proteomics for risk assessment procedures of NPs.

In conclusion, at present and at the state of the art, intrinsic limitations of proteomics seem to exceed advantages, which requires us to carefully consider if proteomics can be considered as a useful tool for ecotoxicological studies on non-target organisms, not only to assess the toxicity and the MoA of NPs (or other contaminants) but also in biomonitoring operations [54,66,67]. Proteomics surely can provide valuable information beyond what can be obtained from the application of classical ecotoxicological tests or biomarkers. For instance, proteomics may improve biomarker measures by integrating multiple markers or by measuring the complexity in specific markers, but at the same time it has not, as yet, practically provide any new biomarker, suggesting that it is simply likely not very useful for biomarker discovery. However, as the contribution of “omics” to ecotoxicology has been a matter of debate for years [54,68], some recent methodological and technical advances encourage the application of novel approaches and analyses. Moreover, considering the power and potentiality of proteomics, they surely deserve to be integrated in further interdisciplinary research relying on holistic approaches to shed light on the toxicity of NPs. For instance, in the case of non-target species with a partially sequenced genome, a better-annotated transcriptomes might represent a valuable starting point to improve proteomics approach. Nanoparticle exposures investigated by proteomics need to be preceded by transcriptomic analyses in order to provide the respective genetic information allowing the identification of the pathways specifically affected by NPs. With such a two-step procedure, it is possible to obtain much more detailed information on the pathways affected, also for non-model organisms. Thus, the integration of proteomics with other “omics” techniques should be useful to understand the toxicity of NPs and to obtain reliable data to be used in a risk assessment of these hazardous contaminants for marine ecosystems.

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