Received: 10 January 2022 | Revised: 11 August 2022 | Accepted: 11 August 2022

DOI: 10.1002/pmic.202200008

REVIEW

Proteomic evaluation of nanotoxicity in aquatic organisms: A review

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Abstract

The alteration of organisms protein functions by engineered nanoparticles (ENPs) is dependent on the complex interplay between their inherent physicochemical properties (e.g., size, surface coating, shape) and environmental conditions (e.g., pH, organic matter). To date, there is increasing interest on the use of 'omics' approaches, such as proteomics, genomics, and others, to study ENPs-biomolecules interactions in aquatic organisms. However, although proteomics has recently been applied to investigate effects of ENPs and associated mechanisms in aquatic organisms, its use remain limited. Herein, proteomics techniques widely applied to investigate ENPs–protein interactions in aquatic organisms are reviewed. Data demonstrates that 2DE and mass spectrometry and/or their combination, thereof, are the most suitable techniques to elucidate ENPs–protein interactions. Furthermore, current status on ENPs and protein interactions, and possible mechanisms of nanotoxicity with emphasis on those that exert influence at protein expression levels, and key influencing factors on ENPs–proteins interactions are outlined. Most reported studies were done using synthetic media and essay protocols and had wide variability (not standardized); this may consequently limit data application in actual environmental systems. Therefore, there is a need for studies using realistic environmental concentrations of ENPs, and actual environmental matrixes (e.g., surface water) to aid better model development of ENPs–proteins interactions in aquatic systems.

KEYWORDS
aquatic organisms, engineered nanoparticles, nanotoxicity, protein expression, proteomics

1 | INTRODUCTION

The increasing production and applications of engineered nanoparticles (ENPs) in numerous household products and industrial applications, have in turn, increased their presence in the aquatic environments. For example, a 25-fold increase in commercialized nanoproducts globally was estimated between 2005 and 2010 [1], and production of half a million tons of ENPs in general by 2020 [1–3]. This increase is also evidenced by increasing reports on detected environmental concentrations of ENPs [4]. As a result, there are concerns about potential deleterious effects of ENPs on human health, and ecological integrity [5–7]. For example, myriad studies have documented ENPs toxicity to aquatic organisms at different levels of biological organization whether at epic, cellular, and/or molecular end-points including on fish [8–13], algae [14, 15], nematodes [16], crustaceans [17–19], plants [20–22], and bacteria [23]. In turn, ENPs

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can impose deleterious ecological impacts by altering or halting the role of these organisms in the ecosystems. For example, the interactions of a nematode roundworm *Caenorhabditis elegans* with ENPs in its ecological settings led to an inhibition of their reproduction, and as a result could not provide ecosystem services as a decomposer [24].

Studies have demonstrated that ENPs can induce deleterious effects by establishing direct contact with aquatic organisms through organs such as gills or gastrointestinal tract (oral exposure) as observed in fish [25, 26], and bacteria [27], or cell membranes as is the case for bacteria [28–30]. Following the contact process, ENPs may then penetrate cell walls, disrupt cell membranes, and/or even in certain cases be internalised through endocytosis pathways into various cell organelles, such as the endoplasmic reticulum, golgi apparatus, and endo-lysosomal system, which in turn may result in inducing oxidative stress [31]. Some widely documented mechanisms of ENPs toxicity includes the release of ions from soluble ENPs, induction of oxidative stress through the production of reactive oxygen species (ROS), and physical interaction with environmental organisms [32–36].

Use of conventional approaches to determine nanotoxicity are limited as they do not elucidate the underlying mechanisms, but only reveal physical impacts, e.g., mortality and reproduction [37–39]. In recent years, "omics" based analytical approaches that can aid to elucidate the toxicity mechanisms of environmental pollutants are increasingly finding wide applications [40, 41], especially as most pollutants occur at low concentrations of ng/l to μg/L including ENPs [42]. Omics approaches include proteomics, genomics, transcriptomics, and metabolomics [43]. In the case of nanotoxicity, proteomics techniques have yielded insights into the ENPs–protein interactions at molecular level, even though the number of studies remain low [44–46]. The proteome of any organism is dynamic and changes greatly in response to external stimulus, and therefore those differentially expressed proteins, and enzymes can be used as specific biomarkers for nanotoxicity ([47].

Thus, the objectives of this review are two-fold, and chiefly to examine the ENP-protein interactions. First, to highlight the mechanisms of ENPs toxicity to aquatic systems with specific emphasis on those that influence protein expression levels. Secondly, identify proteomic approaches applied in the nanoecotoxicity studies as well as an outline of their merits and limitations.

2 | CURRENT LITERATURE ON PROTEOMICS RESEARCH ON AQUATIC ORGANISMS

Herein published literature on use of proteomic techniques to aquatic organisms were collected from sources including Google scholar, Scopus, Web of Science databases, and Science Direct using a set of keywords. Search terms used include proteomics, toxicity, nanoecotoxicity, ENPs, engineered nanomaterials, nanomaterials, and environment. Peer-reviewed articles identified and included in the review were from 2010 to 2022. Nanotoxicity studies on human cell lines, mice, conference papers, abstracts, and reports were omitted. As a result,

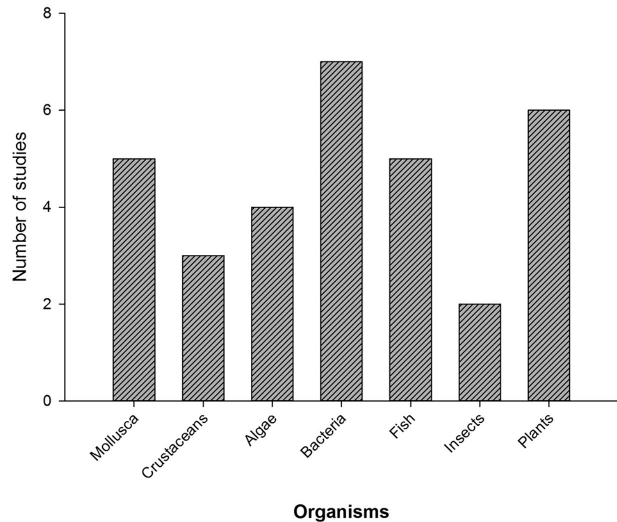

FIGURE 1 Number of proteomic studies on the interactions of ENPs with different classes of taxa in the aquatic systems.

32 peer-reviewed articles were selected for this review (Figure 1), categorised according to organisms studied.

A variety of ecotoxicological studies were performed on various aquatic organisms and they have employed a wide range of proteomic techniques as summarised in Table 1. The studies indicate that various proteomic related end points have been investigated including protein expression levels, protein alterations, and enzymatic activities. Broadly, these studies have highlighted proteomic-related effects associated with nanotoxicity, e.g. the accumulation of protein precursors, expression of envelope proteins, protein digestions, expression of protein thiols, and carbonyls as well as alterations in protein abundance and expressions. Although proteomics have demonstrated the ability to reveal toxicity at molecular level, key results on mechanisms still remain scanty. Furthermore, proteomic studies reported to date are for different organisms and types of ENPs ranging from low to unrealistic high exposure concentrations (0.4 –2.86 $\times$ 10^7 μg/L) (Table 2). As a result, the published data is defined by inconsistences with reference, for example, to exposure concentration ranges, species or ENPs types, and therefore, limits the ability to draw firm conclusions or trends.

3 | FACTORS INFLUENCING PROTEOME-RELATED NANOTOXICITY

During interactions with biological materials in aquatic systems, both the ENPs physicochemical properties and environmental factors play a key role concerning the observed toxicity. In essence, the 'nano–bio' interface has been proposed to govern the nanotoxicity, and determines the nature of physicochemical interactions (e.g., dynamic, kinetic, and other exchanges) between ENPs and the surfaces of various biological components (e.g., proteins, membranes, phospholipids, DNA) [73]. The ENPs inherent controlling factors include size, capping agents, charge and stability influence the properties of the nano–bio interface

TABLE 1 Use of proteomics to investigate the effects of ENPs to organisms at different levels of organizations using various techniques, varying ENPs properties, and exposure media chemistries

Organism group	Organism name	NPs type	PC properties	Duration	Dosage (μg/L)	Proteomic approaches	Endpoint	Target organ/system	Assay/ Environmental factors	Proteomic related effects Results	Effects related to ENP concentration and environmental factors (water chemistry)	Ref
Mollasca	*Mytilus galloprovincialis*	nAg	Size: 50 and 100 nm	3, 6, 12 h	100	1 DE	Thiol oxidation and protein carbonylation	Gills and digestive glands	Oxygenated artificial seawater (Salinity 35%, pH 8, aeration, temperature 16°C,	Accumulation of envelope protein precursors nAg destabilize the outer membrane Expression of cell envelope proteins stimulated by nanoparticle treatment	Free thiol content increased with nAg Presence of DMSO increased thiol concentration Thiol oxidation decreased in the presence of nystatin	[1]
Crustacean	*Daphnia magna*	nAg	40 and 110 nm	24 h	0, 2, 4, 8, 16, 32 and 64	ICP-AES	Protein digestion	Whole organism	NS	Protein digestion upon exposure to nAg	NS	[2]
Plant	*Oryza sativa L.*	nAg	Size: 18.34 nm Shape: Spherical	20 d	0.03, and 0.06	2-DE Nano LC/FT-ICRMS	Protein expression level	whole plant	NS	Abundance of responsive proteins changed (increment and decrement)	NS	[3]
Bacteria	*Mytilus edulis*	nAu	Size:5.3 ± 1 nm	24 h	750	1DE 2DE SDS PAGE	Oxidative stress responses	Whole cell	NS	Decreased thiol-containing proteins	Increased diameter of ENPs caused modest effects	[4]
Crustaceans	*Daphnia magna*	nAg	Size: 3–11.4 nm	24 h	5, 15, 20 and 30	1DE 2DE MALDI/TOF TOFMS	Protein expression levels	Whole organism	Elendt M4 medium	Exposure to nAg increased protein thiol and carbonyl levels	NS	[5]
Bacteria	*Bacillus thuringiensis*	nAg	Size: 18.34 nm Shape: spherical	450 and 550 min	0, 0.001, 0.0025, and 0.005	2-DE NanoLC/FT-ICRMS	Protein expression level	Whole cell	NS	Exposure of *B. thuringiens* to nAg caused an accumulation of envelope protein precursors, indicative of the dissipation of a proton motive force.	NS	[6]
Plant	*Eruca sativa* (rocket)	nAg	Size:14±0.3 nm Coating: poly vinyl pyrrolidone (PVP)	5 d	1.0×10^4	2-DE nanoLC-nESI-MS/MS)	Protein expression level	Seeds	NS	Alteration of endoplasmic reticulum and vacuole proteins Accumulation of sulphur metabolism protein noted. nAg induced jacalin lectin family proteins	NS	[7]

(Continues)

TABLE 1 (Continued)

Organism group	Organism name	NPs type	PC properties	Duration	Dosage (µg/L)	Proteomic approaches	Endpoint	Target organ/system	Assay/ Environmental factors	Proteomic related effects Results	Effects related to ENP concentration and environmental factors (water chemistry)	Ref
Crustaceans	*Daphnia magna*	nAg	Size: 3.0-11.4 nm	24 h	5, 15, 20, 30	2-DE Bioinformatics analysis	Protein expression level	NS	Bradford assay	Increased protein thiol and carbonyl was observed	NS	[8]
Bacteria	*Bacillus cereus*	Nanosized zero-valent ion (nZVI)	Size: <50 nm	3 h	5	2D-DIGE SDS–PAGE Scanning Differential in-gel analysis (DIA) module	Protein expression level	Whole cell	Not specified	Overexpression of oxidative stress response and tricarboxilic acid (TCA) modulation proteins Repression of motility and biosynthesis	NS	[9]
Mollasca	*Mytilus edulis*	nCuO	Size: 100 nm	1 h	0.4, 0.7 and 1.0	2 DE MALDI-TOF/TOF	Protein thiols and carbonyls expression	Gill tissue extracts	Not specified	Decreased protein thiols Protein oxidation shown by increased carboxylation	Increasing nCuO decreased protein thiols and increased protein carbonyls	[10]
Algae	*Phaeodactylum tricornum*	CdSe/ZnS quantum dots	Size: 0.2 µm	4 d	69– 345	2DE SELDI-TOF-MS	Differential protein expression	Whole organism	NS	Exposure to QDs changed protein expression patterns	NS	[11]
Mollasca	*Mytilus galloprovincialis*	nAg	Size: <50 nm and <100 nm	15 d	10	2DE MALDI-TIF-TOF	Protein expression	Gills and digestive glands	NS	Exposure to nAg changed protein expression patterns	NS	[12]
Plant	*Triticum aestivum*	nAg	Size: 5.6 nm	4 h	1×10^3 and 1×10^4	2 DE LC-ESI-MS/MS	Protein expression levels	Seeds	NS	nAg altered expression levels of proteins involved in primary metabolism and cell defence	NS	[13]
Mollusc	Oyster	nZnO $nMnO_2$	Size: 200µM	24 h (nZnO) 48 h ($nMnO_2$)	1.63×10^4 (ZnO) 8.06×10^3 (MnO)		Overall toxicological outcome (Oxidative stress)	Larvae	Artificial sea water conditions	Ion sorption blocked redox-active sites	Organic matter mitigated toxicity of nZnO and $nMnO_2$	[14]

(Continues)

TABLE 1 (Continued)

Organism group	Organism name	NPs type	PC properties	Duration	Dosage (μg/L)	Proteomic approaches	Endpoint	Target organ/system	Assay/ Environmental factors	Proteomic related effects Results	Effects related to ENP concentration and environmental factors (water chemistry)	Ref
Bacteria	*Pseudomonas*	nAg	NS	NS	307.2	SWATH-MS	Protein expression level Antioxidant enzymes activities	Whole organisms	NS	Exposure to nAg altered the content of 59/166 proteins Exposure to nAg increased enzymatic activities of superoxide dismutase and glutathione peroxidase Exposure to nAg affected protein translation	NS	[15]
Algae	*Chlorela vulgaris*	nZnO	NS	24 h	5×10^4, 1×10^5, 2×10^5,	Flow cytometry	Oxidative stress	Whole organism	NS	nZnO decrease glutathione and increased lipid peroxidation Antioxidant enzyme superoxide dismutase (SOD) activity increased with exposure to nZnO	GSH decreased with nZnO concentrations > 100 mg/L LPO increased with nZnO	[16]
Fish	*Danio rerio*	nGO	NS	21 d	10, 1×10^2, 1×10^3	LC-MS/MS	Differentialy expressed proteins	Whole organism	60 mg/L natural salt water	Altered functions and pathways of troponin complex, actin cytoskeleton, monosaccharide transmembrane transporter activity, oxidoreductase activity and focal adhesion	NS	[17]
Fish	*Gilthead seabream (Sparus aurata)*	nAu	Size: 7 and 40 nm	96 h	80	2DE MALDI-TOF/TOF MS	Protein abundance alterations	Liver	Artificial seawater	nAu altered abundance of 26 proteins related to cytoskeleton and cell structure, gluconeogenesis, amino acid metabolism, protein activity (synthesis, catabolism, folding, transport), energy metabolism, stress response.	NS	[18]

(Continues)

TABLE 1 (Continued)

Organism group	Organism name	NPs type	PC properties	Duration	Dosage (µg/L)	Proteomic approaches	Endpoint	Target organ/system	Assay/ Environmental factors	Proteomic related effects Results	Effects related to ENP concentration and environmental factors (water chemistry)	Ref
Plant	*Phaseolus vulgaris L.*	$nCeO_2$	Size: 10–30 nm	15 d	2.5×10^5, 5.0×10^5, 1.0×10^6 2.0×10^6	Hybrid quadrupole time-of-flight (Q-TOF)	Protein abundance	Aerial plant parts	Double distilled water	Increase in Ce induced accumulation of proteins responsible for folding and turnover	NS	[19]
Algae	*Chlorella vulgaris*	nAg	Size: 24 and 29 nm Coatings: Citrate(24 nm) and polyethyleneim (29 nm)	24 h	71.2 ± 13.6 *(citrate)* 51.6 ± 9.6 *(polyethyleneimi*	L-CQ Exactive mass spectrometry	Protein expression	Whole organism	BG 11 (algal culture) media	Negatively charged nAg regulated mitochondrial function related proteins Positively charged nAg affected ribosome function-related proteins	NS	[20]
Mollasca	*Mytillus galloprovincialis*	nAg	Size: <50 and <100 nm	3, 6 and 12 h	100	Bradford method 1DE	Protein quantification	Gill and digestive gland	NS	Both sizes induced protein thiol oxidation and/or protein carbonylation	NS	[21]
Algae	*Phaeodactylum tricornutum*	nCdSe	Size: 5 and 12 nm	7 d	83 (5 nm size) 222 (12 nm size)	Bradford assay NanoLC-MS/MS TripleTOF mass spectrometer	Protein expression	Whole organism	f/2 medium realised with filtered natural sea water	In response to toxicity, nCdSe responded by regulating proteins involved in protection	NS	[22]
Plant	*Nicotiana tabacum L.*	nAg	Size: 50 nm	30 d	2.7×10^3, 5.4×10^3, 8.1×10^3, and 1.08×10^4	2 DE MALDI-TOF/TOF	Protein expression	NS	Solid Murashige and Skoog (MS) nutrient medium	66 proteins were up-regulated and 1 down regulated due to nAg Up-regulated of primary metabolism proteins	NS	[23]
Insect	*Oncopeltus fasciatus*	$nTiO_2$ nAl_2O_3	Size: 30–60 nm	5 d	2.86×10^7 1.43×10^7	Bradford method	Protein expression	NS	NS	$nTiO_2$ increased protein content in adult insects while nAl_2O_3 decreased protein content	NS	[24]

(Continues)

TABLE 1 (Continued)

Organism group	Organism name	NPs type	PC properties	Duration	Dosage (µg/L)	Proteomic approaches	Endpoint	Target organ/system	Assay/ Environmental factors	Proteomic related effects Results	Effects related to ENP concentration and environmental factors (water chemistry)	Ref
Insect	Silkworm (*Bombyx mori*)	nAg	30 nm	155 h	1×10^5 4×10^5	2 DE Mass Spectrometry	Protein expression	Larval midguts	Deionised water	Increase in nAg concentration lead to down-regulation of digestive enzymes, resulting in enzyme superoxide dismutase and protein HSP 1 suppression. this eventually led to oxidative stress	NS	[25]
Bacteria	*Halophilic Bacillus sp. EMB9*	nAg	NS	42 h	107.9	2 DE LCESI-MS/MS	Protein expression level	Whole cell	4% (v/v) mother culture	50% reduction in the number of expressed proteins due to nAg. Of the 261 proteins spots detected, 24 were newly expressed, and 132 suppressed completely after exposure to nAg	NS	[26]
Fish	*Labeo rohita*	nAg nNi nCo_3O_4 nCr_3O_4	Size (nAg): 40 ± 6 nm) Size: (nNi, 43 ± 6 nm) Size: (nCo_3O_4): 60 ± 6 nm Size (nCr_3O_4): 50 ± 5 nm)	21 d	2.5×10^4	Total protein kit using photometric Colorimetric-Biuret method Albumin kit	Protein quantification	Whole organism	NS	Total protein elevation was detected in nNi, nCo_3O_4, and nCr_3O_4 treated groups Increase in albumin level for nCr_3O_4-treated groups High level of globulin recorded for nNi- and nCo_3O_4-exposed groups.	NS	[27]
Fish	*Oncorhynchus mykiss*	nAg	Size: 19.4 Coating: Citrate	0.5–2 h	1×10^5 (stock solution)	Bradford assay SDS-PAGE Electrospray LC-MS/MS) –label free	Protein quantification	Gill cells	10 mM 3-morpholinopropanesu acid (MOPS, pH 7.5)	Inhibition of Na+/K+-ATPase, a key protein in ion regulation in gill cells was inhibited by nAg		[28]

(Continues)

TABLE 1 (Continued)

Organism group	Organism name	NPs type	PC properties	Duration	Dosage (µg/L)	Proteomic approaches	Endpoint	Target organ/system	Assay/ Environmental factors	Proteomic related effects Results	Effects related to ENP concentration and environmental factors (water chemistry)	Ref
ia	*Bacillus subtilis*	Mg-doped nZnO	25 and 73 nm (LB medium) 10 nm (DMEM with 10% of serum)	1 d	5×10^3	LC–MS	Protein expression levels	Whole Cells	Luria-Bertani broth	Exposure of *B. subtilis* to non-lethal doses of nMg–ZnO increased expression of proteins responsible for detoxification of ROS, translation and biofilm formation		[29]
Bacteria	*Bacillus subtillis*	Mg doped ZnO	Size: 25 and 75 nm	1 h	50	LC-MS	Protein abundance	Whole organism	LB medium	Proteins relating to stress response, biofilm formation, motility, chemotaxix, purine metabolism and translation were differentially abundant Decreased levels of YrpD and YncM (sporulation and biofilm) proteins and increased AhhC and YdbP (oxidative stress)	NS	[30]
Fish	*Cyprinus carpio*	nAg		96 h	750	Tandem mass tag LC-MS Bicinchoninic assay (BCA) kit	Protein expression level	Gills	Declorinated tap water	nAg induced 87 differential expression proteins in gills that are involved in signalling mechanisms, cytoskeleton, and arachidonic cid metabolism	NS	[31]
Plant	Strawberry	CeO_2	NS	45 d	6.08×10^4–1.15×10^5	LC-MS coupled with HPLC	Protein expression levels	Whole plant, fruits, flower, pollen	NS	Down regulation of malate related differentially expressed proteins Differential regulation of sugar-associated proteins 146 proteins up-regulated 291 proteins down regulated	NS	[32]

NS – Not specified, 1. [49], 2. [50], 3. [51], 4. [52], 5. [53], 6. [51], 7. [54], 8. [53], 9. [55], 10. [33],11. [56], 12. [57], 13. [58], 14. [34], 15. [44], 16. [35], 17. [59], 18. [60], 19. [61], 20. [46], 21. [49], 22. [62], 23. [63], 24. [64], 25. [65], 26. [66], 27. [67], 28. [68], 29. [69], 30. [70], 31. [71], 32. [72].

TABLE 2 Summary of ENPs concentrations used to investigate their interactions with proteins on aquatic taxa at different levels of biological organization

Organism	ENPs	Exposure concentrations (μg/L)	Number of studies
Molluscs	Ag	10–100	3
	CuO	0.4–1.0	1
	MnO	8.06×10^4	1
	ZnO	1.63×10^4	1
Crustaceans	Ag	2–32	3
Algae	CdSe/ZnS	69–345	1
	ZnO	2.0×10^5	1
	Ag	51.6–71.2	1
	CdSe	8–222	1
Bacteria	Ag	1.0×10^{-1}–3.07×10^2	3
	Au	7.5×10^2	1
	Mg-doped ZnO	5.0×10^3	2
	ZVI	5	1
Fish	Ag	80–1.0×10^5	4
	GO	10–1.0×10^3	1
Insects	Ag	1.0×10^5–4.0×10^5	1
	TiO_2	1.43×10^7–2.86×10^7	1
Plants	Ag	0.03–1.08×10^4	4
	CeO_2	6.0×10^4–1.0×10^6	2

[74], and adsorption of proteins [73]. Furthermore, environmental factors including temperature, pH, organic matter, and ionic strength also alter the nano–bio interface as they influence ENPs aggregation with resultant change to their initial properties [73, 75, 76]. Here, both ENPs physicochemical properties and environmental factors will be discussed with special reference to ENPs-proteome related effects.

3.1 | Size

ENPs size affects their toxicity, solubility, and chemical reactivity [77–79]. Smaller ENPs have larger surface area to volume ratio; hence, they exhibit higher reactivity. The large number of atoms in smaller-sized ENPs on their surfaces, in turn increases surface energy, thereby increasing their adsorption. When ENPs interact with proteins, the strength of protein–ENPs binding is determined by their size, which then ultimately affects the amount of protein absorbed by the ENPs as well as the thickness, composition, and protein activity of the associated protein corona [80, 81].

Investigations by Barreto et al. [60] revealed different proteomic effects on gilthead seabream were dependent on the ENPs size. For example, irrespective of the nAu coating type (citrate or PVP), the 7-nm-sized nAu altered more proteins relative to larger 40-nm-sized ones; hence, the smaller-sized particles were more bioactive. Seven-nm citrate-coated nAu altered the abundances of 13 proteins (nine up- and four downregulated), but 40-nm citrate nAu (six up- and four downregulated) altered abundances of only 10 proteins. Furthermore, Bouallegui et al. [49] documented that nAg < 50 nm induced a reduction in thiols in mussels gills, unlike the larger sizes (>50 nm), implying higher toxicity and bioactivity from the small sizes of nAg.

3.2 | Surface properties

Toxicity of ENPs is influenced by surface properties including charge, area, smoothness, stability, coating type, and the presence/absence of any defects on the surface [74, 82, 83]. This, in turn, influences the affinity as well as the amount of proteins that may adsorb on the ENPs surfaces [74]. The outer ENPs charge, either positive or negative, affects the overall observed toxicity in a given organism under investigation. For example, positively charged ENPs tend to induce higher toxicity as they interact more with biological molecules due to reduced repulsion between cell surfaces, and ENPs [84].

A study by Al-Awady et al. [85] reported that $nTiO_2$ coated with cationic polyelectrolyte were more toxic towards microalgae *C. reinhardtii* relative to those coated with anionic polyelectrolyte. In another study, citrate and polyethyleimine-coated nAg exhibited different toxicity effects on *Chlorella vulgaris*. Citrate-coated nAg regulated mitochondrial-function related proteins, resulting in disruption of several associated metabolic pathways, including amino acid synthesis. However, polyethyleimine-coated $nTiO_2$ adversely affected ribosome-function related proteins, and in turn interrupted pathways of protein

synthesis [86]; hence, surface coating has significant influence on overall interrupted or uninterrupted proteins.

Barreto et al. [60] investigations showed that 7-nm citrate-coated nAu altered abundance of 13 proteins, whereas PVP-coated ones of same size altered abundance of 10 proteins in gilthead seabream. In addition, they observed that for the 40-nm-sized, citrate-coated nAu altered abundance of 10 proteins, whereas PVP-coated ones altered abundance of only seven proteins. Furthermore, Hou et al. [50] observed higher toxicity from citrate-coated nAg relative to PVP-coated nAg on *D. magna*, where PVP-coated nAg affected biological pathways responsible for protein digestion and absorption. The same study also highlighted surface coating contribution to the observed ENPs toxicity was more profound relative to the particle size. Citrate coating was also reported to enhance the toxicity of nAg towards *D. magna* [18]. In the light of foregoing findings, highly variant ENPs surface properties play an important role on the environmental nanotoxicity including at the proteomic level.

3.3 | Shape

The shape of ENPs has been found to influence nano–bio interactions. Although research has highlighted the influence of ENPs shape on their toxicity [74, 87], information on shape-related effects and mechanism at organisms' proteome level remain limited, and the few available studies were carried out in mammalian systems. For example, effects of graphene oxide nanosheets to mammalian cells demonstrated that their shape induced physical damage to the cell membrane owing to graphene oxide nanoparticles ability to adsorb protein molecules on their surfaces, thereby changing the shape of the ENPs [87]. Abdelhamid and Wu [74] further documented that ENPs surface curvature influences the amount of protein binding on the ENPs. Shape also influences the interactions between ENPs and cell layers as well as nanoparticle uptake by cells [88]. Until now, although studies using ENPs in mammalians systems have shown the influence of ENPs shape on interactions with proteins, this remain to be done for the aquatic organisms.

3.4 | Environmental factors

In the aquatic environment, ENPs toxicity is regulated by the abiotic factors of the system [89]. For example, Canesi et al. [90] demonstrated that exposure media chemistry alters the bioavailability, and uptake of ENPs on marine invertebrates with their concomitant interactions with proteins. However, unlike under laboratory conditions, the physicochemical properties of ENPs in natural environments cannot be accurately characterized due to the complexity of the system [91]. This has resulted in limited information on the impact of ENPs physicochemical properties on nanotoxicity mechanisms in the natural environment [92]. Nonetheless, recently Fadare et al. [93] evaluated the effects of natural organic matter (NOM), fulvic acid (FA), and humic acid (HA) on nanoplastic particles on *D. magna*. Results revealed increased protein adsorption to ENPs in the presence of FA; however, HA induced a reduction in protein adsorption. When proteins attach to the surfaces of ENPs in aquatic environment, they form an 'eco-corona', which give the ENPs a new 'biological identity', thus affecting their bioavailability, uptake, and toxicity [94]. For example, the secretion of eco-protein by *D. magna* increased toxicity and uptake of polystyrene nanoparticles [95].

The strong tendency of ENPs to agglomerate in the environment makes it even more difficult to elucidate their mechanisms of action [96]. When they studied the toxicity of nAg towards zebrafish under different environmental exposure conditions, Kim et al. [96] observed agglomeration in 62.5 μM $CaCl_2$ and ultrapure water, but none in the standard zebrafish embryo medium. This implies plausible variability of toxicity mechanisms across different environments. Noventa et al. [34] reported elevated toxicity of nZnO towards oyster larvae in the presence of NOM through oxidative stress. This is because the NOM adsorbed on to surface reactive sites of ENPs, thus modifying their structure and physicochemical properties with resultant changes on the observed toxicity.

Dissolved organic carbon have been observed to enhance toxicity of metallic ENPs in aqueous media towards crustaceans *D. magna* and *Thamnocephalus platyurus*, and protozoan *Tetrahymena thermophile* [97]. Divalent cations (e.g., Ca^{2+}) induced higher agglomeration relative to monovalent cations [98, 99], thus affecting the transport and toxicity of ENPs in the aquatic systems. Conversely, it has also been observed that when ENPs are exposed to light irradiation in the natural environment, their toxicity is enhanced as observed when *E. coli* was exposed to nZnO, nCuO, nCo_3O_4, and $nTiO_2$ [100], or in some cases suppressed as they get passivated as mentioned in the review by Shi et al. [101]. Even though these studies have shed light on the effect of environmental factors on nanotoxicity; there are no accounts for what their influence at cellular and protein level could be. Therefore, an understanding on the influence of inherent ENPs physicochemical properties and exposure media chemistry (e.g., in actual environmental matrices like river or lake water at relevant concentrations) is essential as it can accelerate our ability to model ENPs hazards in aquatic environment.

4 | ENPS-BIOMOLECULES INTERACTIONS AND MECHANISMS OF TOXICITY

ENPs can easily move across biological membranes due to their small size [102]. This can alter biochemical reactions within cells including causing oxidative stress and in certain cases eventual apoptosis [103, 104] (Figure 2). These aspects are partly driven by physicochemical transformations, such as, dissolution, agglomeration, aggregation, and sedimentation, which ENPs undergo and ultimately altering their shape, size, and surface properties [105, 42].

As an example, ionic species are formed following the dissolution of soluble and/or partially soluble ENPs and have been observed to cause ENPs toxicity to aquatic organisms [32]. Vannini et al. [58] demon-

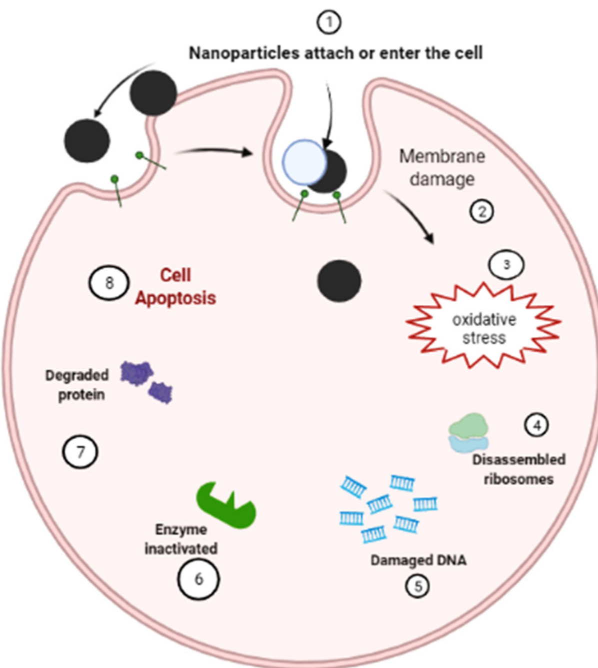

FIGURE 2 ENPs ability to enter or attach to the cell and cause toxicity through different pathways that encompass damage to cell organelles and macromolecules.

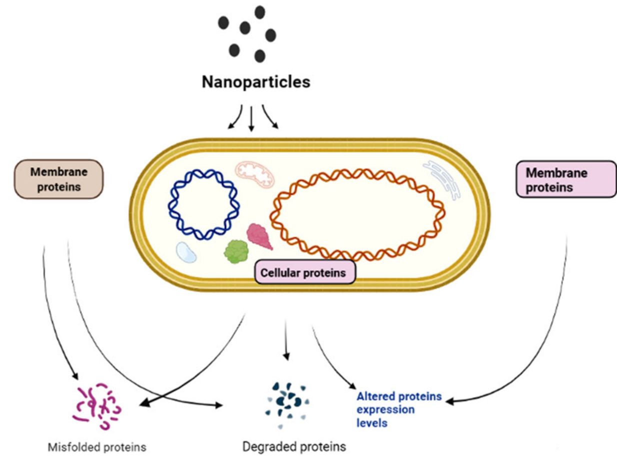

FIGURE 3 ENPs interactions with membrane and cellular proteins causing folding, degradation, and altered expression levels.

strated the toxicity of nAg on wheat seedlings as mainly due to the release of Ag^{+}. Furthermore, toxicity due to both nAg particulates and Ag^{+} have also been observed with the latter effects being more profound as evidenced by an increase in antioxidant enzymes (SOD and GPOXs) in *Pseudomonas* sp. *M1* [44]. Wang et al. [76] investigated the toxicity of soluble (nCuO and nZnO) and insoluble (nFe_2O_3, nCo_3O_4, nCr_2O_3, and nNiO) ENPs concerning their potential inhibition on the bioluminescence of *Photobacterium phosphorium*. Findings indicated that soluble nZnO antibacterial effect was solely due to Zn^{2+}. For the case of nCuO, however, the antibacterial effects were linked to both the released Cu^{2+} and particulates of nCuO. Whereas, for the insoluble ENPs, the antibacterial effects observed were solely attributed to their particulates [76].

Available literature documenting nanotoxicity mechanisms remain largely undefined and highly variant. The most documented mechanisms includes (i) ENPs-cell attachment causing cell wall and cell membrane damage, (ii) ENPs attachment to intracellular organelles and biological molecules (DNA, protein, ribosomes, enzymes), and (iii) the release of ROS [32–36, 106] (Figure 2).

4.1 | ENPs-cell attachment

Attachment of ENPs to cell membranes is a critical initial step that precedes their observed toxicity pathways [107, 108]. Following the initial attachment, ENPs subsequently enter into the cell [109], where they damage cell membranes and interrupts energy transductions [108, 110]. Yue et al. [68] demonstrated that nAg induced toxicity on algae through cell surface attachment. Similarly, Sendra et al. [111] reported that $nTiO_4$ induced toxicity on marine microalgae mediated through membrane damage. In other studies, the accumulation of nAg on cell surfaces of *E. coli* lead to the formation of 'pits' on the cell surfaces [30, 112].

4.2 | ENPs internalisation and protein alterations

Internalisation of ENPs and their cytoplasmic accumulation therein may lead to interactions with cell organelles and macromolecules. For example, reports have documented that variant biomolecules including carbohydrates, proteins (Figure 3), lipids, polysaccharides, and nucleic acids may get attracted to the ENPs, and adhere to their surfaces raising the plausible interactions [81, 108, 113, 114]. Although it is not clear how ENPs enter the cytoplasm, it is hypothesized may occur through damaged cell membranes [115]. Further, ENPs uptake into the cells may occur through processes such as endocytosis, phagocytosis, and adsorption. For example, results of Yue et al. [68] demonstrated that nAg entered into fish gill cells through the endocytosis process.

Despite myriad studies having reported on the entry of ENPs in cells, it remains unclear how the internalized ENPs interacts with diverse biological macromolecules in different cell compartments. It is, however, reported that aquatic organisms response to deleterious effects of ENPs through the release of proteins among other biomolecules. Furthermore, the released proteins may attach to the ENPs with the resultant toxicity often associated with changes in structural organization of proteins [109, 116]. For instance, Sharma et al. [116] reported that Ag^{+} mediated toxicity induced protein leakage by binding to and modifying transport proteins. In addition, the authors highlighted that ENPs can inhibit bacterial respiration owing to their interactions with thiol groups of enzymes especially respiratory enzymes, for example, NADH dehydrogenase [116]. In other works, ENPs were

found to induce structural modifications of proteins and resulting in abnormal protein functions [117, 118]. For example, Wigginton et al. [118] demonstrated that nAg caused loss of enzymatic activity in tryptophanase (TNase) upon their interactions with *E. coli*.

A common response by aquatic organisms to nanotoxicity is the alteration of protein expression levels [119]. Following exposure to nAg, results on proteomic analysis of *Bacillus* sp. EMB9 revealed marked global changes in its intracellular proteome where cells grown in presence of nAg had 50% reduction of expressed proteins, where out of 261 proteins, 24 were newly expressed, and 132 proteins were suppressed upon exposure to nAg [48]. Other proteomic investigations showed that exposure of *E. coli* to nAg caused cellular responses including the excretion of more cell envelope protein precursors, which in turn destabilized the outer membrane and the collapse of the proton motive force [120].

ENPs can interact with proteins and induce conformational changes and examples are summarised in Figure 3. These includes changes on protein structures, adducts formation, phosphorylation status alteration, thiols alteration, and the conversion of side chains to aldehyde or ketone groups [121, 122]. The substantial structural changes, for example, unfolding patterns and reduction in free protein α-helical content have been observed to occur when proteins bind to ENPs [84]. Taking these results together points to potential impact on the downstream functions of proteins.

The interactions summarised in Figure 3 might be the reason why aquatic organisms respond to nanotoxicity through alteration of their protein expression levels [123, 124]. For example, proteomic analysis on *E. coli* revealed alterations in the expression patterns of envelope and heat shock proteins following exposure to nAg [120]. Mirzajani et al. [51] documented an increase of protein precursors indicating dissipation of a protein motive force following exposure of *Oryza sativa* to nAg. The *Pseudomonas aeruginosa* exposed to nAg yielded 27 and 32 up- and downregulated proteins, respectively, which were linked to membrane damage and generation of ROS mechanisms [125]. Moreover, proteomic profiling of *Paracoccus denitrificans* exposed to nAg revealed inhibition of proteins responsible for catalytic processes, electron transfer and metabolic processes [126].

4.3 | ENPs interactions with cell organells and release of ROS

Oxidative stress is a result of imbalance between production and accumulation of ROS due to direct contact between ENPs and cells/cell organelles, and/or ionic species following the dissolution of ENPs [32]. As such, oxidative stress is a key factor in intracellular ENPs-induced toxicity [32]. Other works have also shown ROS can induce damaging biological responses including cell wall damage which enhances membrane permeability [127–129] and protein carbonyl levels [130].

Release of ROS have been demonstrated to induce membrane lipid peroxidation [76] as well as damage amino acids residues of proteins including Tir, Phe, Trp, Met, and Cys; thus, leading to the formation of carbonyl residues. Bouallegui et al. [49] observed differential redox-based protein changes such as thiol oxidation and carbonyl formation at the onset of oxidative stress following exposure of *Mytillys galloprovincialis* to nAg. Their results indicated that the mussels activated pathways to mitigate against the toxicity of nAg by reducing proteins. A similar response was observed on *Daphnia magna* following exposure to nAg [53]. Furthermore, the results of Katsumiti et al. [131] pointed to oxidative stress as the main mechanism of nAg toxicity to mussel haemocytes and gill cells as evidenced by observed increase in catalase activity.

Several studies have demonstrated the role of ROS on the toxicity of ENPs at proteome level as it causes oxidative modification of proteins [58, 132]. For example, Vannini et al. [58] observed changes in proteins essential for redox regulation, sulphur metabolism, endoplasmic reticulum, and vacuole alteration following exposure of *Eruca sativa* plant to nAg. Oxidation of proteins showed a decrease in protein thiols and an increase in carbonylation following exposure of *Mytilus edulis* mussels gills to nCuO [33]. Using redox proteomics, the authors identified six unique proteins as targets of oxidative stress where their expression was altered by the nCuO. Results showed two proteins (actin and triosephosphate isomerase) were targets of thiol oxidation and three (alpha-tubulin, tropomyosin, and Cu–Zn superoxide dismutase) for carbonylation, with four of these six proteins being important cytoskeletal components which are known redox targets [133].

In plants, ROS and free radicals have been observed to interfere with chlorophyll, and in turn, concomitant interruption on photosynthesis. For example, Deng et al. [134] documented a reduction in photosynthesis on *Phaeodactylum tricornutum* diatoms following exposure to $nTiO_2$ and $nCeO_2$. Also, an accumulation of ROS in chloroplasts of *Karenia brevis* after exposure to $nTiO_2$ have been reported [77]. Similarly, Sosan et al. [135] observed an accumulation of ROS specifically the H_2O_2 partially generated by NADPH oxidase following exposure of *Arabidopsis thaliana* plant to nAg.

Overall, although many published works have reported distinct mechanisms as the basis of the observed deleterious effects, however, the mechanisms generally occur in synchrony. For example, a study by Zhao et al. [136] has shown the observed toxicity of ENPs to *Chlamydomonas reinhardtii* after exposure to nAg was due to multiple mechanisms including internalisation, release of ROS, and increased cell membrane permeability. Hence, future works should consider investigations for multiple mechanisms to gain better understanding of key triggers of nanotoxicity and plausible dominant pathways.

5 | TECHNIQUES FOR ANALYSIS OF ENP-PROTEOME INTERACTIONS

Due to the complexity of interactions between proteins and ENPs, different analytical techniques, and high throughput methods are needed to evaluate bio–nano interactions. Proteomics techniques can provide comparative and quantitative analysis of the proteins composition, architecture, and dynamics associated with pollutants

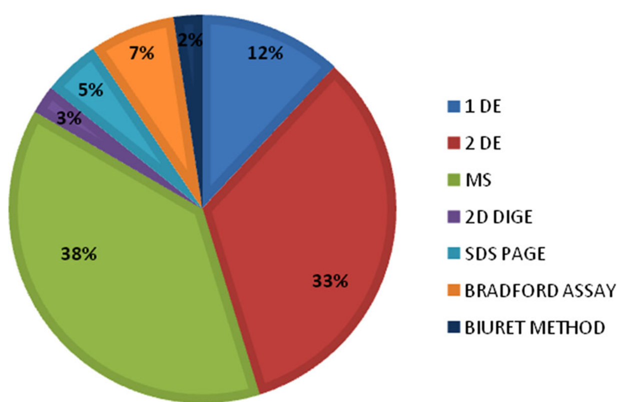

FIGURE 4 Distribution of studies for different proteomic approaches with MS (Mass spectrometry) as the most commonly used, and then followed by gel electrophoresis. 1 DE: 1 dimensional electrophoresis; 2 DE, two dimensional electrophoresis; MS, mass spectrophotometry; 2D DIGE, two dimensional difference gel electrophoresis; SDS PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

including ENPs [137–140]. From the reviewed studies, results indicate that gel-electrophoresis and mass spectrometry-based techniques or a combination of both are the most widely used for the analysis of ENPs–proteome interactions in aquatic organisms (Figure 4). Among the studies reviewed herein, 44% were done using a combination of gel electrophoresis and mass spectroscopy in an attempt to obtain better proteomic insights. Even though rarely used in nanoecotoxicity domain, other techniques including circular dichroism (CD) spectroscopy, Fourier transform infrared spectroscopy, Raman spectroscopy, fluorescence spectroscopy, size-exclusion chromatography, isothermal titration calorimetry, rheology, and surface plasmon resonance (SPR) spectroscopy can also be used to investigate protein–ENP interactions [141]. Table 3 sets out the, strengths and weaknesses of proteomics techniques identified to have been used to elucidate protein–ENP interactions in aquatic organisms.

5.1 | Gel-based proteomics techniques

5.1.1 | 1D Gel electrophoresis

One-dimensional gel electrophoresis (1DE) separates protein samples based on their molecular weights, and is mostly used for moderately complex protein mixtures. However, 1DE has limitations (Table 3) such as low resolution for protein separation, it is laborious and has a limited dynamic range and detection sensitivity of samples when analysed with a mass spectrometer [151]. Besides these drawbacks, the technique has been applied in proteomic studies for ENPs in aquatic organisms. Tedesco et al. [52] used 1DE to separate proteins when they profiled *Mytilus edulis* exposed to gold ENPs (nAu) where the results indicated a decrease in thiol-containing proteins. Bouallegui et al. [49] investigated the toxicity of nAg to *M. galloprovincialis*; they did separation and quantification of proteins using 1DE and Bradford assay, respectively. Furthermore, these techniques were complemented with the use of typhoon scanner and densitometry techniques in order to better improve quantification of protein thiols and carbonyl.

5.1.2 | 2D Gel electrophoresis

Two-dimensional gel electrophoresis (2DE) is a classic, and most commonly used technique in proteomic studies to separate and visualise proteins [138, 152]. The technique separates protein samples perpendicularly using two dimensions; namely, the isoelectric point focusing and molecular weight. In nanotoxicity studies, Gomes et al. [57] used the 2DE to discriminate differentially expressed proteins in *M. galloprovincialis* exposed to nAg. Results demonstrated that 2DE offered better protein resolution relative to 1DE.

To increase its efficiency, researchers supplant 2DE with mass spectrometry in order to achieve protein identification [138, 153–155]. For example, Mirzajani et al. [51] used 2DE in conjunction with NanoLiquid Chromatography/Fourier transform-ion cyclotron resonance mass spectrometry (NanoLC/FT-ICR MS) to observe protein expression levels in *Oryza sativa L.* following exposure to nAg. The authors observed an accumulation of precursors for oxidative stress tolerance proteins and protein degradation. Vannini et al. [54] used 2DE prior to Liquid Chromatography-ElectroSpray Ionization-tandem Mass Spectrometry (nanoLC-nESI-MS/MS) analysis. In turn, they could construct proteomic profiling of *Eruca sativa* plant exposed to nAg, and the results indicated alteration of endoplasmic reticulum and vacuole proteins.

More advanced 2DE techniques have been employed to investigate the nanoecotoxicity of aquatic systems primarily to reduce gel-to-gel variation, and improve reproducibility in protein quantification. For example, 2D fluorescence difference gel electrophoresis (2D-DIGE) was used to study proteomic stress responses of *B. cereus* after exposure to nanosized zero-valent iron (nZVI) [55]. They reported overexpression of oxidative stress response proteins. Similarly, Saccà et al. [156] applied the same approach to visualise proteins when assessing the molecular stress responses of nZVI and later reported alterations in redox-proteins expression levels.

5.1.3 | Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is used for size-specific separation and analysis of proteins, in which the 12-carbon tail enables the SDS to interact hydrophobically with non-polar regions of protein molecules [157]. The buffer composition and permeability of gels used for SDS-PAGE are varied based on the size of proteins of interest. Bouallegui et al. [49] used SDS-PAGE and observed protein thiol oxidation and formation of carbonyls in the gills of *M. galloprovincialis* following exposure to nAg. In another study, Tedesco et al. [52] applied the SDS-PAGE to study the effect of nAu to *Mytilus edulis*, and results demonstrated a decrease in thiol containing proteins. The thiols prevent oxidative modification of proteins [158].

TABLE 3 Strengths and limitations of proteomics techniques when used for nanoparticles research

Techniques	Strengths	Limitations	Complementary/Modifications to suit ENPs
2 DE	Rapidly separates several proteins in a proteome [1] Generates large volumes of information from the simultaneous resolution of thousands of proteins [1] Can separate and detect post translational modified proteins [2]	Offers no measure of protein expression level, therefore, unsuitable for clustered data analysis [1] Silver staining used is not suitable for quantitative analysis [1] The silver staining is not compatible with protein identification methods such as mass spectrometry [1] Not possible to analyse the entire proteome [2]	2D master maps [3] Peptide sequencing [3]
Denaturing polyacrylamide gel electrophoresis (PAGE)	Allow for resolution of many proteins in a single gel due to excellent resolving power [4] Identification and quantification of the protein composition of the corona [5]	Not suitable for quantitative analysis [2, 4]	Used in combination with LC-MS/MS
Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)	Useful for high throughput comparative proteomics [1] Multiple separations can be run in parallel [1] High quality 2D spot patterns [1] Up to 10 000 distinct protein and peptide spots can be separated from complex biological samples [1] Can give information about proteins adsorbed onto nanoparticles [5] Used for comparison hence it is difficult to obtain quantitative results [3]	The labelling used affect protein migration and may lead to false positive or negative results [5] Requires multiple LC-MS/MS Mobility on gel affected variations in proteins of interest; therefore molecular weight cannot be accurately determined [3] Limited sensitivity [3] Need optimisation for protein bands of interest as it can only detect between 1 and 50ng of a single protein band [3]	
Difference gel electrophoresis (DIGE)	Can control for non-biological variation [5] It has an internal control which allows for repetitive measurements and quantitative multivariable analysis in protein expression [5] Enables comparison of untreated and nanoparticle-treated samples within the same gel [5] Identification of differentially expressed proteins is possible [5] Can assess protein abundance differences [6] Advanced form of 2 DE which allows accurate and reproducible quantification of multiple samples [6] Can directly visualise hundreds to thousands of proteins [6] Minimal changes in protein abundance can be detected [6] Spot signal and intensity can easily be matched and normalised [2]	Difficult to detect and identify low abundant proteins due to the dynamic range of proteins [5]	

(Continues)

TABLE 3 (Continued)

Techniques	Strengths	Limitations	Complementary/Modifications to suit ENPs
Mass spectrometry	High degree of sensitivity and specificity in protein detection [4] Larger dynamic range as protein signals are detected directly [4] Enable novel insights into the modulation of particle surfaces by biological fluids (formation of the protein corona) and subsequent particle-induced cellular responses [5] Can identify nature and properties of the protein corona, and effects on intracellular signalling and cellular responses [7]	In more complex protein mixtures it is challenging to resolve peptides to acquire mass spectral information to distinguish between proteolytic proteins and post translational modifications [4] Possible interferences from sample contamination with analytes from silver stained gels.[1] Little is known about specific interactions of nanoparticles with cell surface proteins and intracellular organelles [7]	^{18}O-labeling [7]
Label free liquid chromatography – mass spectrometry	Can determine the composition of the hard human plasma protein corona [8] Can determine the dependence of protein corona on nanoparticle size [8] Highly sensitive [8] Less affected by the various caveats of 2D-Page, spot excision and protein identification by mass spectrometry [8] It offers high analytical quality [8] Enables simultaneous identification and relative quantification of proteins in a complex mixture with high reproducibility and sensitivity [8]		
Equilibrium dialysis	Can isolate nano-particle-protein complexes [9] Determine total protein binding to small particles [9]		
Size-exclusion chromatography	Can isolate nano-particle-protein complexes [9] Can analyse protein kinetics without in a less disruptive manner [9]		
Matrix-Assisted Laser Desorption/Ionization Time-of-Flight MS (MALDI-ToF-MS)	Can identy proteins whose expression was stimulated by nanoparticles [10] Accurately measures peptide masses less than 50 ppm [10] Offers sensitivity and specificity [10]	Not suitable to sequence peptides in proteolytic digests, unless the peptides are derivatised prior to analysis [10]	
UV-vis spectrophotometry	Can characterise and visualise the protein corona [5]		
Fluorescence microscopy (viz.: confocal laser scanning microscope, super resolution microscope, single-particle tracking microscope)	Enables studies on cellular processing of nanoparticles showing great details on spatial and temporal changes [5]	Shows little and only fragmentary description of most protein networks and hierarchical complexes coordinating formation of the protein corona, cellular uptake and intracellular trafficking [5]	

(Continues)

TABLE 3 (Continued)

Techniques	Strengths	Limitations	Complementary/Modifications to suit ENPs
$^{16}O/^{18}O$ chemical labelling	Coupled with 2DE, the technique can be used to study bioaccumulation of nanoparticles, and show effects of ENPs on living organisms [5]		
Fourier transform mass spectrometry (FTMS)	High resolution mass analysis [5] Can quantitatively investigate intrinsic changes in proteome upon exposure to nanoparticles [5]		
Triple TOF/QQQ technology	High resolution mass analysis [5] Can quantitatively investigate intrinsic changes in proteome upon exposure to ENPs [5]		
SILAC (Stable Isotope Labelling by amino acids)	Can do comparative analysis of the cellular effects of nanoparticles treatment in vitro [5]		
On-nanoparticle digestion	Ability to isolate protein corona from ENPs, simple, and offers high yield [5]		
Biological variation analysis (BVA) module	Automatically match protein spots from different gels and highlights those with statistically significant differences [6]		
Differential In-gel Analysis (DIA) module	Detect spots automatically [6]		
Immunoblotting	Used for verification of protein abundance changes (DIGE results) both qualitative and quantitative results		
Western blotting	Can give information about proteins adsorbed onto nanoparticles [3]	Used for comparison hence it is difficult to obtain quantitative results [3] Mobility on gel affected variations in proteins of interest, therefore molecular weight cannot be accurately determined [3] Limited sensitivity [3] Need optimisation for antibodies and conjugated substrate of choice [3]	

(Continues)

TABLE 3 (Continued)

Techniques	Strengths	Limitations	Complementary/Modifications to suit ENPs
Isothermal titration calorimetry (ITC)	Can analyse protein binding kinetics in a less disruptive manner [3]		
Surface plasmon resonance (SPR)	Can analyse protein binding kinetics in a less disruptive manner [3]		
Hydrophilic interaction liquid chromatography (HILIC)		High degree of orthogonality [11]	Samples should be pre-fractioned offline with HILIC before an online RP-LC-MS/MS analysis [11] Used ammonia evaporation perpendicular to the electrospray needle, which reduced the average charge-state of iTRAP labelled peptides [11]
Reversed phase chromatography		High degree of orthogonality [11]	Samples should be pre-fractioned offline with HILIC before an online RP-LC-MS/MS analysis [11] Technique use ammonia evaporation perpendicular to the electrospray needle, which reduces the average charge-state of iTRAP labelled peptides [11]

1. [142]; 2. [143]; 3. [144]; 4. [145]; 5. [139]; 6. [45]; 7. [146]; 8. [147]; 9. [148]; 10. [149]; 11. [150]; HILIC, Hydrophilic Interaction Liquid Chromatography.

5.1.4 | Mass spectroscopy-based techniques

Mass spectrometry approach entails identification, characterisation, and profiling of proteins through matching peptides to protein identities [138, 146]. Protease enzymes digest proteins to peptides at predetermined sites. First, a unique peptide mass fingerprint (PMF) is created by measuring the peptides masses and identified in a protein database. The peptides will then be confirmed using mass spectrometry, whereby peptides are fragmented to yield a peptide fragment fingerprint (PFF) containing amino acids sequences [159]. To date, MS based proteomics has contributed significantly towards the characterization and identification of proteins in biological samples [160] and may partly account for its wide application in the nanotoxicity domain (Figure 4). Discussed below are variant MS-based techniques as applied in the domain of proteomics to elucidate the effects of ENPs to aquatic organisms.

5.1.5 | Matrix-assisted laser desorption ionization–time of flight

Matrix-assisted laser desorption ionization–time of flight (MALDI–TOF) is a MS-based technique and is used for spot identification and quantification of up- and downregulated protein spots. The technique entails excision, tryptic digestion in gel, and finally an analysis of the peptide mixture processes [161–163]. In MALDI-TOF, sample surface is coated with a matrix, for example, α-cyano-4-hydroxycinnamic acid, 2,5-dihydroxybenzoic acid (2,5-DHB), and sinapinic acid to aid analyte extraction [164]. Identification of peptides is done using computational scoring of correlations between predicted and measured peptides. In a study by Bouallegui et al. [49], MALDI-TOF was used to identify 12 differentially expressed proteins in mussels exposed to nAg. The redox-sensitive proteins were produced at the onset of oxidative stress.

When they studied interaction of serum proteins and carbon nanotubes (CTBs), Du et al. were able to identify proteins bound to CTBs using MALDI-TOF in combination with atomic force microscopy (AFM) [165]. Wigginton et al. [118] applied MALDI-TOF to assess the affinity of *E. coli* proteins to bare and carbonate-coated nAg. They reported that the proteins displayed high affinity for both surface modifications. Furthermore, using MALDI-TOF, Gomes et al. [57] identified differentially expressed proteins in mussels gills *M. galloprovincialis* after exposure to the particulates and ionic species where nAg toxicity was observed to be mediated through oxidative stress-induced cell signalling cascades. MALDI-TOF/TOF MS was employed to assess the toxicity of $nTiO_2$ on *D. magna* where overexpression of vitellogenium (Vtg)-like proteins was established to be involved in redox balance [166]. However, to date the application of MALDI-TOF in nanoecotoxicology domain is limited since most aquatic organisms are poorly captured in sequence databases, except for daphnia (http://wFleaBase.org), which is widely and mostly used as the model organism [167].

The various studies cited above demonstrate the suitability of MALDI-TOF to provide insight on nanotoxiticy at molecular level. The technique can also be applied to study different ENPs and environmental organisms.

5.1.6 | Surface enhanced laser desorption ionisation time of flight-mass spectrometry

Surface enhanced laser desorption ionisation time of flight-mass spectrometry (SELDI-TOF-MS) is a combination of chromatography- and mass spectrometry-based techniques, generally used for quick analysis of protein profiles and mostly applied in biomarker studies [168–170]. In essence, SELDI-TOF-MS is a modified MALDI-TOF technique, and entails the use of protein arrays known as protein chips to identify protein expression levels in distinct samples based on protein molecular weights [149]. The technique can analyse protein mixtures on chromatographic arrays and produce a spectra based on mass-to-charge ratio of the proteins and binding affinities [171]. Scebba et al. [172] used SELDI-TOF-MS technology, and observed differentially expressed proteins in marine diatoms following exposure to CdSe/ZnS quantum dots. The results showed the technique's ability to identify potential biomarkers of CdSe/ZnS nanotoxicity. Although this approach appears to have significant potential for application in the nanotoxicity domain, it has however not been widely used based on the published literature. This could be due to the need for and yet limited availability of chromatographic surfaces that captures oxidative modifications to enable investigation of oxidatively modified proteins [173]

5.1.7 | Inductively coupled plasma mass spectrometry

Inductively coupled plasma mass spectrometry (ICP-MS) technique can detect metals in biological samples through ionisation of samples using inductively coupled plasma followed by mass spectrometer to analyse metal ions [174]. ICP-MS is used to quantify proteins and peptides using phosphorus and sulphur present in their structure as natural tags [175]. Where natural tags are not available, specific labelling reagents are used. For example, stable isotope labelling by amino acids in cell culture (SILAC), culture-derived isotope tags (CDIT), enzymatic labelling with ^{18}O from heavy-oxygen water, isotope-coded affinity tags (ICAT), isobaric tag for relative, and absolute quantitation (iTRAQ) [176] Unlike the soft ionisation techniques like MALDI that are used for structural characterisation and identification, ICP-MS offers high quality qualitative elemental data. For example, Yan et al. [125] used ICP-MS to identify silver binding proteins in *P. aeruginosa* following exposure to nAg. Silver binding proteins were then applied to a gel slab, excised, washed, and trypsin digested before the analysis was done using nanoscale LC-electrospray ionization-QTOF MS/MS. Results showed that 27 proteins were upregulated, 32 downregulated and 5 silver binding proteins identified. However, this technique is not commonly applied in proteomic nanoecotoxicology domain, which may be due to the costs of its operation.

5.1.8 | Liquid chromatography–mass spectrometry (LC-MS/MS)

The Liquid chromatography–mass spectrometry (LC-MS/MS) is a gel-less technique, and entails the digestion of proteins with a proteases such astrypsin, chymotrypsin, Glu-C, or LysN. The resultant peptides are separated using high performance liquid chromatography (HPLC), and then characterized using tandem mass spectrometry [177]. Proteomic databases are then used to identify the proteins digested. From the LC–MS/MS derived results, numerous non-redundant proteins may be identified [160]. Magnetic multi-walled carbon nanotubes (MWCTs) have been used in conjunction with LC-MS to successfully detect high amounts of mycotoxins on maize samples [178]. Canesi et al. [90] used HPLC-MS/MS system to investigate the formation of protein corona between haemolymph proteins and cationic polyesterene ENPs in marine bivalve haemocytes. Results showed the formation of hard protein corona that comprised only of putative C1q domain containing protein.

To elucidate the toxicity of nAg on germinating wheat seedlings, Vannini et al. [58] applied HPLC to observe protein regulation in different cell compartments in combination following protein separation using 1DE. Alterations in proteins responsible for redox regulation and sulphur metabolism after exposure to nAg were observed. In another study, Vannini et al. [58] also employed LC-ESI–MS/MS to observe altered protein expression profiles after wheat seedlings were exposed to nAg. Moreover, by use of LC-MS/MS following the exposure of *Oryza sativa* to nAg, a dose dependent accumulation of protein precursors for proteins involved in oxidative stress tolerance, Ca^{2+} regulation, signalling, transcription, protein degradation, cell wall and DNA/RNA/protein direct damage, cell division and apoptosis was observed [51].

The above cited studies verify the undisputed suitability of various proteomic approaches in studying nanoecotoxicity, despite their limitations as summarised in Table 3. By employing proteomics, altered proteins can be identified, as well as down or up regulation of various proteins. The information is key for identification and development of protein biomarkers for nanoecotoxicity monitoring and research.

5.2 | Complementary non-proteomic approaches towards understanding nanoecotoxicity

Even though this review focused on the application of proteomics to understand nanotoxicology, researchers have also used other approaches to study nanotoxicity. The common ones includes other 'omics' techniques, for example, toxicogenomics, metallomics, transcriptomics, metabolomics, and collectively regarded as 'exotoxicogenomics' [179]. These tools provide a more comprehensive molecular insights of biological systems [41]. For example, toxicogenomics approach was shown to identify nanotoxicity-altered genes and protein activities at cellular level. For example, Dai et al. [180] used polymerase chain reaction (PCR) to study toxicity of CuO nanoparticles in plants. They were able to show insights into molecular mechanisms and identified genetic biomarkers of oxidative stress related to nanotoxicity. In another study, Kang et al. [40] employed PCR to study toxicity of graphene oxide quantum dots on algae (*C. vulgaris*). They reported up- and downregulation of various genes responsible for metabolism, photosynthesis, and biosynthesis that were linked to nanotoxicity of graphene oxide. This field of transcriptomics therefore reveals unique differentially-expressed gene profiles that can potentially also be used as biomarkers for biomonitoring purposes [47].

The recommendation of proteomics should therefore be considered with caution, or better still be combined with other approaches, for example, metallomics, to give a more wholistic analysis of metalloproteins profiles within cells, and eventually enable identification of new biomarkers. Integration of two or more omics approaches gives more sensitive and comprehensive details on NP-induced toxicity. The heterogeneous and massive data generated is also sometimes coupled with computational methods like machine learning (ML), which has proved to be very useful. It is however observed that fewer studies have used omics tools to evaluate nanotoxicity. These could probably due to the fact that the approaches generate large amounts of data, which is often difficult to interpret. [36].

It is also imperative to acknowledge that proteomic alterations do not immediately translate or cause observed or apparent toxicity at whole organism level. Rather, the adverse consequences are likely to be observed later in the lifespan of the organisms. For example, various types of ENPs have been observed to impair proteins in *C. elegans*, and the effects were linked to accelerate aging and heightened risks to diseases [181].

6 | CONCLUDING REMARKS AND RECOMMENDATIONS

Despite numerous studies on nanotoxicity to date, there remains significant knowledge gaps regarding mechanisms of toxicity at cellular level, particularly in the natural aquatic environments. Here, we have highlighted data deficits on the effects of ENPs at proteome level, yet it is critical to account for their effects even when exposed to sub-concentrations. Furthermore, most studies have been conducted at phenotypic end-points mostly in synthetic exposure media, and this limitation is attributed to lack of standardised protocols and techniques for nanotoxicity studies. Hence, use of proteomic analysis can provide additional information at molecular level. Studies have shown that proteomic approaches can reveal key molecular pathways associated with ENPs adverse outcomes. In addition, proteomic analysis using both gel- and mass spectrometry-based approaches can reveal potential mechanisms, unlike in the case of conventional testing methods [182]. The physicochemical characteristics of ENPs and environmental factors can influence the transport and toxicity of ENPs in the aquatic environments, however, from the reviewed studies herein only handful attempts were made to correlate these properties

with the observed biological effects. Hence, studies on the influence of ENPs inherent physicochemical properties and media properties on the toxicity of ENPs at proteome level are recommended as is part of 'omics' essential to understand at molecular level the basis of the observed toxicity.

Since aquatic organisms alter their protein expression level in response to nanotoxicity in an attempt to maintain cellular homeostasis; proteomics therefore offers a pathway to bridge knowledge gaps and accelerate an understanding of protein–ENPs interactions in the aquatic organisms. However, existing proteomics techniques may require modifications to enhance their suitability to detect and monitor effects induced by of ENPs to aquatic organisms especially arising from challenges linked to complex exposure media properties.

Therefore, here we outline recommendations to enhance generation of proteomics data to improve modelling of ENPs hazards in the aquatic environmental systems in pursuit to balance societal benefits of nanotechnology, and undesirable ecological implications. These includes:

(1) Need for long-term studies using proteomics to understand the ENPs–proteins interactions in different environmental matrixes (e.g., dam, lakes, rivers), and at realistic exposure concentrations as well as potential trans-generational effects. This could aid in better understanding the basis of ENPs toxicity to aquatic systems.
(2) Influence of both inherent ENPs physicochemical properties and exposure media chemistry to the processes such as ENPs transformation, aging, and concomitant influence on nanoecotoxicity especially in endeavour to develop proteomic-based biomarkers.
(3) Identify best possible approaches that could be coupled with proteomics techniques to complement its shortcomings. For example, because proteomics generate large volumes of data that is often difficult to analyse, bioinformatics can be applied to analyse the data. Researchers could focus more on identifying other techniques that can be coupled with proteomics to advance the field of nanotoxicology, or better still, find ways of overcoming shortcomings brought about by proteomics and increase its sensitivity.
(4) To better improve the use of proteomics in elucidating the toxicity of ENPs in aquatic organisms, we would require development of testing and standardized protocols. Therefore, we propose the use of green synthesis of ENPs, and their interactions with proteins to establish a base line for use as reference for other forms of ENPs. Such approach could aid to model the ENPs–proteins interactions in the environment. Unlike, chemical and physical ENPs synthesis methods, biosynthesized nanoparticles are environmentally friendly.
(5) At present, reported ENPs-proteins interactions are mostly in vitro, and the effect of co-contaminants are not considered. Hence, there is need to apply the proteomic techniques where mixtures of ENPs as well as with other non-ENPs contaminants are taken into account as that is more likely scenario in the actual environmental systems.

ACKNOWLEDGEMENTS

The financial support from the Botswana International University of Science and Technology (BIUST) to (G.T., L.P.K.), and University of Pretoria (N.M.), and Water Research Commission (N.M.).

CREDIT STATEMENT

Gosaitse Tubatsi: Investigation, methodology, data curation, formal analysis, validation; Writing – Original draft; Writing – Review & editing. Lemme Prica Kebaabetswe: Formal analysis, funding acquisition, validation; Writing – Review & editing, supervision. Ndeke Musee: Conceptualization, formal analysis, funding acquisition, project administration, resources, validation; Writing – Review & editing, supervision. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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How to cite this article: Tubatsi, G., Kebaabetswe, L. P., & Musee, N. (2022). Proteomic evaluation of nanotoxicity in aquatic organisms: A review. *Proteomics*, *22*, e2200008. https://doi.org/10.1002/pmic.202200008