-Omics Approaches in Studies of Polystyrene MNP Toxicity

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The investigation of the toxicity mechanism of micro- and nanoplastics (MNPs) is a topic of major concern for the scientific community. The use of transcriptomics, proteomics, and metabolomics has suggested that the main pathways affected by polystyrene (PS) MNPs are related to energy metabolism, oxidative stress, immune response, and the nervous system, both in fishes and aquatic invertebrates.

Keywords: microplastics ; nanoplastics ; polystyrene ; -omics approaches ; transcriptomics ; proteomics ; metabolomics ; mechanism of toxicity ; fish ; aquatic invertebrates

1. Introduction

Plastic pollution is a fast-rising environmental threat. Due to plastics' low cost, durability, and flexibility, their use has increased worldwide, leading to an augmentation of their release into the marine environment. Most of the plastic debris found in the seas originates from land-based sources [1]. Once in the natural environment, plastic can be degraded into micro- (MPs, <5 mm) and nanoscale sizes (NPs, <1 μm) [2][3] by weathering, sunlight radiation, and biodegradation processes [4][5][6][7][8]. MPs and NPs (MNPs) can be also classified into primary and secondary based on their sources. Primary MNPs are those that enter the environment in their original small size associated with specific applications and consumer products (e.g., cosmetics, clothing fibers, drug delivery, ink for 3D printers), whilst secondary MNPs are a consequence of macro/microplastics degradation [4][9][10][11]. The formation of smaller particles leads to alterations in the physical-chemical proprieties, surface area, and size of MNPs, wherein, once the nanoscale is reached, the strength, conductivity, and reactivity will differ substantially from those of macro-/micro-sized particles [12][13][14][15]. Obviously, as the size of the plastic particle decreases, biological reactivity, on the other hand, increases. Thus, it is crucial to comprehend the burden of MNPs' availability and their biological impact on aquatic biota [14][16].

Up to now, polystyrene (PS) ha sbeen chosen as a proxy for MNPs due to the fact that it is one of the most largely used non-biodegradable plastics worldwide and, unlike other polymers, it shows a greater stability in sea water suspension with low styrene release [17]. Several studies have been conducted to evaluate the lethal and sublethal effects of PS MNPs on aquatic biota, reporting fertility, growth, and reproduction abnormalities [18][19][20][21][22][23][24][25][26][27][28], as well as metabolism disorders, oxidative stress, and immune and nervous system dysfunction [13][29][30][31][32][33][34][35][36]. Consequently, one of the main challenges today is to understand the mechanism of the toxicity of MNPs correlated to the lethal/sublethal effects studied so far. With this aim, the aquatic ecotoxicology field can benefit significantly from using the -omics approaches, which are emerging systemic and holistic tools for the global identification of the processes and pathways involved in the normal and abnormal physiological states, that allow not only the study of the mode of action of chemicals, but also the prediction of their toxicological effects on a given biological system [37]. -Omics approaches permit the production of large-scale datasets, measuring simultaneously the changes in gene expressions, proteins, and metabolites (by application of transcriptomics, proteomics, and metabolomics, respectively) occurring at the molecular, cell, tissue, and whole-organism levels [38][39]. These approaches allow the characterization of complex signal pathways and correlation of gene/protein expression, rather than focusing on the modulation of individual genes/proteins. Among others, -omics technologies include: (i) transcriptomics, which is used to study the whole set of RNA transcripts and to identify general and specific transcript biomarkers as transcriptional consequences related to natural environmental factors or the mode of action of environmental pollutants in an organism [38][40]; (ii) proteomics, which is used to study the whole set of proteins in order to evaluate any alterations in their function and/or structure in an organism after changes in the environmental conditions [41]; and (iii) metabolomics, which is used to study the whole set of cell metabolites, and has been employed in the past several years with the purpose of unveiling the molecular and biochemical mechanisms underlying the response, sensitivity, tolerance, and adaptation of aquatic organisms to environmental challenges or

pollution [42][43]. Transcriptomic studies dominated until 2016, whereas a shift towards proteomics, and mostly metabolomics, including multi-omics studies, is now apparent [44].

2. -Omics Approaches in Studies of PS MNP Toxicity

2.1. Transcriptomics

Over the past decades, transcriptomics has predominantly been applied for environmental risk assessment by evaluation of the health status of aquatic animals [45]. It determines the changes in gene expression by measuring the level of mRNA after studying the whole set of transcripts, also named the transcriptome, present in an organism. Indeed, the quantitative real-time polymerase chain reaction (qRT-PCR) is the simplest and most widely used technique to conduct a transcriptomic analysis. Although the relative expression of selected genes is easy to undertake, as the amount of the gene studied is compared to the amount of a control reference gene, qRT-PCR can quantify only a limited number of genes, with the requirement for prior knowledge of target genes. To cope with these limitations and to target thousands of single mRNAs in a single run, microarrays and RNA-sequencing (RNA-seq) have therefore been used lately. In particular, the last mentioned technique uses high-throughput sequencing methodologies to detect the presence and quantity of RNA in a biological sample with the aim of analyzing the whole cellular transcriptome. In brief, the method consists of isolating total RNAs from biological samples and then performing its reverse transcription to obtain double-stranded cDNA. After that, cDNAs are sequenced as short reads, aligned, and mapped against a known genomic reference sequence. In recent years, RNA-seq has been successfully used to assess differential responses in a variety of aquatic species since it is effectively able to analyse whole transcriptomes, generating data on more differentially expressed genes (DEGs), which, through bioinformatics, will give information about the major pathways affected following a stress condition [44]. A description of the effects of PS MNPs at the transcription level in fishes and aquatic invertebrates is reported in **Table 1**.

Table 1. Table summarizing the effects of PS MNPs at transcript level evaluated by transcriptomics in fishes and aquatic invertebrates.

FISHES								
Method Used	**Organism Tested**	**PS MNPs Size**	**Concentration Tested**	**Time of Exposure**	**Organ/Tissue Target**	**Life Stage**	**Effect**	**References**
qRT-PCR	*Danio rerio* (zebrafish)	10 µm	200 particles/mL	120 hpf	Whole organism	Larvae	↑ *sod1*, *sod2*, *cat*, *gst* and *cyp*	[35]
qRT-PCR	*Danio rerio* (zebrafish)	500 nm	0.1, 1 and 10 ppm	96 hpf	Whole organism	Larvae	↑ *p53*, *cas-3* and *cas-9*; ↓ *bcl* and bdnf	[46]
qRT-PCR	*Danio rerio* (zebrafish)	30 nm	0.1, 0.5 and 3 ppm	120 hpf	Whole organism	Larvae	↑ *sod1*, *sod2*, *cas-1*, *cas-8*, and *il1β*; ↓ *hsp70*, *bcl-2*, *ache*, DNA repair genes *gadd45α* and *rad51*	[47]
qRT-PCR	*Danio rerio* (zebrafish)	1 µm	1000 µg/L (around 1.91 × 10^7 particles/L)	96 hpf	Whole organism	Larvae	↑ *il1β*; ↓ *cat*	[48]
qRT-PCR and RNA-seq	*Danio rerio* (zebrafish)	5 µm	20–100 µg/L	21 d	Liver	Adult	↑ *aco* and *fabp6*; ↓ *cpt1*, *ppar-α*, *acc1*, *fas*, *srebp1α*, *ppar-α*. KEGG pathways analysis revealed carbon, lipid and amino acid metabolism effect	[49]
RNA-seq	*Danio rerio* (zebrafish)	size ranging from 25 to 90 µm	100–1000 µg/L	20 d	Liver	Adult	Alteration in pathways related to immune response and lipid metabolism, i.e., sterol biosynthetic process, steroid metabolic process and fatty acid metabolic process	[50]
RNA-seq	*Danio rerio* (zebrafish)	50 and 200 nm,	100–1000 ppb	5 d	Whole organism	Larvae	Nervous system development and function pathways	[51]

FISHES								
Method Used	**Organism Tested**	**PS MNPs Size**	**Concentration Tested**	**Time of Exposure**	**Organ/Tissue Target**	**Life Stage**	**Effect**	**References**
qRT-PCR	*Oncorhynchus mykiss* (rainbow trout)	100–400 µm	500–2411 particles/fish/day	4 w	Intestine	Adult	No change in immune response related genes	[52]
qRT-PCR	*Oncorhynchus mykiss* (rainbow trout)	0.2, 1, 20, 40 and 90 µm	2×10^5 particles/L	2 h	Gills	Adult	↑ *ifny* gene exposed to 0.2 and 40 µm beads; ↓ *il1β* (bead size 1 µm), *s100a* (bead size 40 µm) and *saa* (1, 40 and 90 µm)	[53]
qRT-PCR	*Oryzias melastigma* (marine medaka)	plain PS, carboxylated PS: PS-COOH and aminated PS: PS-NH_2 with a size of 1 µm	0.02 mg/L	7 d	Whole organism	Larvae	↓ *cox1* and *cox2* by PS, PS-COOH and PS-NH_2; ↓ *cyp1a*, multifunction gene (ATPase) by PS-NH_2 and PS-COOH, respectively. No impairment of oxidative stress genes in all the treatments	[54]
RNA-seq and qRT-PCR	*Oryzias melastigma* (marine medaka)	0.05, 0.50, and 6.00 µm	0.1; 1×10^3; 1×10^6 particles/mL	19 d	Whole organism	Larvae	↓ inflammatory and immune-related signaling pathways (Hippo, B cell receptor, RIG-I-like receptor, and inflammatory mediator regulation of the TRP-channels-signaling pathway); heart development (↓ *gata4 and nkx2.5*, and ↑ bmp4) hatching enzyme (*hce* and *lce*)	[55]
			AQUATIC INVERTEBRATES					
qRT-PCR	*Artemia franciscana* (brine shrimp)	50 nm PS-NH_2	1 µg/mL	14 d	Whole organism	Adult	↑ *clap* and *cstb* genes	[56]
RNA-seq	*Artemia franciscana* (brine shrimp)	5 µm	1 mg/L	14 d	Whole organism	Adult	KEGG enrichment analysis mapped into arrhythmogenic right ventricular cardiomyopathy, viral myocarditis, hypertrophic cardiomyopathy, phagosome, fluid shear stress, atherosclerosis and regulations of actin cytoskeleton, with most of the DEGs correlated with ROS activity and apoptosis activity	[57]
qRT-PCR	*Artemia franciscana* (brine shrimp)	50 nm PS-NH_2	1 µg/mL	48 h and 14 d	Whole organism	Neonates and adult	Time-dependent ↑ *clap* and *cstb* genes and *hsp60* and *hsp70*	[58]

FISHES								
Method Used	**Organism Tested**	**PS MNPs Size**	**Concentration Tested**	**Time of Exposure**	**Organ/Tissue Target**	**Life Stage**	**Effect**	**References**
RNA-seq	*Ciona robusta* (ascidian)	50 nm PS-NH_2	10 and 15 µg/mL	22 hpf	Whole organism	Embryos	↓ genes involved in glutathione metabolism (*glutamate--cysteine ligase catalytic subunit-like transcript variant X1 and X2*; *glutathione S-transferase omega-1-like*), immune defense (*integumentary mucin C.1-like transcript variant; mucin-5AC transcript variant; interferon-induced protein 44-like; plasminogen-like*), nervous system (*acetylcholinesterase-like; sco-spondin*), transport by aquaporins (*aquaporin-like*) and energy metabolism (*succinate--CoA ligase [ADP/GDP-forming] subunit alpha mitochondrial-like; 6-phosphofructo-2-kinase/fructose-26-bisphosphatase 1 transcript variant; glycoside hydrolase transcript variant*)	[24]
qRT-PCR	*Daphnia magna* (water flea)	50 nm	0.05, 0.5 µg/L	21 d	Whole organism	Adult	↓ *cat* after exposure of 21 d to 0.5 µg/mL	[59]
qRT-PCR	*Daphnia pulex* (water flea)	75 nm	0.1, 0.5, 1, 2 mg/L	21 d	Whole organism	Adult	*Sod*, *gst*, *gpx* and *cat* initially ↑ and then inhibited. ↑ *hsp* in all the treatment groups	[60]
RNA-seq	*Daphnia pulex* (water flea)	~70 nm	1 mg/L (5.32×10^8 particles/mL)	96 h	Whole organism	Neonates	Alterations in oxidative stress (arachidonic acid metabolism, glutathione metabolism, and porphyrin and chlorophyll metabolism), immune response (drug metabolism–cyp450 and other enzymes, metabolism of xenobiotics by cyp450, glutathione metabolism, hippo signaling pathway, and adherens junction) and energy metabolism pathways (starch and sucrose metabolism, pentose and glucuronate interconversions, galactose metabolism, fructose and mannose metabolism, carbohydrate digestion and absorption, and glycolysis/gluconeogenesis)	[61]
RNA-seq	*Daphnia pulex* (water flea)	75 nm	1 mg/L	21 d	Whole organism	Adult	Alteration in genes involved in chitin metabolism, trehalose transport and metabolism, growth-related genes, long-chain fatty acids metabolism, defense mechanisms, and sex differentiation	[62]
qRT-PCR	*Litopenaeus vannamei* (whiteleg shrimp)	100 nm	200 and 2000 mg/kg	14 and 28 d	Hepatopancreas	Adult	↑ Beta-glucan binding protein, *LPS*/β-glucan binding protein, and *hsp90* genes. ↑ *TLR* gene	[63]

2.2. Proteomics

Proteomics is used to analyse translational and post-translational variations in proteins. The main analytical strategies for separation and identification of the proteome are based on two different approaches: the top-down and the bottom-up approach. Top-down approaches rely on the use of intact proteins for direct separation and identification, typically performed by liquid chromatography–tandem mass spectrometry (LC-MS/MS). These two strategies present different advantages for the complete analysis of proteoforms. However, challenges regarding solubility, separation, and ionization efficiency still remain to be addressed. In contrast, proteomic approaches that use a bottom-up workflow minimize

FISHES								
Method Used	Organism Tested	PS MNPs Size	Concentration Tested	Time of Exposure	Organ/Tissue Target	Life Stage	Effect	References
RNA-seq	*Meretrix meretrix* (marine clam)	100 nm PS-NH_2 200 nm PS-COOH	2 mg/L	7 d	Digestive gland	Adult	↑ energy homeostasis imbalance (e.g., lipid metabolism, PPAR signaling pathway, protein digestion and absorption, pyruvate metabolism, and glycolysis. Impairment of immune system (e.g., NLRs, NF-κB signaling pathway, TLR signaling pathway, phagosome, lysosome, and apoptosis)	[64]
qRT-PCR	*Mytilus galloprovincialis* (Mediterranean mussel)	50 nm PS-NH_2	0.150 mg/L	24 and 48 hpf	Whole organism	Embryos	↓ *cs*, *ca*, and *ep* genes	[25]
qRT-PCR	*Mytilus galloprovincialis* (Mediterranean mussel)	3 µm	50–500 particles/mL	24 and 48 hpf	Whole organism	Embryos	↑ *ep*, *ca*, and *cs* genes; ↑ *mytc* and *mytb* genes; ↓ *gusb*, *hex*, *ctsl* genes	[65]
qRT-PCR	*Mytilus galloprovincialis* (Mediterranean mussel)	50 nm PS-NH_2	10 µg/L	First exposure 24 h, rest period 72 h, second exposure 24 h	Hemocytes	Adult	↑ *epp*, *lyso*, *amps*, *mytb*, *mytc*, ↑ *frep*	[66]
qRT-PCR	*Paracentrotus lividus* (sea urchin)	50 nm PS-NH_2	3 µg/mL	24 and 48 hpf	Whole organism	Embryos	↑ *cas8*	[19]
qRT-PCR	*Paracentrotus lividus* (sea urchin)	50 nm PS-NH_2	3 and 4 µg/mL	24 and 48 hpf	Whole organism	Embryos	↑ *hsp70*, *p38 Mapk*, univin and *cas8*	[20]
RNA-seq	*Pinctada margaritifera* (black-lip pearl oyster)	6 and 10 µm	0.25–2.5–25 µg/L	2 months	Mantle	Adult	Alteration in energy, stress, and immune-related genes. ↓ *cyp2d11*, *gst1*, *sult1c4* and *abcb1*, *cpk* and *actin* gene	[67]
RNA-seq	*Procambarus clarkia* (red swamp crayfish)	0.10 µm	1.43×10^{11} particles/L	72 h	Hemocytes and hepatopancreas	Adult	In hemocytes, ↑ 8 DEGs involved in gene transcription and translation. In hepatopancreas, differential expression of only 3 genes (*cyp49a1* and two unknown genes)	[68]
qRT-PCR	*Sterechinus neumayeri* (Antarctic sea urchin)	40 nm PS-COOH 50 nm PS-NH_2	1 and 5 µg/mL	6 and 24 h	Coelomocytes	Adult	↑ antioxidant genes at 1 µg/mL PS-COOH. ↓ *sod* at 1 µg/mL PS-NH_2. ↑ *mt* at both the concentrations tested PS-NH_2. ↑ the immune-related gene *NF-κB* and *LBP/BPI* by PS-NH_2	[30]

problems by analyzing peptides prepared by enzymatic proteolysis. In this strategy, however, greater sample separation power is required [69][70]. The main proteomic approach that has been established in recent years consists of protein separation in gels and mass spectrometry analysis. Gel separation of proteins is used for the separation and global quantification of proteins. It commonly relies on using an older technique of two-dimensional gel electrophoresis (2D-GE). However, the loss of all membrane proteins, the appearance of multiple proteins in a single spot, and the presence of a single protein in multiple spots constitute some important limitations in the use of this technique. As a result, the most frequently used method for measuring cellular proteins has become liquid chromatography coupled with mass spectrometry (LC-MS or LC-MS/MS), because of its sensitivity, selectivity, accuracy, speed, and throughput [71][72][73][74]. Hence, proteomics has recently been applied to study the metabolic pathways' modifications, biodistribution, and bioaccumulation caused by plastic molecules in aquatic environments, showing huge potential [75]. A description of the effects of PS MNPs at proteomic level in fishes and aquatic invertebrates is reported in **Table 2**.

Table 2. Table summarizing the effects of PS MNPs at protein level evaluated by proteomics in fishes and aquatic invertebrates.

FISHES								
Method Used	Organism Tested	PS MNPs Size	Concentration Tested	Time of Exposure	Organ/Tissue Target	Life Stage	Effect	References
ELISA	*Danio rerio* (zebrafish)	50 and 500 nm	0.1, 1, 10 mg/L	12 and 72 hpa	Whole organism	Larvae	Slight ↑ Il-10, Tnf-α and Nf-κB at the lowest dose. ↓ Il-10, Tnf-α, FgF20a and Nf-κB at the higher dose	[76]
WB	*Danio rerio* (zebrafish)	5 µm	50 ng/mL	7 d	Whole organism	Larvae	↑ iNOS and Nf-κB	[77]
AQUATIC INVERTEBRATES								
WB	*Brachionus koreanus* (marine rotifer)	0.05 and 0.5 µm	10 µg/mL	24 h	Whole organism	Neonates	↑ phosphorylation of JNK and p38 Mapk related to ROS level	[78]
LC-MS/MS	*Crassostrea gigas* (Pacific oyster)	2 and 6 µm	0.023 mg/L	60 d	Oocytes	Gamets	↓ arginine kinase and ↑ severin	[18]
LC-MS/MS	*Daphnia magna* (water flea)	Mean particle size 13.03 ± 7.75 µm	101.6 mg/L	19 d	Whole organism	Adult	Changes in 41 proteins, mostly those related to sulfation, chitin-binding and cuticle's structural integrity. The less abundant proteins are related to pigment binding, response to stimuli, response to ROS, response to oxidative stress, and response to oxygen-containing compound	[79]

↑: increase; ↓: reduction; qRT-PCR: quantitative real-time polymerase chain reaction; RNA-seq: RNA-sequencing; hpf: hours post-fertilization; d: days; w: weeks; ppm: parts per million; ppb: part per billion; sod: superoxide dismutase; *cat*: catalase; *gst*: glutathione S-transferase; *cyp*: cytochrome P450; *p53*: tumor protein p53; *cas*: caspase; *bcl-2*: B-cell lymphoma 2; *bdnf*: brain-derived neurotrophic factor; *il1β*: interleukine 1-β; *aco*: acyl-CoA oxidase; *fabp6*: fatty acid binding protein 6; *cpt1*: carnitine palmitoyltransferase 1; *ppar-α*: peroxisome proliferator-activated receptor-α; *acc1*: acetyl-CoA carboxylase 1; *fas*: fatty acid synthase; *srebp1α*: sterol regulatory element binding protein 1α; *ppar-γ*: peroxisome proliferator-activated receptor-γ; *cox*: cyclooxygenase; *hsp*: heat shock protein; *ache*: acetylcholinesterase; *gadd45α*: growth arrest and dna damage inducible alpha; *rad51*: rad51 recombinase; *ifnγ*: interferon-gamma; *s100a*: s100 calcium binding protein a1; *saa*: serum amyloid A; *RIG-I*: retinoic acid-inducible gene I; *TRP*: transient receptor potential; *bmp4*:

bone morphogenetic protein 4; *hce:* high choriolytic enzyme; *lce:* low high choriolytic enzyme; *gpx*: glutathione peroxidase; *NLRs*: NOD-like receptor signaling pathway; *cs*: chitin synthase; *ca*: carbonic anhydrase; *ep*: extrapallial protein; *mytc*: myticin C; *mytb*: mytilin B; *gusb*: β glucorinidase; *hex*: hexosaminidase; *ctsl*: catepsine L; *p38 Mapk*: mitogen-activated protein kinase; *epp*: extrapallial protein precursor; *lys*: lysozime; *amps*: antimicrobial peptides; *frep*: fibrinogen-related proteins; *sult1c4*: sulfotransferase 1C4; *abcb1*: ATP binding cassette subfamily B member 1, *cel*: bile salt-activated lipase; *mt*: metallothioneine; *NF-kB*: nuclear factor kappa-light-chain-enhancer of activated B cells; *LBP/BPI*: lipopolysaccharide-binding protein and bactericidal/permeability-increasing protein; *LPS*: lipopolysaccharide; *TLR*: toll like receptor.

Method Used	Organism Tested	PS MNPs Size	Concentration Tested	Time of Exposure	Organ/Tissue Target	Life Stage	Effect	References
RPLC/MS	***Daphnia pulex*** **(water flea)**	**500 nm**	**1 mg/L**	**14 d**	**Whole organism**	**Adult**	**Changes in 89 proteins, including those involved in IFT-body assembly, nuclear-transcribed mRNA catabolic process, ATP-dependent chromatin remodeling, energy metabolism and unfolded protein responses**	**[80]**
LC-MS/MS	***Daphnia pulex*** **(water flea)**	**Mean particle size 71.18 nm**	**0.5–2 mg/L**	**21 d**	**Whole organism**	**Adult**	**327 proteins ↓, including those involved in cell signaling, immune function, detoxification, energy metabolism, ECM-receptor interaction pathways, and glutathione metabolism**	**[81]**
nLC-MS/MS	***Dreissena polymorpha*** **(Zebra mussel)**	**1 and 10 µm size**	**4 × 10^6 particles/L mixtures**	**6 d**	**Gills**	**Adult**	**78 proteins ↓ and 18 proteins not expressed. Effect of catalytic activity (27%), nucleotide binding, proteins involved in structural molecule activity (12%) and protein binding (11%), proteins related to RNA (5%) and metal ion (4%) bindings**	**[82]**
LC-MS/MS	***Litopenaeus vannamei*** **(Pacific white shirmp)**	**100–200 µm**	**1 mg/L**	**14 d**	**Haemolymph**	**Adult**	**47 proteins ↓, including those belonging to extracellular, plasma membrane and lysosomal localization, and related to T cell receptor signaling pathway, epithelial cell signaling in *Helicobacter pylori* infection, and phospholipase D signaling pathway**	**[83]**

FISHES								
Method Used	**Organism Tested**	**PS MNPs Size**	**Concentration Tested**	**Time of Exposure**	**Organ/Tissue Target**	**Life Stage**	**Effect**	**References**
Nano HPLC MS/MS	*Paracentrotus lividus* (sea urchin)	45 µm	$10, 50, 10^3, 10^4$ particles/L	72 h	Coelomocytes	Adult	↑ proteins involved in endosome transport via multivesicular body sorting pathway and in establishment of protein localisation, and proteins involved in catabolic processes	[34]
NMR	*Paracyclopina nana* (marine copepod)	0.05 µm	10 µg/mL	24 h	Whole organism	Neonates	Oxidative stress induction (↓ ROS level) and ↑ phosphorylation of the proteins p38 Mapk, ERK and Nrf2	[88][89]
LC-MS/MS	*Tigriopus japonicus* (marine copepod)	6 µm	0.28 µg/mL	Two generations (F1 and F2)	Whole organism	Adult	↑ proteins involved in several cellular biosynthesis and cellular energy storage in F1 generation. Transgenerational proteome plasticity in F2 generation with elevated energy metabolism and stress related defense	[35][36][43][92][93][94][95] [96][97][98][99][100][101]
^{1}H NMR	*Danio rerio* (zebrafish)	5 µm	50 and 500 µg/L	21 d	Intestine	Adult (5 months)	Changes in 34 metabolites. ↑ alanine, proline, propylene glycol, alanine, glutamine, leucine, ornithine, carnitine, threonine, TMAO; ↓ lysine, phenylalanine, tyrosine, linoleic acid, palmitic acid, triglycerides.	[31]
GC-MS	*Danio rerio* (zebrafish)	100 nm	250 and 2 × 10^4 items of PS MNPs in 50 mL	72 hpf	Whole organism	Embryo	Changes in 508 metabolites. Disorders in unsaturated fatty acids, linoleic acid, taurine, hypotaurine, nicotinate, nicotinamide, alanine, aspartate, glutamate.	[22]
GC-MS	*Danio rerio* (zebrafish)	5–50 µm	100 and 1000 µg/L	7 d	Whole organism	Embryo	Changes in 78 (5 µm) and 121 (50 µm) metabolites. Disorders in carbohydrates, fatty acids, amino acids, nucleic acids and others.	[102]

2.3. Metabolomics

Metabolomics is the most recent discipline among the -omic sciences. It studies the metabolome, a term coined by Oliver in 1998 [86], indicating the entire set of metabolites present in a biological system. Metabolomics is a powerful bioanalytical technique that allows the systematic identification and quantification of all endogenous metabolites with low molecular weight (<1500 Da), which may vary according to the physiological state, developmental stage, or pathological condition of cells, tissues, or organs, or of the whole organism under examination [87]. Therefore, analysis of complex metabolite data along with uni- and multivariate statistics, as well as mapping of altered pathways, enables the mechanistic understanding of biological phenotypes and discovery of biomarkers or drug targets for a variety of conditions [88]. To obtain the global metabolic profiling of a given organism or biological sample in relation to external stimuli, two metabolomic analytical platforms, namely, nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS), are commonly employed. Although NMR is relatively less sensitive than MS, it offers numerous benefits to the metabolomics field since it is highly reproducible and quantitative, non-selective and non-destructive, does not require sample preparation or separation, and enables the identification of unknown metabolites in complex biological mixtures [89]. On the contrary, MS is a highly sensitive technique able to detect thousands of metabolites, prior to separation using liquid or gas chromatography (LC or GC, respectively) [90]. Overall, both of the two metabolomics platforms, in combination with powerful chemometric software for multivariate data analysis, which is necessary to deconvolute the huge amount of data produced following a metabolomic experiment [88][91], allow the simultaneous determination and comparison of a wide range of metabolites, offering numerous advantages to clarify the organism–environment interactions [35][36][43][92][93][94][95] [96][97][98][99][100][101]. A description of the effects of PS MNPs at metabolomic level in fishes and aquatic invertebrates is reported in **Table 3**.

Table 3. Table summarizing the effects of PS MNPs at metabolite level evaluated by metabolomics in fishes and aquatic invertebrates.

↑: increase; ↓: reduction; ELISA: Enzyme Linked Immunoassay; WB: Western Blot; LC-MS/MS: Liquid Chromatography–Tandem Mass Spectrometry; RPLC/MS: Reversed-Phase Liquid Chromatography Tandem Mass Spectrometry; nLC-MS/MS: nano-Liquid Chromatography Mass Spectrometry; Nano HPLC MS/MS: nano-High Performance Liquid Chromatography and Mass Spectrometry; hpa: hours post amputation; hpf: hours post fertilization; d: days; Il-10: cytokines Il-1β, Tnf-α: tumor necrosis factor; NfkB: nuclear factor kappa-light-chain-enhancer of activated B cells; FgF20a: fibroblast growth factor 20a; iNOS: nitric oxide synthase; ROS: reactive oxygen species; ECM: extracellular matrix; p38 Mapk: mitogen-activated protein kinases; ERK: extracellular signal-regulated kinases; Nrf2: nuclear factor erythroid 2 related factor 2; JNK: jun n-terminal kinases.

FISHES								
Method Used	**Organism Tested**	**PS MNPs Size**	**Concentration Tested**	**Time of Exposure**	**Organ/Tissue Target**	**Life Stage**	**Effect**	**References**
LC-MS/MS	*Danio rerio* (zebrafish) *Perca fluviatilis* (perch)	5–12 µm	1, 50 e 100 mg	21 d	Gills and liver	Adult	Changes in 33 metabolites. Zebrafish gills: ↓ phenylalanine, carnitine, proline, salicylic and lactic acid, choline. Perch gills: ↑ phenylalanine, salicylic acid; ↓ acetyl-carnitine, alanine, glutamic and pyruvic acid. Zebrafish liver: ↑ adenine, adenosine, glutamine; ↓ hypoxanthine, uridine, deoxyadenosine, valine, arginine, phenylalanine, asparagine, proline. Perch liver: ↑ arginine, succinic acid, adenosine; ↓ hypoxanthine, oxoglutaric acid, citrulline, creatinine, adenine	[103]
LC-MS	*Gobiocypris rarus* (rare minnow)	1 µm	200 µg/L	4 w	Liver	Subadult (3 months)	Changes in 41 metabolites. ↑ glyceraldehyde; cytosine, glucose, fructose, mannose; ↓ mannitol 1-phosphate, acetyl-phenylalanine, mannonate	[104]
UPLC-Q-TOF-MS	*Oreochromis mossambicus* (tilapia)	100 nm	20 mg/L and recovery	7 d	Whole organism	Larvae	Changes in 203 metabolites. Disorders in fatty acyls, carboxylic acids and their derivatives, organooxygen compounds, keto acids and their derivatives.	[105]
LC-MS	*Oreochromis niloticus* (red tilapia)	0.3, 5 and 70–90 µm	100 µg/L	14 d	Liver	Adult	Changes in 31 (0.3 µm), 40 (5 µm) and 23 (70–90 µm) metabolites. Disorders in amino acids, fatty acids, glycerophosphoethanolamines, glycerophosphoserines, glycerophosphocholines, purine nucleosides, eicosanoids.	[106]
^{1}H NMR	*Oryzias javanicus* (Javanese medaka)	5 µm	100, 500 and 1000 µg/L	21 d	Gut	Adult	Changes in 9 metabolites. ↑ glucose, lactate, alanine, glutamate, glucoronate, valine, anserine, 2-hydroxyvalerate, creatine.	[107]
GC-MS	*Oryzias melastigmas* (marine medaka)	10 and 200 µm	10 mg/L	60 d	Liver	Adult (8 months)	Changes in 83 metabolites. ↑ disaccharides, trisaccharides, fatty acids, fatty acid methyl and ethyl esters; ↓ monosaccharides, organic acids, amino acid.	[108]
LC-MS/MS	*Sebastes Schlegelii* (marine jacopever)	5 µm and 100 nm	0.23 mg/L	15 d	Liver	Juvenile	Changes in 345 metabolites. Disorders in essential amino acids, omega-3 fatty acids, intermediate products of glucose metabolism and TCA intermediates. ↓ gluconic acid, cis-aconitate, malic acid, tyrosine, targinine, glycerol phospholipid	[109]
LC-MS	*Xiphophorus helleri* (swordtail fish)	1 µm	1×10^{6} microspheres/L (B) and 1×10^{7} microspheres/L (C)	72 h	Liver	Adult (3 months)	Changes in 37 (B) and 103 (C) metabolites. ↑ 3-hydroxyanthranilic acid, histidine, citrulline, linoleic acid, pantothenate, xanthine.	[110]

FISHES								
Method Used	**Organism Tested**	**PS MNPs Size**	**Concentration Tested**	**Time of Exposure**	**Organ/Tissue Target**	**Life Stage**	**Effect**	**References**
AQUATIC INVERTEBRATES								
LC-MS/MS	*Crassostrea gigas* (Pacific oyster)	6 and 50/60 µm	1×10^4 particles/L	14 d	Gills	Adult	Changes in 22 metabolites. ↑ asparagine, phenylalanine, glutathione, glucose-6-phosphate, carbohydrates, lactose, mannose; ↓ N-palmitoyl taurine, fatty acids.	[111]
^{1}H NMR	*Daphnia magna* (water flea)	53 nm (PS-NH_2), 62 nm (PS-COOH)	3.2 µg/L	37 d	Whole organism	Adult	Changes in 15 metabolites. ↑ alanine, asparagine, glutamate, glutamine, isoleucine, leucine, tyrosine, valine, lactate, methionine sulfoxide; ↓ glycine, TCA, glycerol, nucleic acids, isopropanol.	[112]
LC-MS/MS	*Litopenaeus vannamei* (Pacific white shrimp)	2 µm	0.02 to 1 mg/L	72 h	Hepatopancreas	Post-larvae	Changes in 119 metabolites. ↑ amino acids and dipeptides, e.g., taurine, aspartic acid and alanine; ↓ glycerol and fatty acids, e.g., 3-methyladipic acid and leucinic acid.	[113]
^{1}H NMR	*Mytilus galloprovincialis* (Mediterranean mussel)	3 µm	50 particles/mL	72 h	Gills and hepatopancreas	Adult	Changes in 10 (in gills) and 18 (in hepotopancreas) metabolites. Gills: ↑acetoacetate, ATP/ADP, mytilitol, betaine, taurine, homarine; ↓ alanine, glycine, succinate, acetylcholine. Hepatopancreas: ↑ isoleucine, leucine, valine, alanine, dimethylglycine, tyrosine, lactate, glycogen, glucose, betaine, taurine, homarine; ↓ glutathione; ↓ glycine, acetoacetate, succinate, malonate, hypotaurine.	[32][36]

↑: increase; ↓: reduction; hpf: hours post-fertilization; h: hours; d: days; w: weeks; ^{1}H NMR: proton Nuclear Magnetic Resonance; GC-MS: Gas Chromatography-Mass Spectrometry; LC-MS/MS: Liquid Chromatography-Tandem Mass Spectrometry; UPLC-Q-TOF: Ultra-high Performance Liquid Chromatography with Quadrupole Time-of-Flight; TMAO: trimethylamine N-oxide; TCA: tricarboxylic acids.

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