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To be or not to be plastics? Protein modulation and biochemical effects in zebrafish embryos exposed to three water-soluble polymers

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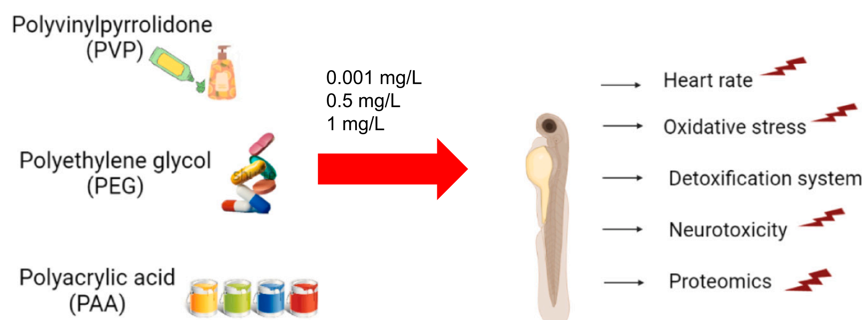
HIGHLIGHTS

- Zebrafish embryos were exposed to different water-soluble polymers (WSPs).
- Biomarkers and proteomics were applied to evaluate the WSP toxicity.
- Biomarkers were affected by the exposure to polyacrylic acid and polyvinylpyrrolidone.
- WSPs significantly modulated some proteins, mainly related to genetic processes.
- The environmental hazard of WSPs should be reconsidered.

GRAPHICAL ABSTRACT



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ABSTRACT

Water-soluble polymers (WSPs) are a particular category of polymers that, due to their capability to be soluble in water, come out of the classic definition of plastic and therefore also from its regulation and control, representing a possible new environmental problem considering the number of consumer products in which they are contained. For this reason, the aim of this study was to evaluate the possible adverse effects of three of the most used WSPs (polyacrylic acid - PAA, polyethylene glycol - PEG, polyvinylpyrrolidone - PVP), administered at relevant environmental concentrations (0.001, 0.5 and 1 mg/L) to *Danio rerio* (zebrafish) embryos up to 120 h post fertilization. To assess the WSP toxicity at the molecular, cellular and organism level we used an integrated ecotoxicological approach of both biomarkers and high-throughput technology based on gel-free proteomics. The main results showed how all the three WSPs up-regulated many proteins (up to 74 in specimens exposed to 1 mg/L PVP) with a wide range of molecular functions and involved in numerous cellular pathways of exposed specimens. On the other hand, the measurement of biomarkers showed how PAA and PVP were able to activate the antioxidant machinery following an over-production of reactive oxygen species, while PEG produced no significant changes in the biomarkers measured. Based on the obtained results, the use and application of WSPs should be revised and regulated.

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1. Introduction

The problem of the release of plastics into the environment as well as the consequent ecosystem impacts of macro-, micro- and nanoplastics, begins to be well known by both scientific community and especially citizens, bringing governments and international institutions to seek urgent solutions. However, a further possible environmental concern, represented by the so-called synthetic water-soluble polymers (WSPs), also known as "liquid plastics", is widely overlooked yet. The WSPs are compounds able to dissolve, disperse or swell in water and whose polymeric chains contain hydrophilic groups (nonionic, anionic, cationic or amphoteric) as substituents or incorporated into the backbone (Kadajji and Betageri, 2011). Due to their physicochemical characteristics, WSPs do not fit with the conventional definitions of synthetic polymers, based on solid state and insolubility in water (Hartmann et al., 2019), rather fall into the class of persistent and mobile substances and potentially toxic compounds (Neumann and Schliebner, 2017), which are under discussion for Registration, Evaluation and Authorization of Chemicals (REACH) regulation (Duis et al., 2021). The uses of WSPs range from numerous home applications, as in washing agents, cosmetics, personal care products, and pharmaceuticals to many industrial applications as in the textile industries or as flocculants for wastewater and drinking water production (Koltzenburg et al., 2014). Other applications that can release WSPs directly into the environment are due to their use in paints, coatings and building materials (Knapen and Van Gemert, 2009), as well as in the formulations of pesticides, fertilizers and other products used during crop cultivation (Xiong et al., 2018). As WSPs are not currently registered under REACH, the quantities produced and used in Europe are not known, but it is possible to estimate them using polymer synthesis data, which are instead under REACH registration (Huppertsberg et al., 2020). This estimate indicates a European production ranging from about 10^3 t/y for polyvinylpyrrolidone (PVP) to about 10^6 t/y for polyethylene glycol (PEG), which are quantities comparable to those of surfactants. These high production volumes, together with the numerous applications in which WSPs are used, highlight a considerable probability of release into the environment, where degradation is more slowly than in industrial or water treatment processes (Arp and Knutsen, 2020). Another characteristic that determines a greater or lesser presence of WSPs into the ecosystems is their solubility in water which depends strictly by their molecular weight (MW), as polymers with high MW are in general less soluble and slower in degradation.

From the ecotoxicological point of view, only recently few studies addressed the evaluation of the hazard of WSPs towards several biological models: Mondellini et al. (2022) highlighted as concentrations of five different WSPs ranging from 1 mg/L to 50 mg/L were unable to produce acute toxicity in terms of mortality, immobilization or heart rate alterations in *Daphnia magna*, while they identified some chronic effects (body weight variations and decrease in number of offspring) after 21 days of exposure, but always obtained at concentrations above 5 mg/L. The study by Hatami et al. (2019) showed that 10 mg/L of PEG administered for 21 days to common carp (*Cyprinus carpio*) modified significantly ($p < 0.05$) some biochemical parameters, while a PEG concentration of 5 mg/L was not toxic. Rozman and Kalcikova (2021) highlighted as 100 mg/L of an acid-based WSP had no effect on the motility of *Daphnia magna* exposed for 48 h, but the same concentration inhibited the bioluminescence by 73 % in the bacterium *Allivibrio fischeri*. All these studies are characterized by the use of high WSP concentrations in laboratory experiments. On the other hand, it is known that data on WSP contamination are almost completely missing, as currently only three studies report the concentrations measured in the environment for two WSPs. PEG was detected in UK water courses in a concentration range from 2.1 to 33.5 μg/L (Sainju et al., 2023), and with 21.9 μg/L in fresh falling snow in Montréal (Canada; Wang et al., 2021). An older study by Antić et al. (2011) showed PVP levels of about 180 μg/L in the Rur River (Germany) near the effluent of a wastewater treatment plant (WWTP), while a concentration of PVP ranging from 0.9 mg/L to 7.1 mg/L was measured in samples from the sewage canal of the WWTP of Aachen (Germany). Also, the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) recently reported as the monitoring of these polymers in the environment is an extremely difficult task due to the lack of specific analytical methods (ECETOC, 2020).

In this context, we carried out an extensive study exposing embryos of *Danio rerio* (zebrafish) up to 120 h post fertilization (hpf) to three different concentrations (0.001, 0.5 and 1 mg/L) of PVP, PEG and polyacrylic acid (PAA), which are three of the most used WSPs (Halake et al., 2014). We have chosen lower concentrations than the studies mentioned above, closer to the only environmental data available so far (0.18 mg/L; Antić et al., 2011). This has led us to consider more sensitive endpoints than apical tests, as it is necessary when assessing the hazard of emerging contaminants, whose environmental concentrations are often lower than those of effect of classic tests for the ecotoxicity assessment (Sanderson and Solomon, 2009). To have the widest possible picture of the risk associated to the selected WSPs, we initially considered their mode of action by evaluating the variations in the swimming behavior of zebrafish larvae (Nigro et al., 2023), while in this study we will present the results obtained by proteomic analysis and a biomarker suite to also investigate their mechanisms of action. Therefore, to evaluate the chronic toxicity of WSPs, we selected some biochemical endpoints to assess molecular and cellular effects (oxidative stress, detoxifying performance and neurotoxicity), as well as the impact at the organism level linked to the evaluation of the heart rate of each individual embryo. In addition, the most sensitive, but also time- and cost-consuming, high-throughput methodology based on the gel-free proteomics was applied only to specimens exposed to the highest concentration (1 mg/L) of the three WSPs.

The application of proteomics in ecotoxicology (ecotoxicoproteomics) coupled with the measurement of many biomarkers represents a valid strategy, as it can integrate the diagnostic and prognostic information obtained from a biomarker suite to the variation of proteins from the whole proteome of the selected biological model, correlating phenotypic and molecular data and document the toxicity mechanisms of pollutants (Gouveia et al., 2019).

This is the first comparative study between three different WSPs conducted with concentrations that could be representative of their environmental levels by evaluating possible effects through an integrated ecotoxicological approach.

2. Methods and materials

2.1. Preparation of WSP testing solutions

The WSP standard powders, purchased by Sigma-Aldrich (Merk Life Science), presented the following molecular weights (MWs): ~450,000 Da for PAA (CAS number: 9003-01-4), 1900–2200 Da for PEG (CAS number: 25322-68-3) and 10,000 Da for PVP (CAS number: 9003-39-8). For each WSP, we prepared a 250 mg/L stock solution in reconstitute zebrafish water containing 0.1 % methylene blue, 0.1 g/L sodium bicarbonate ($NaHCO_3$), 0.1 g/L Instant Ocean®, and 0.2 g/L calcium sulphate ($CaSO_4$). Solutions were heated to guarantee the complete WSP solubilization. From the stock solutions, we performed serial dilutions in zebrafish water to obtain the exposure concentrations of 0.01, 0.5 and 1 mg/L, selected based on our previous studies (Nigro et al., 2022, 2023). Before the use, the solutions were aerated overnight to reach the oxygen saturation.

2.2. Zebrafish exposures

Zebrafish fertilized eggs were obtained by the facility of the Department of Earth and Environmental Sciences of the University of Milan Bicocca, according to the Italian laws, rules and regulations (Legislative Decree no. 116/92; authorization n. 0020984 - 12/02/

2018). We exposed the embryos, to the three different WSP concentrations, from the eggs' fertilization to 120 hpf. To obtain the requested amount of biological material for the evaluation of the selected endpoints, we performed 5 different exposures with zebrafish embryos. For each group, we exposed 20 specimens in triplicate, for a total of 60 specimens *per* treatment.

Embryos were placed in Petri dishes with 50 mL of WSP testing solutions, in static conditions and at 28 °C. We daily checked the eventual acute effects (coagulation of eggs, lack of somite formation and heart breath), according to the guideline 236 of the Organization for Economic Cooperation and Development (OECD, 2013), as well as sublethal anomalies (scoliosis, development delay, edemas and malformations) according to Schiwy et al. (2015). Dead embryos were removed from the Petri dishes and at the end of exposures the specimens were processed for both proteomic and biomarker evaluations. Lastly, regarding the WSP measurement in the exposure media, the nominal concentrations were investigated through the ^{1}H NMR (nuclear magnetic resonance) spectroscopy in Milli Q® water. Control of the concentration throughout the exposure period was not possible due to the strong interference detected in the reconstitute zebrafish water due to the presence of salts. The results reported in our previous study (Nigro et al., 2023) aimed to assess the behavioral effects of WSPs on zebrafish larvae at the same concentrations tested in this research.

2.3. Gel free proteomics

The method used for the proteomic analysis is in-depth reported in our previous studies (Magni et al., 2019, 2021). Proteomics was conducted on 3 pools of 20 specimens *per* treatment (control and highest tested concentration of 1 mg/L). The organisms were homogenized through a potter in a solution of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 20 mM at pH 7.5, sucrose 320 mM, ethylenediaminetetraacetic acid (EDTA) 1 M at pH 8.5, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetra-acetic acid (EGTA) 5 mM at pH 8.1, sodium orthovanadate (Na_3VO_4) 1 mM, β-glycerophosphate 10 mM, sodium fluoride (NaF) 10 mM, sodium pyrophosphate (NaPPi) 10 mM, phenylmethylsulfonyl fluoride (PMSF) 1 mM in ethanol, dithiothreitol (DTT) 5 mM and protease inhibitors (Roche) in Milli Q® water. After homogenization, the samples were centrifuged at 15,000*g* (S15 fraction) for 10 min at 4 °C. The first protein quantification was performed at the EnSight™ multimode plate reader (PerkinElmer) using the Bradford (1976) method, and 200 μg of proteins were subsequently precipitated using methanol/chloroform/Milli Q® water (4:1:3 *v/v*). The samples were centrifuged at 15,000*g* at 20 °C for 15 min and the obtained pellets were re-suspended in a solution of urea 8 M in Tris-HCl 50 mM with sodium chloride (NaCl) 30 mM at pH 8.5 and protease inhibitors (1 tablet, Roche). The samples were centrifuged again at 14,000*g* at 4 °C for 30 min. After this step, the second protein quantification was performed using the Bradford (1976) method and 10 μg of proteins were processed for reduction and alkylation. In detail, DTT 50 mM in ammonium bicarbonate (AMBIC) 50 mM was added to samples, then incubated for 30 min at 52 °C under stirring (600 rpm). Subsequently, iodoacetamide (IANH2) 100 mM in AMBIC 50 mM was added to the samples and incubated for 20 min at room temperature (RT). The obtained samples were digested using trypsin (Trypsin Sequencing Grade, Roche, Italy) in AMBIC 50 mM and incubated over-night at 37 °C under stirring (400 rpm). Then, 25 μL of each sample was purified using Zip Tips (μ-C18; Millipore, Milan, Italy) and the obtained eluate was concentrated with a Speedvac and reconstituted with 20 μL of 0.1 % formic acid. The protein characterization was performed at the facility of the University of Milan UNITECH OMICS, injecting 5 μL of each sample, in triplicate, in a Dionex Ultimate 3000 nano-LC system (Sunnyvale CA, USA) connected to Orbitrap Fusion™ Tribrid™ Mass Spectrometer (Thermo Scientific) equipped with nanoelectrospray ion source. Peptides were pre-concentrated onto an Acclaim PepMap 100–100 μm × 2 cm C18 (Thermo Scientific) and separated on EASY-Spray column ES802A, 15 cm × 75 μm ID packed with Thermo Scientific Acclaim PepMap RSLC C18, 3 μm, 100 Å using mobile phase A (0.1 % formic acid) and mobile phase B (0.1 % formic acid in acetonitrile, *v/v*) at a flow rate of 0.300 μL/min. To prevent sample carryover, one blank was run between samples. The spectra were collected with the following setting: over an *m/z* range of 375–1500 Da at 120,000 resolutions, operating in the data dependent mode, cycle time of 3 s between master scans, collision energy of 35 eV and positive polarity. Data elaboration was performed using the Proteome Discoverer Software 2.5 (Thermo Scientific), the *Danio rerio* database (sp_incl_isoforms TaxID = 7955_and_subtaxonomies) and trypsin as enzyme.

2.4. Biomarker suite

Since the methods for biomarkers are described in our previous studies (Magni et al., 2018; Parenti et al., 2019), we reported only a brief description of them. As performed for proteomics, for biomarker evaluation we pooled 20 specimens *per* treatment ($n = 3$ pools of 20 specimens), homogenized in 250 μL of phosphate buffer 100 mM at pH 7.4, with potassium chloride (KCl) 100 mM, EDTA 1 mM, DTT 1 mM and protease inhibitors (1:100 *v/v*). Subsequently, we centrifuged the samples at 15,000*g* (S15 fraction) for all biomarkers, except for the samples for monoamine oxidase (MAO) activity measurement that were centrifuged at 1000*g* (S1 fraction), for 30 min at 4 °C (Magni et al., 2018). The protein content, used to normalize the biomarker measurements, were quantified in the supernatants using the Bradford (1976) method. Regarding the oxidative stress, the kinetic of superoxide dismutase (SOD) was evaluated spectrophotometrically (6715 UV–Vis spectrophotometer, Jenway) at 550 nm for 2 min through the reduction inhibition of cytochrome C 10 μM due to the superoxide anion formed by the complex xanthine oxidase-hypoxanthine 50 μM. The reactive oxygen species (ROS) levels were measured using the dichlorofluorescein-diacetate (DCFH-DA 10 mg/mL in dimethyl sulfoxide, DMSO). For each S15 fraction, 20 μL of samples were added to a 96-well plate and incubated for 5 min at 37 °C. Subsequently, we added 100 μL of phosphate buffer saline (PBS) and 8.3 μL of DCFH-DA and incubated for 30 min at 37 °C. ROS quantification was conducted by reading the fluorescence at the EnSight™ multimode plate reader (PerkinElmer) with λex 485 nm and λem 530 nm.

Moving to the detoxifying enzymes, the ethoxyresorufin-O-deethylase (EROD) activity was measured following the protocol described by Parenti et al. (2019) using the EnSight™ multimode plate reader (PerkinElmer) at λex 535 nm and λem 590 nm and a temperature of 37 °C. The homogenate was added to a 96-well plate in 50 mM Tris buffer at pH 7.4, with bovine serum albumin (BSA) and nicotinamide adenine dinucleotide phosphate (NADPH), and the reaction substrate ethoxyresorufin. The standard concentration range of resorufin curve was between 0.001 and 1 μM and was obtained by diluting the stock solution in 15 % methanol (MeOH). EROD activity was calculated from relative fluorescence units such as resorufin product, quantified with the standard resorufin curve. The glutathione-S-transferase (GST) activity was measured spectrophotometrically (6715 UV–Vis spectrophotometer, Jenway) using glutathione (GSH) 20 mM, and 20 mM 1-Cl-2,4-dinitrobenzene (CDNB) in ethanol and reading the absorbance at 340 nm for 1 min against blank. Regarding neurotoxicity, we evaluated the acetylcholinesterase (AChE) activity in the S15 fraction using the Ellman reagent (Ellman et al., 1961), which contains 1 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) in 100 mM Tris-acetate at pH 7.4, and acetylthiocholine 1 mM in Tris-HCl 50 mM as substrate. We assessed the absorbance at 30 °C for 15 min (read interval of 1 min) at 412 nm. The kinetic of MAO was measured in S1 fraction using 1 mM tyramine as substrate, 10 μM dichlorofluorescein diacetate in a 140 mM NaCl, 10 mM HEPES and sodium hydroxide (NaOH) buffer at pH 7.4, 1 mg/mL peroxidase and 10 mM of 3-amino-1,2,4-triazole. We measured the fluorescence for 3 min (read interval of 42 s) with λex 485 nm and λem 528 nm. Lastly, the heart rate was evaluated on 15 organisms *per*

treatment. Each embryo was placed in a water drop on a glass slide and subsequently filmed using a video camera connected to an optical microscope (Basler acA1300-60gm GigE camera). Videos were recorded for 10 s and heartbeats were counted during this time.

2.5. Statistical approach

The eventual significant differences, treated *versus* control, were evaluated using the one-way ANOVA followed by the Fisher LSD *post-hoc* test ($p < 0.05$). The eventual outliers were removed using the box-plot graphs.

For proteomics, only proteins with an abundance ratio (AR), compared to control, at least of 2-fold change (<0.5 for down regulated proteins and >2.0 for up regulated proteins), and with a $p < 0.01$ were considered modulated by the treatment.

3. Results

3.1. Proteomics

Gel-free proteomics analysis was able to identify 249 total proteins in common among both the three WSP treatments and controls. Using the two cut-offs based on the 2-fold changes and statistically significant differences between treated and controls, we identified 20 proteins modulated by the exposure to 1 mg/L PAA (16 up-regulated and 4 down-regulated; Table S1), which correspond to about 8 % of the 249 common identified proteins, 64 modulated proteins by 1 mg/L PEG (58 up-regulated and 6 down-regulated; 25 % of common identified proteins) and 74 modulated proteins by 1 mg/L PVP (59 up-regulated and 15 down-regulated; 30 % of common identified proteins) as shown in Fig. 1A.

Not only PEG and PVP were the two WSPs that had the highest impact on the proteome of the exposed organisms, but it is clear from the Venn's chart that they affected much of the same proteins, as they modulated 38 proteins in common, while PAA showed only one modulated protein in common with PEG and PVP, respectively (Fig. 1B). Further confirmation that these substances have different effects on zebrafish is highlighted by the only 17 proteins modulated by all the three WSPs (7 % out of the 249 common identified proteins). In this context, PAA was able to selectively modulate 1 protein, PEG modified the regulation of 8 specific proteins and PVP impacted the expression of 18 proteins not in common with the other two WSPs (Fig. 1B).

We have categorized the different proteins modulated by the three WSPs using the UniProt bioinformatics database (Fig. 2). As can be seen, PAA modulated mainly proteins involved in protein binding and transport (25 % of the total), and in genetic processes (25 %), that instead represents the class of proteins most impacted by PVP (24 %) and PEG (24 %), for which the effects on proteins involved in protein binding and transport represent a much smaller portion, equal to 7 % and 10 % respectively (Fig. 2). Lastly, it is interesting to note that PVP and PEG were able to modulate a very specific class of proteins, linked to photoreceptor activity, in which all proteins were up-regulated.

3.2. Biomarkers

The only organismic endpoint measured showed a significant effect of treatment ($F_{9,140} = 8.20$; $p < 0.01$). In particular, PAA and PVP resulted in a significant ($p < 0.05$) increase in heart rate for all the concentrations tested, while PEG resulted in a dose-dependent increase, but not significant compared to controls (Fig. 3A). The highest effect was caused by 1 mg/L of PVP which increased the heart rate by about 20 % compared to the baseline value, while 0.001 mg/L of PAA increased the heart rate by about 7 %.

Moving to molecular and cellular endpoints, we observed a significant effect of treatment for ROS level ($F_{9,20} = 4.0576$; $p < 0.01$), highlighting that PAA and PVP were also able to increase ROS production in a highly significant way ($p < 0.01$), specifically for the highest concentration of PAA and for the two lowest concentrations of PVP. On the contrary, 1 mg/L of PVP resulted in only a slight *non*-significant increase in ROS levels compared to controls (Fig. 3B). As with the previous biomarker, PEG did not show significant ROS overproduction, although we noticed a more than twofold increase in their levels for the highest administered concentration.

The increase of ROS is also confirmed by data obtained for SOD, which represents the first enzyme of the antioxidant chain that controls the homeostasis of oxidative stress caused by ROS overproduction. We observed a significant effect of treatment on SOD activity ($F_{9,20} = 3.951$; $p < 0.01$), with a significant increase ($p < 0.05$) for the two lowest concentrations of PVP and for the two highest concentrations of PAA (Fig. 3C), coherently with the observed ROS levels with the exception of 0.5 mg/L PAA concentration (Fig. 3B). Given the absence of a significant increase in ROS, the PEG showed no significant change compared to baseline SOD values (Fig. 3C).

PAA and PVP were also able to modify AChE activity, even if in the opposite way. In particular, we observed a significant effect of treatment ($F_{9,20} = 3.951$; $p < 0.01$) on the AChE activity, with a significant increase ($p < 0.05$) induced by PAA only for the highest concentration, while PVP caused a significant decrease in AChE activity for the intermediate concentration of 0.5 mg/L (Fig. 4A).

Another endpoint related to the evaluation of possible neurotoxic effects is the measurement of MAO activity which, unlike what was observed for AChE, did not show any significant difference from baseline levels (Fig. 4B), indicating that the WSPs tested do not seem to be able to modulate the activity of such monoaminergic neurotransmitter inactivators.

No WSP has been able to significantly change the GST activity, which points out the possible activation of phase II detoxification system (Fig. 4C).

The EROD activity also did not respond to any of the three WSPs (Fig. 4D), indicating that these molecules are not counteracted even by phase I detoxification.

4. Discussion

4.1. Proteomics

Despite being a rather complex and expensive high-throughput technique, proteomics is increasingly used for the identification of stress signatures since many proteins resulting from physiological events can be modulated by several natural and anthropogenic stressors (Raposo de Magalhães et al., 2020).

Considering the protein modulation due to the three different WSPs exclusively from a quantitative point of view, we can point out that PVP was the one that determined the greatest effect on the proteome of zebrafish, as 1 mg/L PVP was able to produce a modulation in about a third of the common identified proteins, followed by PEG, which instead modulated just over a quarter of the proteins in common and, lastly, by the PAA which determined the modulation of <10 % of the common identified proteins (Fig. 1A). Interestingly, most of the proteins modulated by all the three WSPs were up-regulated, a feature that would seem to suggest a response to substances recognized as potentially dangerous by the exposed organisms rather than a random effect on proteome, which would determine on the contrary both a generalized up- and down-regulation of proteins. This observed protein up-regulation could be an indication of the General Adaptation Syndrome (GAS) which is constituted by a series of biochemical and physiological changes, that it is present in all higher organisms, fish included (Haque et al., 2019), to fight both natural stress (*e.g.* poor water quality, parasites, thermal changes) and pollutant exposure (Roberts et al., 2010). Although these changes may be different, it is possible to recognize three phases that make up the GAS: the first, called alarm reaction, consists in the overproduction of catecholamines and cortisol which represents the first

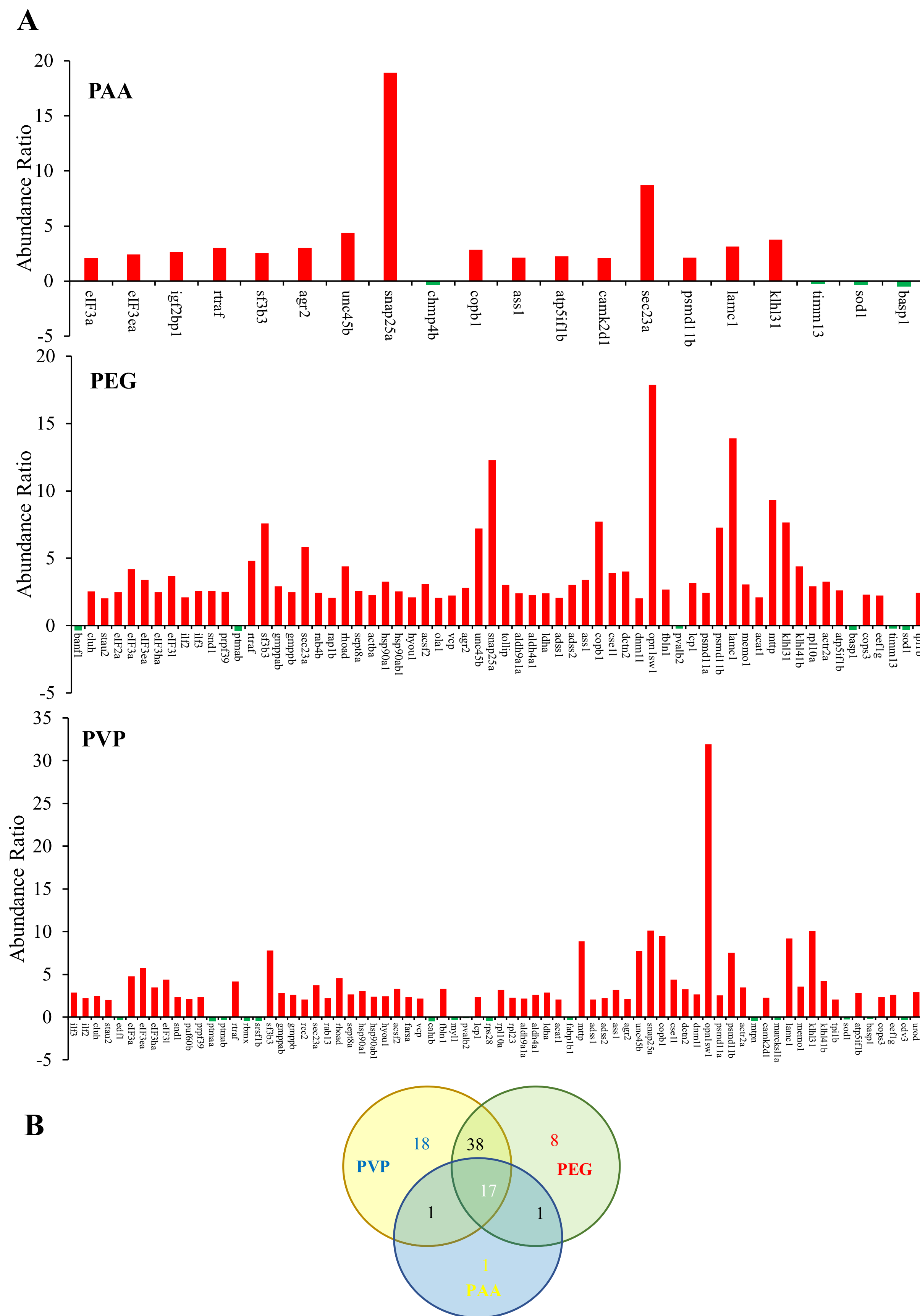

Fig. 1. Gel-free proteomics analysis results; (A) Abundance ratio of modulated proteins by 1 mg/L of the three WSPs compared to controls (red = up-regulation; green = down-regulation). (B) Venn's diagram showing proteins modulated by individual WSPs and in common with each other.

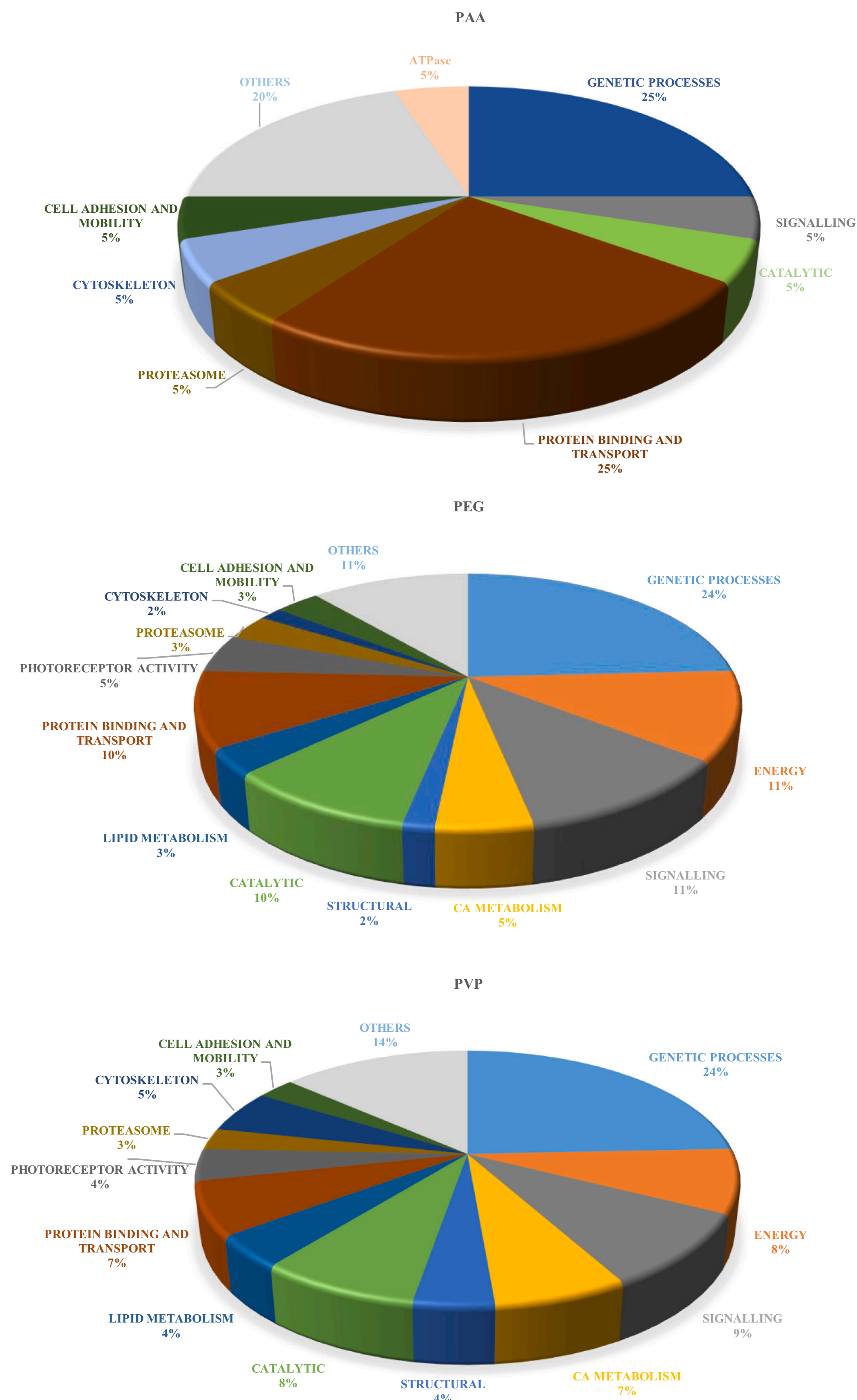

Fig. 2. Protein classes modulated by the three WSPs tested (1 mg/L) with their relative percentages.

response to acute and chronic stress, respectively. The second response, in which protein and biochemical changes are included, consists in the physiological and behavioral adjustments to stress conditions by the modulation of proteins, ion and metabolite levels involved in many molecular, cellular and metabolic responses. Lastly, in the third response, activated only by chronic stress, effects occur at the level of the whole organism that can lead to inhibitory events on reproduction, immunity, and growth and eventually to mortality (Haque et al., 2019).

The modulated proteins did not show the activation of specific cellular pathways, as the analysis performed by a protein-protein

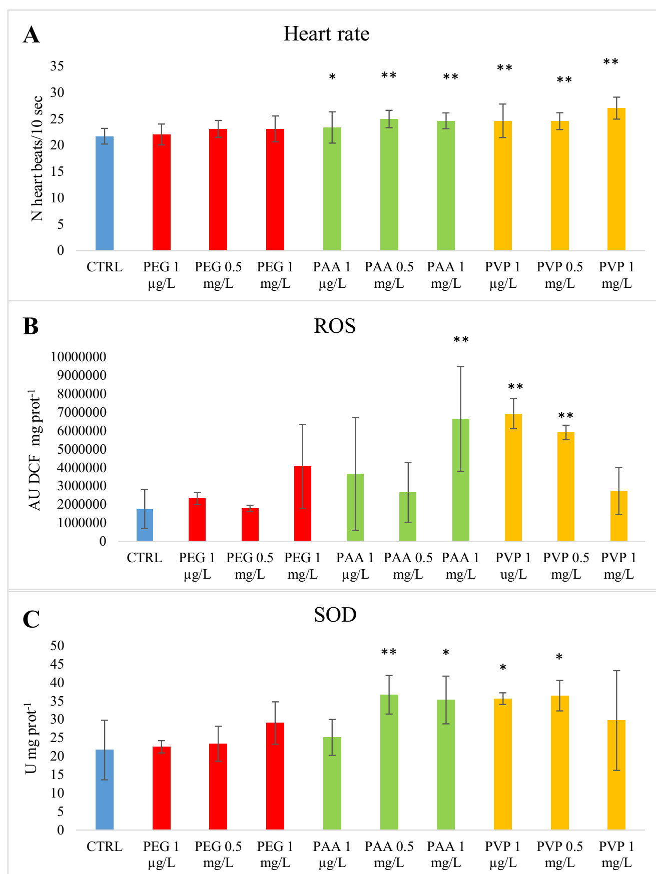

Fig. 3. Effects of different concentrations of the three WSPs on heart rate and oxidative status of *Danio rerio*, data are expressed as mean ± standard deviation, * means significant differences between treated and controls ($p < 0.05$), while ** means significant differences between treated and controls ($p < 0.01$). (A) Heart rate (N beats/10 s), (B) Concentrations of ROS, (C) Activity of SOD.

interaction networks functional enrichment analysis (STRING© freeware) did not detect connections that allow to define the activation of proteins referable to any common metabolic pathways. However, from the qualitative analysis of the proteomic dataset, it is possible to highlight the classes of proteins modulated by all the three WSPs and the relative relationships (Fig. 5). For instance, of the 17 modulated proteins in common among the three WSPs (Fig. 1B), most belong to proteins involved in genetic processes (Fig. 5), more precisely DNA-binding proteins (DBPs) and RNA-binding proteins (RBPs; Table S1). The formers are a large class of proteins that interact in different ways directly with DNA to act in histones' organization and compaction, transcription regulation, DNA replication, recombination and modification (Jen and Travers, 2013), while RBPs normally interact with RNA by some RNA-binding domains to regulate its metabolism and function, even if it has been discovered that a reverse mechanism can also occur, in which the RNA can bind to RBPs to affect their fate and function (Hentze et al., 2018). Interestingly, PAA modulated RBPs, while PEG and PVP also modified DBPs (Table S1). In this context, all the three WSPs were able to modulate different eukaryotic translation initiation factors (eIFs; Table S1) which are fundamental proteins for the mRNA translation and they are primary targets of many signaling pathways to regulate gene expression (Hao et al., 2020). Choundhuri et al. (2010) showed that the eIF3 class is involved in specific developmental programs during vertebrate embryogenesis and, more specifically, that eIF3ha regulates development of the brain, heart, vascular and lateral line in zebrafish. Thus, the up-regulation of several eIF3 proteins (Fig. 1A) could indicate specific effects on the development of exposed embryos or a homeostatic

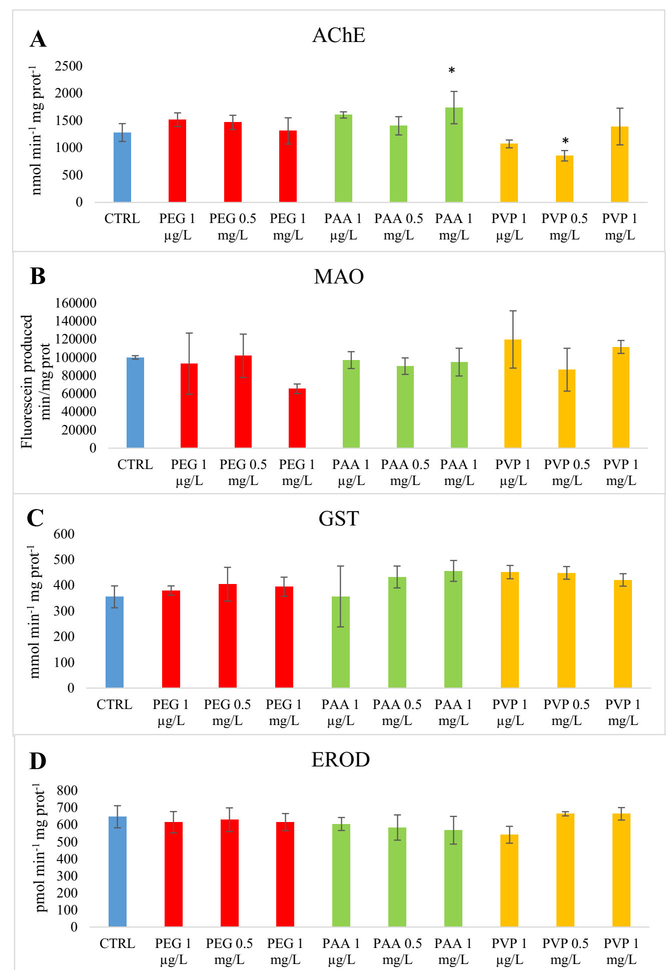

Fig. 4. Evaluation of the WSPs neurotoxicity and of their effects on the detoxifying enzymes, data are expressed as mean ± standard deviation, * means significant differences between treated and controls ($p < 0.05$). (A) AChE activity, (B) MAO activity, (C) GST activity, (D) EROD.

response to its possible variation due to WSPs.

Linked precisely to the signaling pathways that also act on RBPs, we have observed an up-regulation of several signaling proteins, namely the GTP-binding proteins, especially regarding exposures to PVP and PEG (Figs. 2 and 5). They are regulatory proteins that act as molecular switches and control a wide range of biological processes, as receptor signaling, intracellular signal transduction pathways, and protein synthesis. Their activity is regulated by factors that control their ability to bind and hydrolyze guanosine triphosphate (GTP) to guanosine diphosphate (GDP; Krauss, 2008). For example, the transport protein Sec23a, which is the only one of these GTP-binding proteins that has been modulated (up-regulated) by all the administered WSPs (Table S1), is specifically involved in the development of vertebrate skeleton (Fromme et al., 2007), as it is one of the five core proteins that are responsible for the first step of the transport of proteins from the endoplasmic reticulum (ER) to their final destination through the Golgi complex (Sarmah et al., 2010). More specifically, Lang et al. (2006) showed that Sec23a is essential for the craniofacial chondrocyte maturation in zebrafish.

It is interesting to note that 4 of the 17 proteins (24 %) modulated by all the three WSPs are involved in protein binding and transport (Table S1), which also represents the protein class most modulated by the PAA (Fig. 2). In particular, these 5 proteins are the Anterior gradient protein 2 homolog (Agr2), Protein unc-45 homolog B (Unc45b), Synaptosomal-associated protein 25-A (Snap25a) and Coatomer subunit

beta (Copb1). In detail, Agr2 is a crucial protein for terminal differentiation of intestinal goblet cells in zebrafish embryos (Chen et al., 2012) that are responsible for the production and maintenance of the protective mucus blanket (Yang and Yu, 2021). Furthermore, also Unc45b is involved in several developmental processes in zebrafish embryos, as it plays a role in sarcomere formation during muscle cell development and myoblast differentiation (Comyn and Pilgrim, 2012), myofiber attachment, motility and craniofacial development (Wohlgemuth et al., 2007), as well as it is necessary for normal early lens development (Hansen et al., 2014), which confirms that eye development is one of the specific targets of synthetic polymers as well reported below.

Snap25a is an intracellular protein belonging to the SNARE (SNAp Receptor) protein complex which is crucial to synaptic vesicle exocytosis of neurotransmitters and to regulate intracellular calcium dynamics (Antonucci et al., 2016). The two isoforms Snap25a and Snap25b differ depending on the stage of development, as the first is active mainly during the embryonic phase, as in our case, while the second represents the main isoform during postnatal stages (Bark et al., 1995). Interestingly, this protein is involved in several human neurological syndromes, as attention-deficit/hyperactivity disorder, schizophrenia and bipolar disorder (Antonucci et al., 2016 and references therein).

Lastly, Copb1, which is part of the coatomer vesicular complex, is generally located in the Golgi membrane and cytoplasmic vesicles and it is another crucial protein for zebrafish embryos, as it is involved in both the development of the notochord and somite (Coutinho et al., 2004).

A separate discussion deserves some proteins that are involved in the development and maintenance of the eye. Although numerically they are few, the proteins modulated by PVP and PEG involved in photoreception activity (Fig. 2) have a crucial importance, not only because they could indicate problems in eye development or vision, but also because they confirm the results obtained in our previous article on zebrafish embryos in which it was observed that the eye represents a target for NPs (Parenti et al., 2021). In this context, the short-wave-sensitive 1 Opsin-1 (Opn1sw1) is the protein with the highest degree of up-regulation for PVP and PEG (Fig. 1A). Opsins belong to the superfamily of G-protein coupled receptor proteins (GPCR) involved in multiple cellular signaling processes and in particular to phototransduction because the visual pigments in vertebrates consist of an opsin protein moiety bound to a light-sensitive chromophore (Arshavsky et al., 2002). Since Opn1sw is the blue cone photoreceptor pigment, the observed modulation of this protein could be one of the possible explanations for the hypoactivity in the swimming behavior during the light phases observed in the same specimens used in the present study (Nigro et al., 2023), even if Wang et al. (2014) observed an opposite effect by the exposure to retinoic acid which created a significant increase in the expression of opn1sw gene in zebrafish larvae at 72 hpf accompanied by hyperactive swim behavior.

The other two proteins involved in the photoreception activity, always modulated only by PVP and PEG, are the dynactin subunit 2 (Dctn2) and dynamin-1-like (Dnm1l; Table S1). The first one, also known as Dynamitin (p50), is part of a protein complex whose function is to participate in the dynein/dynactin complexes involved in the transport of a multitude of intracellular cargos, but also in the determination of nuclear position in several cellular types, including zebrafish photoreceptors (Tsujikawa et al., 2007). Interestingly, Jing and Malicki (2009) have shown that a mutation of the zebrafish ale oko locus, that encodes Dynamitin (p50), can cause an extremely rapid photoreceptor degeneration. Dnm1l protein is instead a small GTP-protein able to directly or indirectly regulate mitochondrial dynamics to affect the size, shape, and distribution of neurons (Reddy et al., 2011). Ko et al. (2016) found as the imbalance between the phosphorylation sites of this protein in humans can cause various neurodegenerative diseases including glaucoma, while Al Ojaimi et al. (2022) showed as pathogenic variants of DNM1L gene create both an encephalopathy, characterized by delayed psychomotor development and hypotonia, and an optic atrophy with slowly progressive visual loss. Moreover, other ocular dysfunctions caused by these mutations result in dyschromatopsia (blue–yellow), central scotoma, a slow decrease in visual acuity, and optic nerve atrophy.

The whole dataset seemed to highlight that the three WSPs are able to selectively up-regulate different proteins involved in embryogenesis. This aspect represents a landmark for future studies focused on the evaluation of phenotypic effects related to exposure to these emerging contaminants.

4.2. Biomarkers

As can be seen from the entire dataset obtained by the biomarker suite, PVP and PAA confirm their effects on exposed zebrafish, as

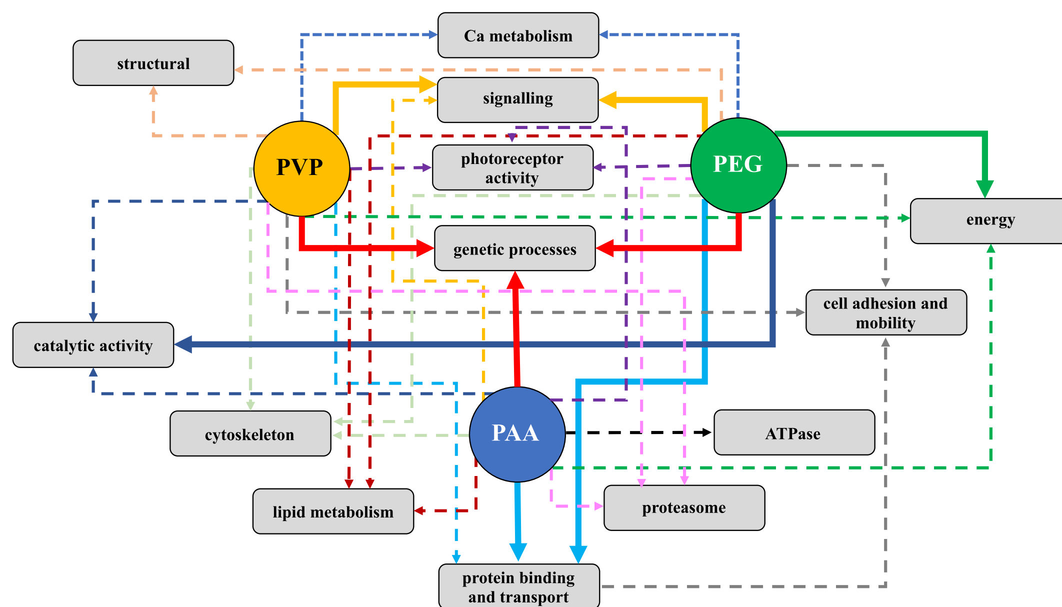

Fig. 5. Network of the different cellular pathways affected by protein modulation due to the 3 WSPs (1 mg/L). Same color = common effect on a specific cellular pathway. Bold lines = modulation of >10 % of the proteins involved in this pathway by each individual WSP. Dotted lines: modulation of <10 % of the proteins involved in this pathway by each individual WSP.

already highlighted not only by proteomics, but also by the results obtained previously through the evaluation of behavioral changes in the larvae swimming (Nigro et al., 2023). The only result in contrast is referred to PEG, as we did not observe significant changes for any biomarker compared to baseline levels. This could be a further confirmation that the three WSPs have different MoAs and, that to highlight all the effects on the chosen biological model, it is necessary to turn to a multidisciplinary approach based on the measurement of many endpoints selected on different levels of the biological organization. It is possible to hypothesize that, while for PVP and PAA the measured biomarkers are among the preferential targets of these two compounds, we have not had the chance to highlight on which cellular pathways the PEG selectively acts.

From a general point of view, we can point out that the evaluation of the heart rate was the biomarker more sensitive, as a significant increase was observed ($p < 0.05$) for all the three concentrations tested of both PAA and PVP (Fig. 3A). The heart rate of zebrafish embryos is about 120–180 beats/min, very similar to that of humans (Baker et al., 1997) and comparable to our controls. The significant increase observed for PVP and PAA and, although minor and not significant, also for PEG (Fig. 3A) could be due to several factors. A possible cause can be the cardiotoxicity of WSPs and/or effects on the cardiac system development (McGrath and Li, 2008) that is particularly sensitive to environmental contaminant exposure (Sarmah and Marrs, 2016), as clearly demonstrated in zebrafish embryos by Zhang et al. (2013) after a phenanthrene exposure. This will eventually be investigated with much more in-depth analyses and with longer exposures than those used by us. A second hypothesis could be a confirmation of GAS as a stress response against these environmental pollutants since the increase of heart rate is one of these typical responses. Indeed, stressed organisms usually require more oxygen to carry out their metabolic processes and, to compensate for the increased oxygen requirement, hyperventilation and consequently a heart rate rise may occur (Mercy and Prabha, 2022). These responses are mediated, as in the rest of vertebrates, by the activation of two hormonal axes: the sympatho-chromaffin (SC) axis and the hypothalamic-pituitary-interrenal (HPI) axis (Wendelaar Bonga, 1997). The first is precisely the one responsible for activating the rapid stress response involving the cardio-respiratory system by increasing ventilatory and heart rate, as well as heart stroke volume and blood perfusion in gills and muscles (Balasch and Tort, 2019). Such evidence has been found in zebrafish embryos after the combined exposure to microplastics (MPs) and Cd (Zhang et al., 2020), as well as in great pond snails (*Lymnaea stagnalis*) exposed to different concentrations of four progestogens (Svigruha et al., 2021).

Moving to cellular and molecular biomarkers, PVP and PAA were able to induce a ROS overproduction, unbalancing the redox potential and increasing oxidative stress (Fig. 3B). This effect is typical of exposure to different kind of synthetic polymers, as shown in a recent paper by Zhang et al. (2021), in which a ROS increase in zebrafish larvae exposed to weathered MPs of polylactic acid (PLA) was found, and by Shengchen et al. (2021) who showed that polystyrene MPs caused a ROS overproduction in mice myoblasts. Our result represents the first confirmation that even WSPs are able to cause a ROS overproduction, with a consequent rise of oxidative stress, in a model-organism also used in human studies. The ROS overproduction is also confirmed by the activation of the antioxidant chain (Fig. 3C), of which SOD is the first enzyme that is activated to counterbalance the over-production of the superoxide radical ($O_2^{-}\bullet$). This means that the increase in ROS caused by PAA and PVP was such as to activate the SOD, but also that its catalytic activity was not sufficient to keep the amount of ROS around the baseline levels. This is also one of the typical effects caused by synthetic polymers: Wang et al. (2022) have recently shown how polystyrene NPs of 100 μm and 20 μm increased SOD activity by 20 % and 8 %, respectively, as well as 5 μm polystyrene MPs increased SOD activity in the liver of zebrafish after seven days of exposure (Lu et al., 2016). Since no data on this is present until now regarding the activation of the first line of antioxidant enzymes due to WSPs, this result is another novelty due to our exposure tests. Interestingly, among the proteins modulated by tested WSPs, we also find the SOD protein, which is in all cases downregulated (Fig. 1A), suggesting a decrease in its expression. Actually, this is not in contradiction with the observed SOD activity increase, since proteomics was performed only for the highest concentration for all the three WSPs and, as can be seen from Fig. 3C, SOD activity did not increase significantly for either PVP or PEG exposures. Regarding its increase in organisms exposed to 1 mg/L PAA (Fig. 3C), it is important to note that proteomics measures the protein expression, while the performed biochemical assay evaluates the SOD activity and these two biological processes could be not strictly related.

Our data do not seem to be exhaustive with respect to the neurotoxic potential of WSPs because, while AChE activity has been modulated by both PVP and PAA (Fig. 4A), MAO activity has not been significantly changed by any of the three WSPs (Fig. 4B). The result obtained for the modulation of the first catalytic enzyme is also rather particular, since while we observed a significant ($p < 0.05$) decrease in AChE activity for 0.5 mg/L of PVP, a significant ($p < 0.05$) increase in this activity was measured for 1 mg/L of PAA (Fig. 4A). The first effect, which causes an accumulation of the neurotransmitter choline (ACh) in the synaptic cleft of motoneurons, modifying muscle contraction, has also been detected in several biological models exposed to MPs and NPs, suggesting their probable neurotoxic effect, although their MoA is still completely unknown (Hu and Palic, 2020). For instance, zebrafish embryos exposed until 120 hpf at several mixtures of NPs administered alone or in combination with 17 α-ethynylestradiol (EE2) showed a significant decrease of AChE activity ranging from 27 % to 40 % in comparison to controls (Chen et al., 2017), while an inhibition of AChE activity was noticed in hemolymph of the Mediterranean mussel (*Mytilus galloprovincialis*) exposed to polystyrene NPs (110 ± 6.9 nm) for 96 h (Brandts et al., 2018). However, MPs are also able to determine an increase in AChE activity, as we have also observed for PAA, as shown in the recent article by Chen et al. (2020), in which AChE activity increased in earthworms (*Eisenia fetida*) exposed to high concentrations (1.0 and 1.5 g/kg) of low-density polyethylene MPs, while Santos et al. (2022) recently observed how virgin MPs of 1–5 μm were able to determine a significant increase in AChE activity in zebrafish exposed for 30 d, whose causes are still unknown.

Another possible marker for the evaluation of the neurotoxicity of a substance is represented by the dosage of glial fibrillary acidic protein (Gfap), which is the major intermediate filament protein of astrocytes, and which is suggested by the U.S Environmental Protection Agency (U. S. EPA) to evaluate eventual injuries to the central nervous system (McGrath and Li, 2008). Although Gfap was detected by proteomic analysis in all the three WSP treatments, we did not observe any statistically significant changes compared to controls, again suggesting the absence of a neurotoxic effect. In conclusion, from the conflicting data in our possession, it is not possible to affirm that the three WSPs are also neurotoxic substances, referring this possibility to other studies specifically projected to highlight this effect.

Lastly, the lack of significant changes in EROD and GST activities, typical detoxification enzymes of Phase I and II, respectively, can be well explained by the characteristics of WSPs because, being water-soluble substances, they are not recognized by these enzymatic systems that serve to try to make lipophilic compounds more easily excretable. We can therefore say that these two biomarkers can be considered a sort of negative control for the WSP effects since their activation would certainly have been a surprise and an effect difficult to explain.

4.3. Final remarks

The merging of datasets provided both by this study and by the previous one (Nigro et al., 2023), which had evaluated some changes in swimming behavior for the same zebrafish larvae, represents the first multi-level ecotoxicological study performed both with environmentally

relevant concentrations of three of the most used WSPs and measuring contemporarily some endpoints at different levels of the biological organization, as suggested by many studies to improve the Environmental Risk Assessment (ERA; Rudén et al., 2016 and citations therein; Sumpter et al., 2022).

Although it is very difficult finding a relationship between all the endpoints measured, we can assert that all the three WSPs were able to determine different types of adverse effects in a biological model, as zebrafish, useful to predict the xenobiotic impact also for humans (Nagel, 2002; Yang et al., 2009; Dai et al., 2014). However, the entire dataset showed that there are differences in the mode of action of the three WSPs, as PVP and PEG have been able to significantly ($p < 0.05$) modify numerous movement parameters, while PAA seems to determine only mild swimming behavioral effects (Nigro et al., 2023). Even the molecular and cellular investigation performed in this study showed that PVP confirms to be the most harmful tested WSP for zebrafish embryos, as it modulated a greater number of proteins than the other two compounds, as well as determined significant ($p < 0.05$) variations in several biomarkers. The proteomic approach confirms also the behavioral results obtained for the other two WSPs, as not only the number of proteins modulated by PEG is similar to that determined by PVP, but even the main changed protein class was the same (genetic processes), while PAA is still confirmed as the least reactive WSP, since it modulated only about 31 % of the total proteins changed by PEG and 27 % of PVP. All this seems to corroborate the suggested toxicity scale from Nigro et al. (2023): PVP $>$ PEG $\gg$ PAA. But, if we consider results from the biomarkers' suite, this toxicity scale seems to be belied by the fact that PEG and PAA showed an opposite ecotoxicological behavior, since the first one showed no evident effects related to the measured biomarkers, while the second modulated the same endpoints on which PVP acted, although less effectively. This confirms once again how it is necessary to consider effects on multiple levels of biological organization and how all the three WSPs chosen cannot be considered non-hazardous environmental contaminants as considered so far. As recently pointed out by Wang et al. (2023), in the face of a worldwide production of $>$36.3 million tons of polymers in liquid formulations (PLFs) that is expected to double in the next years, the fate and environmental concentrations, as well as the possible (eco)toxicological risk of WSPs remain poorly understood. Thus, bearing in mind the wide use of these compounds and their consequent release into the environment, we consider crucial their possible re-evaluation of (eco)toxicological hazard, a discussion already started within the European Union considering that WSPs are among the persistent and mobile substances and potentially toxic compounds that are currently being reviewed regarding the possible future REACH registration.

5. Conclusions

Data from this study clearly showed many effects of the three WSPs tested both at the level of the whole proteome and on different endpoints measured within the biomarker suite, although with different modes and mechanisms of action. The proteomic approach seems to have indirectly shown a response of exposed organisms against the intake of these emerging contaminants, through the activation of GAS, also confirmed by the increase in heart rate.

Therefore, our data clearly indicate that it is necessary at least to reconsider the possible hazard of these compounds, as well as to give greater information to citizens, as for example recently done for MPs, especially considering the fact that even people more prone to ecological issues and willing to a more conscious use of plastic products, utilize and release in the environment, often unknowingly, these WSPs present in many common-used products. Other studies are necessary in this way to develop a suitable analytical method for WSP monitoring, a fundamental aspect to provide environmentally relevant concentrations for the future investigations on the WSP toxicity.

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CRediT authorship contribution statement

Andrea Binelli: Conceptualization, Investigation, Writing – original draft, Funding acquisition, Supervision. **Lara Nigro:** Methodology, Investigation, Formal analysis, Validation, Data curation, Writing – review & editing. **Riccardo Sbarberi:** Formal analysis. **Camilla Della Torre:** Writing – review & editing. **Stefano Magni:** Conceptualization, Investigation, Methodology, Writing – original draft, Funding acquisition, Project administration, Software, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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