Journal of Hazardous Materials 469 (2024) 134000

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Unveiling the multilevel impact of four water-soluble polymers on *Daphnia magna*: From proteome to behaviour (a case study)

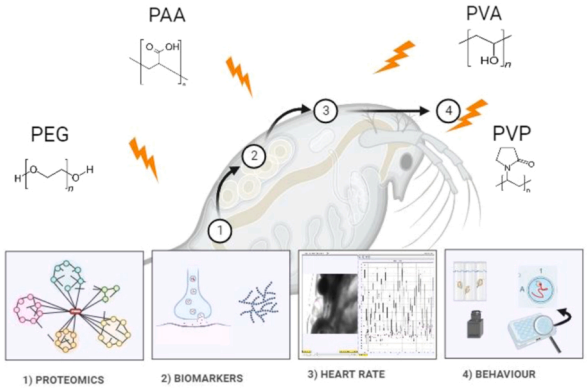
Lara Nigro [a], Stefano Magni [a,*], Marco Aldo Ortenzi [b], Stefano Gazzotti [b], Camilla Della Torre [a], Silvia Giorgia Signorini [a], Riccardo Sbarberi [a], Andrea Binelli [a]

[a] *Department of Biosciences, University of Milan, Via Celoria 26, Milan 20133, Italy*
[b] *Department of Chemistry, University of Milan, Via Golgi 19, Milan 20133, Italy*

HIGHLIGHTS

- *D. magna* was exposed to different water-soluble polymers (WSPs).
- Biomarkers, behaviour and proteomics were applied to evaluate the WSP toxicity.
- PEG induced mortality and neurotoxicity on the specimens exposed.
- All the WSPs affected metabolic pathways and energy allocation in *D.magna*.
- Further investigations on the risk assessment of these WSPs are needed.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Synthetic polymers
Freshwaters
Chronic toxicity
Ecotoxicology
Ecology

ABSTRACT

The ubiquitous presence of water-soluble polymers (WSPs) in freshwater environments raises concerns regarding potential threats to aquatic organisms. This study investigated, for the first time, the effects of widely used WSPs -polyvinyl alcohol (PVA), polyvinylpyrrolidone (PVP), polyacrylic acid (PAA), and polyethylene glycol (PEG)- using a multi-level approach in the freshwater biological model *Daphnia magna*. This integrated assessment employed a suite of biomarkers, evaluation of swimming behaviour, and proteomic analysis to investigate the effects of three environmentally relevant concentrations (0.001, 0.5, and 1 mg/L) of the tested WSPs from molecular to organismal levels, assessing both acute and chronic effects. Our findings reveal that exposure to different WSPs induces specific responses at each biological level, with PEG being the only WSP inducing lethal effects at 0.5 mg/L. At the physiological level, although all WSPs impacted both swimming performance and heart rate of *D. magna* specimens, PAA exhibited the greatest effects on the measured behavioural parameters. Furthermore, proteomic analyses demonstrated altered protein profiles following exposure to all WSPs, with PVA emerging as the most effective.

* Corresponding author.
E-mail address: stefano.magni@unimi.it (S. Magni).

https://doi.org/10.1016/j.jhazmat.2024.134000
Received 15 January 2024; Received in revised form 27 February 2024; Accepted 8 March 2024
Available online 15 March 2024

1. Introduction

Over the last decade, the pervasive presence of synthetic polymers in the environment has emerged as one of the foremost challenges confronting both society and the scientific community in addressing environmental pollution [1]. The advancement of modern technologies, alongside the widespread utilization of these materials across diverse industrial sectors, has elicited considerable apprehension regarding the potential hazards posed by plastic pollutants to the environment, organisms, and human health. Consequently, there has been an escalating emphasis on monitoring the discharge of plastic items into ecosystems, primarily focusing on specific solid polymer particles [2].

However, certain types of polymers found in numerous common products have been excluded from the definition of "plastic" as this term is typically reserved for solid compounds based on a dimensional scale, which categorizes them according to size, distinguishing between macro-, micro, and nanoplastics [3]. This definition excludes water-soluble polymers (WSPs), the primary components of what are often termed "liquid plastics". WSPs represent a significant category of synthetic polymers with a pivotal role in human society, constituting up to 6% of the global polymer market [4,5]. They find extensive use across various applications, including personal care products, pharmaceuticals, fertilizers, flocculants, and numerous other industrial sectors [6]. Despite their widespread use, WSPs are not subject to regulation as chemicals and they have been excluded from the Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) thus far, owing to their perceived low environmental impact due to their high molecular weight [7]. However, their exemption from REACH registration is currently under review.

Information regarding their production volume remains unavailable, with estimations reliant on the production of their educts [8]. Consequently, WSPs have been released into the environment both directly and indirectly, resulting in their ubiquitous presence in ecosystems [7, 9]. Recent studies have begun to focus on detecting WSPs; however, limited information is available regarding their environmental concentrations due to analytical challenges in their detection [7]. Antic et al. [10] quantified the presence of polyvinylpyrrolidone (PVP) in effluents from wastewater treatment plants (WWTPs) in Düren, Germany, with concentrations ranging from 0.9 mg/L to 7.1 mg/L, as well as in river water impacted by urban wastewater emissions, with levels around 0.18 mg/L. Recently, Vidovic et al. [11] uncovered the presence of poly (N-vinylcaprolactam) in WWTP effluents of Aachen, Germany, reaching concentrations up to 0.070 mg/L. Additionally, the emission of polyethyleneimine detected in influent and effluent waters from various treatment plants in Germany ranged from 0.08 mg/L to 0.55 mg/L [12]. Lastly, Wang et al. [5] measured the presence of polyethylene glycol (PEG) in fresh snowfall in Montréal, Canada, at a concentration of 21.9 μg/L, while Sainju et al. [13] found PEG concentrations in the range of 2.1–33.5 μg/L in English rivers. Studies on the ecological risk assessment of WSPs are relatively scarce, and many significant effects have been observed at high exposure concentrations, often exceeding measured environmental levels. For instance, Rozman and Kalcíková [14] found that 100 mg/L of acrylate copolymers affected the bioluminescence and oxygen consumption in nitrifying bacteria *Allivibrio fischeri*, whereas the same concentration had no impact on the motility of *Daphnia magna* over a 48-hour exposure period. Weston et al. [15] reported no significant acute effects of polyacrylamide (PAM) on aquatic organisms such as *Hyalella azteca, Chironomus dilutes, Ceriodaphnia dubia, Pimephales promelas*, and *Selenastrum capricornutum* (Lethal Concentration 50 - LC_{50} - values exceeding 100 mg/L), while *D. magna* showed higher sensitivity (LC_{50} = 14 mg/L). Concerning polyacrylic acid (PAA), low toxicity has been reported for *D. magna* (LC/EC_{50} (48 h) > 200 mg/L) and fish (LC/EC_{50} (96 h) > 200 mg/L; [9]), while Mondellini et al. [16] reported effects on the reproduction of *D. magna* after exposure to PAA, polyvinyl alcohol (PVA), PEG, and PVP only at concentrations above 5 mg/L. More data on PEG ecotoxicity are available; for example, Hatami et al. [17] reported that 10 mg/L of PEG induced alterations in *Cyprinus carpio* during a 21-day exposure, while Nascimento et al. [18] showed how a short exposure to 5 and 10 mg/L of PEG induced neurotoxicity in the tadpole *Physalaemus cuvieri*. Duis et al. [6] reported low acute and chronic toxicity in some crustaceans, while Siniakova et al. [19] noted changes in the luminescence intensity and colour of *Obelia longissimi* in the presence of the same polymer. Studies discussing PVA toxicity predominantly focuses on exceedingly high concentrations that are rarely encountered in natural environments. The LC_{50} for *Ceriodaphnia dubia* after 48 h of exposure was found to be 238.32 mg/L, with the Lowest Observed Effect Concentration (LOEC) observed in the range of 9.87 to 172.64 mg/L [20,21]. Alonso-López et al. [22] investigated PVA effects on the sea urchin *Paracentrotus lividus*, revealing high LOEC values ranging from 3.33 to 10 g/L. However, studies examining 28-day exposures found no toxicity in the amphipods *Hyalella azteca* and *Leptocheirus plumulosus* at concentrations of 5.55 and 0.70 mg/L, respectively. Similarly, a 96-hour exposure of PVA to the fathead minnow *Pimephales promelas* and sheepshead minnow *Cyprinodon variegatus* showed no measured toxicity [21].

Therefore, studies addressing the limited available environmental levels of WSPs remain scarce. To address this gap in knowledge, we conducted prior investigations focused on evaluating the effects of four commonly used WSPs (PEG, PVP, PVA, PAA) at various biological levels on *Danio rerio* larvae [23–25]. These studies enabled us, for the first time, to characterize numerous effects of these WSPs administered at environmentally relevant concentrations and to propose a potential toxicity scale. As a logical extension and one of the objectives of this study, we aimed to assess the effects of these four WSPs on an invertebrate model organism, the water flea *D. magna*. Building upon toxicity data obtained from the previous biological model utilized, our hypothesis was to ascertain whether effects could manifest across different biological levels (molecular, physiological, and organismic) following exposure to these emerging contaminants, even in an experimental invertebrate model. In detail, specimens were exposed to the same four WSPs selected for *Danio rerio* larvae for a duration of 14 days, at concentrations of 0.001 mg/L, 0.5 mg/L, and 1 mg/L, which closely approximate the few available environmental levels mentioned above. The effects of WSP exposures on *D. magna* were assessed using a multi-level approach aimed at investigating the possible mode of action and facilitating comparisons with our previous research on *D. rerio* [23–25]. To this end, we maintained all previously measured endpoints, with the addition of glycogen measurement, given our observation of an energy stock shift in *D. rerio* larvae [25]. At the organismic and physiological levels, we analysed the effects on heart rate and swimming performance, including horizontal swimming, vertical migration, acceleration, mobility, thigmotaxis, and phototaxis. Additionally, we selected biochemical endpoints to assess effects at the molecular and cellular levels. Specifically, we measured the activity of monoamine oxidase (MAO) and acetylcholinesterase (AChE) to evaluate potential neurotoxic effects involved in neurotransmitter metabolism associated with organism movement. Furthermore, we measured glycogen content (GLY) to assess potential alterations in energetic metabolism. Additionally, a high-throughput methodology based on gel-free proteomics was applied to *D. magna* exposed to the highest concentration (1 mg/L) of all the WSPs.

This study represents the first multi-level research to evaluate the potential effects of this group of new environmental contaminants on multiple levels of biological organization using one of the most widely used invertebrate models in ecotoxicology. It also serves as a crucial study for comparing the modes of action of such WSPs in both a freshwater vertebrate and invertebrate model.

2. Materials and methods

2.1. Preparation of WSP testing solutions

The WSP standard powders of PAA (CAS number: 9003-01-4), PEG (CAS number: 25322-68-3), PVP (CAS number: 9003-39-8), and PVA (CAS number: 9002-89-5) were purchased from Sigma-Aldrich (Merck Life Science, Milan, Italy). The molecular weights (MWs) of these powders were approximately 450,000 Da for PAA, 1900–2200 Da for PEG, 10,000 Da for PVP, and 89,000 - 98,000 Da for PVA.

To prepare the four WSP stock solutions (250 mg/L), reconstituted water was used according to the protocol outlined in the *Daphnia magna* Reproduction Test of the Organisation for Economic Cooperation and Development (OECD) guideline 211 [26]. Additionally, the solutions were heated to ensure complete solubilization of the WSPs, following the procedure detailed by Nigro et al. [23,24]. These stock solutions were then diluted to achieve the three exposure concentrations (0.001 mg/L, 0.5 mg/L, and 1 mg/L) based on previous research [23,24]. Before use, the solutions were aerated and maintained at 20 °C.

2.2. *D. magna* exposures

We utilized *D. magna* obtained from ChemService Controlli e Ricerche s.r.l. breeding. Toxicity tests involved exposing daphnids (age <24 h) to three different concentrations (0.001 mg/L, 0.5 mg/L, and 1 mg/L) of all the WSPs for a duration of 14 days under semi-static conditions (with renewal occurring 3 times *per* week). Specimens were housed in 50 mL beakers filled with pre-aerated reconstituted water (oxygenation >5.00 mg/L and pH maintained within the range of 6–9), in accordance with OECD guideline 211 [26]. Each treatment included 10 daphnids (5 specimens *per* beaker) at 20 °C, under a photoperiod of 16 h of light (1500 lx) and 8 h of darkness. The exposure was conducted in triplicate, with viability and immobilization recorded daily.

Throughout the exposure period, organisms were provided daily with a suspension of the green alga *Raphidocelis subcapitata*, initially at a concentration of 8×10^6 cells/individual/day until the *D. magna* specimens reached 8 days of age, then increased to 16×10^6 cells/individual/day. Additionally, yeast *Saccharomyces cerevisiae* (0.01 g/L) was added three times a week, following the protocol outlined by De Felice et al. [27]. Heart rate measurements were conducted after 7 days of exposure, while behavioural analysis was performed at the conclusion of the exposure period. Following behavioural analyses, the same specimens were frozen at –80 °C for subsequent biomarker measurements.

2.3. Polymer quantification

The nominal concentration of WSPs in MilliQ water solutions was determined using a two-step procedure. The analysis was conducted on 1 L of the 1 mg/L solution for each tested WSP. In the first step, the complete evaporation of the water was achieved using a rotary evaporator apparatus. This evaporation of the solvent allowed for the recovery of the solute, enabling the determination of its weight. The second step focused on assessing and verifying the structure of the recovered WSP through ^{1}H NMR analysis. Spectra were recorded on a Bruker Ultrashield 400. Samples were prepared by dissolving the recovered WSP in 0.7 mL of DMSO-d6. The results for PAA, PEG, and PVP are detailed in our previous study [24], while the same analysis was performed for the first time for PVA in this study. In this regard, the amount of recovered PVA was assessed to be 0.92 mg, close to the weighted amount, confirming the reliability of the methodology as previously discussed. The chemical integrity of the recovered PVA was evaluated through ^{1}H NMR analysis (see Fig. S1).

2.4. Gel free proteomics

Tests on *D. magna* for proteomic analysis were conducted solely at the highest concentration (1 mg/L) of all the WSPs. The procedure for the exposure was the same described above, except for the number of specimens per treatment (15 *per* treatment, with 5 *per* beaker). The proteomic analysis method is extensively detailed in our prior studies [25, 28, 29]. Proteomics were performed on 3 pools, each comprising 11 specimens *per* treatment. The animals were homogenized using a potter in 150 μL of a lysis solution containing 20 mM 4-(2-hydroxyethyl)–1-piperazineethanesulfonic acid (HEPES) at pH 7.5, 1 M ethylenediaminetetraacetic acid (EDTA) at pH 8.5, 320 mM sucrose, 1 mM sodium orthovanadate (Na3VO4), 5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N'',N''-tetra-acetic acid (EGTA) at pH 8.1, 10 mM sodium fluoride (NaF), 10 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF) in ethanol, 10 mM sodium pyrophosphate (NaPPi), 5 mM dithiothreitol (DTT), and protease inhibitors (Roche) in Milli Q water. The homogenized samples were centrifuged at 15,000 g (resulting in the S15 fraction) for 10 min at 4 °C. Initial protein quantification was conducted using the Bradford method [30], followed by the precipitation of 200 μg of proteins using a mixture of methanol, chloroform, and Milli-Q water (in a ratio of 4:1:3 *v/v*). Subsequently, the precipitated proteins were reconstituted by centrifuging the samples at 15,000 g for 15 min at 20 °C. The resulting pellets were dissolved in a solution containing 8 M urea in Tris-HCl (50 mM) with NaCl (30 mM) at pH 8.5, along with protease inhibitors. The resulting samples were centrifuged at 14,000 g at 4 °C for 30 min. Following this step, a second protein quantification was performed using the Bradford method [30], and 10 μg of proteins were processed for reduction and alkylation. At this stage, DTT at a concentration of 50 mM in ammonium bicarbonate (AMBIC) 50 mM was added to the samples, which were then incubated at 52 °C for 30 min under stirring (600 rpm). Subsequently, the samples were treated with iodoacetamide (IANH2) at a concentration of 100 mM in AMBIC 50 mM and allowed to incubate at room temperature (RT) for 20 min. Digestion of the resulting samples was carried out using trypsin (Trypsin Sequencing Grade, Roche, Italy) in AMBIC 50 mM, followed by overnight incubation at 37 °C with continuous stirring at 400 rpm. Following digestion, 25 μL of each sample underwent purification using Zip Tips (μ-C18; Millipore, Milan, Italy), and the eluted material was concentrated using a speedvac system before being reconstituted in 20 μL of 0.1% formic acid.

Protein characterization was conducted at the UNITECH OMICs facility of the University of Milan. For analysis, 5 μL of each sample was injected in triplicate into a Dionex Ultimate 3000 nano-LC system (Sunnyvale CA, USA), which was connected to a Thermo Scientific Orbitrap Fusion™ Tribrid™ Mass Spectrometer equipped with a nano-electrospray ion source. Peptides were initially concentrated onto a Thermo Scientific Acclaim PepMap 100 - 100 μm x 2 cm C18 column, followed by separation on an EASY-Spray column ES802A, which measured 15 cm × 75 μm ID and was packed with Thermo Scientific Acclaim PepMap RSLC C18 particles (3 μm, 100 Å). The separation was accomplished 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 any sample contamination, a blank run was performed between each sample. Spectra were acquired in positive polarity mode across a mass-to-charge ratio (*m/z*) range of 375–1500 Da, with at 120,000 resolutions. The instrument operated in data-dependent mode with a cycle time of 3 s between master scans and a collision energy of 35 eV. Data processing was conducted using Thermo Scientific Proteome Discoverer Software 2.5, utilizing the *Daphnia magna* database and trypsin as the enzyme.

2.5. Biomarkers

For biomarker evaluation, specimens (n = 3 pools of 10 specimens *per* treatment) were homogenized in 130 μL of phosphate buffer (100 mM, pH 7.4), containing potassium chloride (KCl) at 100 mM, EDTA at 1 mM, DTT at 1 mM, and protease inhibitors (1:100 *v/v*). Subsequently, half of the homogenate was centrifuged at 15,000 g (resulting in the S15

fraction) for 15 min at 4 °C to measure AChE and GLY content [31,32], while the remaining homogenate was centrifuged at 1000 g (resulting in the S1 fraction) for 20 min at 4 °C to assess MAO activity [23]. Protein content, used to normalize the biomarker results, was quantified in the supernatants using the Bradford method [30] with the EnSight™ multimode plate reader (PerkinElmer).

2.5.1. Biomarker of neurotoxicity

The assessment of MAO activity followed the procedure outlined by Magni et al. [29,33]. MAO activity in the S1 fraction was determined by measuring its kinetics using 1 mM tyramine as the substrate. The measurement was conducted in a buffer solution containing 140 mM NaCl and 10 mM HEPES-NaOH at pH 7.4, supplemented with 10 μM dichlorofluorescein diacetate. Additionally, 1 mg/mL peroxidase and 10 mM of 3-amino-1,2,4-triazole were added. Fluorescence intensity was recorded for 3 min at excitation and emission wavelengths of 485 nm and 530 nm, respectively, using the EnSight™ multimode plate reader (PerkinElmer). The results were expressed as Fluorescein produced min/mg prot.

AChE activity in the S15 fraction was measured following the protocol described by Ellman et al. [34]. Samples were supplemented with 100 mM phosphate buffer at pH 7.4, 5 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), and 1 mM acetylthiocholine (AscH) as the substrate. Absorbance was measured for 15 min at a read interval of 1 min, at a wavelength of 412 nm. The results were expressed as nmol/min/mg prot.

2.5.2. Biomarker of energetic metabolism

The GLY analysis was conducted following the sulfuric acid technique outlined by Dubois et al. [35], employing glucose standards ranging from 0 to 2 mg/mL. In brief, samples were treated with a solution containing 5 mg/mL glucose, 5% phenol (*v/v*), and 98% sulfuric acid (H_2SO_4). Absorbance was measured at 492 nm, and the results were expressed in mg/g FW (mg *per* g of Fresh Weight).

2.6. Heart rate assay

According to prior studies, the heartbeat rate of *D. magna* has been identified as an early and sensitive indicator of pollutant-induced harm [36], particularly for this species [37]. Consequently, a heart rate assay was performed on 7-day-old specimens, with 10 organisms *per* treatment gently positioned on a glass slide within a drop of prepared water, allowing them 2 min to acclimate to the lighting conditions of an optical microscope (Leica Microsystems CMS GmbH), as outlined by Xu et al. [38]. Subsequently, 15 s of video footage were recorded for each individual using a Basler acA1300–60gm GigE camera attached to the microscope eyepiece. The GigE camera was connected to EthoVision XT 11.5 software (Noldus IT, Wageningen, Netherlands) via a Power PoE single injector (Ace series) for video download. Tracker software was then utilized to measure heart rate, quantified by the number of heart contractions within a 10 s interval.

2.7. Behavioural alteration on D. magna

2.7.1. Horizontal swimming

The influence of the four WSPs on the swimming abilities of *D. magna* was evaluated using the DanioVision™ video tracking system (Noldus IT, Wageningen, Netherlands). High-speed infrared cameras captured video data in three separate experiments, with a sampling rate of 30 frames *per* second.

For each experimental group, we utilized all specimens previously exposed to each treatment (10 specimens *per* group, 30 organisms for each treatment). Following exposure, each specimen was placed in an individual well of a 24-multiplate containing 3 mL of "*Daphnia* water" (refer to paragraph 2.1). After a 10 min acclimation period, they underwent two consecutive cycles of light and dark phases, each lasting 5 min. The light phase chosen had an intensity of 2200 lx, akin to measurements recorded from spring to summer on the surface of an oligotrophic lake [39], to better simulate the environmental conditions suitable for *D. magna*. Data were collected every 30 s, and various behavioural parameters were analyzed using EthoVision XT 11.5 software (Noldus IT, Wageningen, Netherlands).

In detail, we conducted measurements on various parameters related to the organism movement. Firstly, we determined the "distance moved," which refers to the distance covered by the organism from its previous position to its final one, expressed in mm. Next, we examined "acceleration", indicating bursts of rapid movement, calculated by dividing the difference in velocity (mm/s) by the time difference between the current and previous samples (s). Another parameter we assessed was "mean mobility", representing the percentage change in position (%) of the detected animal between the current and previous positions. This measure offers insight into the overall movement patterns exhibited by the organism. Additionally, we recorded "cumulative duration", which represents the total time (s) during which the animal occupied specific areas of the well, such as the center or boundaries. This measurement aimed to highlight any potential thigmotactic behaviour displayed by the organism. The distance moved and acceleration were analysed both as an average of the trend of all the specimens every 30 s, and as an average of the total movement of the specimens during the light and dark phases, to better identify responses to changes in light conditions. Only the averages of the total movement of the specimens during the light and dark phases were analysed for the other parameters (mobility and thigmotaxis).

2.7.2. Vertical migration

Considering *D. magna* ability to move vertically in the water column in response to changes in photoperiod, we evaluated the vertical migration and positive/negative phototaxis ratio of *D. magna* specimens. An experimental chamber was constructed by arranging nine cylindrical glass cuvettes (5 × 1; height x diameter), each containing one individual. Within each group, *D. magna* specimens previously exposed to a specific treatment were evenly distributed among the cuvettes, with 10 specimens *per* group and a total of 30 animals for each treatment. The cuvettes were filled with 3 mL of water without any tested contaminants. To provide light stimuli at an intensity of 2200 lx, a visible light LED lamp (4000 K) was positioned on top of the cuvettes, placed at a distance of 25 cm. The dark condition, with an intensity of 80 lx, was achieved by positioning the lamp at a distance of 2 m from the chamber, following the methodology outlined in our previous study [23]. Before video recording, the animals underwent a 10 min acclimation period. Specifically, the specimens were subjected to two cycles of 5 min in darkness (80 lx), followed by 5 min of light at an intensity of 2200 lx (measured using a HoldPeak 881d Digital lx meter positioned on top of the water column). Each experiment had a total duration of 20 min. Video tracking was performed using a Basler acA1300–60gm GigE camera, following the protocol outlined in Nigro et al. [23]. The camera recorded at 25 frames *per* s (fps), and the movement of each animal was analysed using EthoVision XT 12 video tracking software. Individual tracks were acquired every 30 s and were analysed using the EthoVision XT software (Noldus IT, Wageningen, Netherlands) to measure the "distance moved" by each animal. Additionally, considering the potential impact of certain chemicals on phototaxis [40], we also evaluated the negative phototaxis response of *D. magna* to light by analysing the "cumulative duration," which represents the total time (in seconds) during which the animal occupied the lower area of the vial. To conduct this analysis, the experimental chamber (the same one used for vertical migration assessment) was divided into two separate zones (lower = zone 1; upper = zone 2) using EthoVision XT 11.5 software. The distance moved was analysed both as the average trend of all the specimens every 30 s and as an average of the total movement. Meanwhile, the cumulative duration was represented only as the average of the total time spent in zone 1.

2.8. Statistical analysis

The STATISTICA 7.0 software was utilized to conduct all statistical analyses. For the behavioral parameter represented as the average trend of all specimens every 30 s (with treatment and time as variables), significant differences between treated and control groups were assessed using the two-way analysis of variance (two-way ANOVA) (Data shown in Table S1). In contrast, significant differences ($p \leq 0.05$) between treated and control groups for the averages of all behavioral parameters and for the analyses of vitality, heart rate, and biomarkers of neurotoxicity and energetic metabolism were determined using one-way ANOVA (with treatment as the variable) followed by the Bonferroni *post-hoc* test ($p \leq 0.05$). To compare the dose-response of each WSP, a simple regression analysis among the treatments was conducted to identify significant correlations ($p \leq 0.05$). Any outliers were identified and removed using Box and Whiskers plots. Factorial analysis through the principal component procedure was performed using all the measured endpoints and vitality for each of the tested WSPs to assess which biomarkers explain the variance of all used endpoints. Regarding proteomics, only proteins exhibiting an abundance ratio (AR) with a minimum 2-fold change compared to the control (<0.5 for down-regulated proteins and >2.0 for up-regulated proteins), and with a p-value < 0.01, were considered as modulated by the treatment.

3. Results

3.1. Acute toxicity

We observed significant ($F_{3,8} = 12.9000$; $p < 0.05$) differences in vitality compared to the control group, specifically in *D. magna* specimens exposed to 0.5 mg/L of PEG (Fig. S2). Consequently, chronic effects concerning this treatment group were not examined. However, for the other specimens, where no lethal effects were observed, all chronic effects were evaluated.

3.2. Proteomics

We identified 1123 different common proteins among the four WSP treatments and control groups. By applying two cut-offs based on 2-fold changes and statistically significant differences ($p < 0.01$) between treated and control samples, we pinpointed specific protein modulations induced by exposure to WSPs (Table S2). Specifically, exposure to 1 mg/L PAA led to the modulation of 69 proteins (52 up-regulated and 17 down-regulated) (Table S2; Fig. 1), constituting approximately 6% of the 1123 common proteins. Similarly, after exposure to 1 mg/L PVA, 86 proteins were found to be modulated (53 up-regulated and 33 down-regulated) (Table S2; Fig. 1), accounting for approximately 8% of the identified proteins. For 1 mg/L PEG, 64 proteins displayed modulations (51 up-regulated and 13 down-regulated) (Table S2; Fig. 1), representing 6% of the total. Lastly, exposure to 1 mg/L PVP resulted in the modulation of 52 proteins (25 up-regulated and 27 down-regulated) (Table S2; Fig. 1), accounting for 5% of the identified proteins. The Venn diagram (Fig. 2) illustrates the varying effects of these WSPs on *D. magna*, as only 14 proteins were modulated by all three WSPs (1.3% of the 1123 commonly identified proteins). In contrast, PAA selectively modulated 22 proteins, PVA affected 28 proteins, PEG altered the regulation of 7 specific proteins, and PVP influenced the expression of 12 proteins not shared with the other WSPs. Additionally, PEG and PAA demonstrated more proteins in common (13 out of the 14 mentioned) than the other WSPs, followed by PVA and PEG, which shared the modulation of 10 proteins, as PVA and PVP. Lastly, PAA and PVA shared 7 proteins, while PVP and PEG shared only 3.

Further analysis categorized the proteins modulated by the four WSPs based on their molecular function, utilizing the UniProt bioinformatics database (Fig. 3). It can be observed that proteins with catalytic, binding, transport, and ribosomal functions are predominantly modulated by all WSPs. PAA mainly modulated proteins involved in catalytic processes (39% of the total), followed by ribosomal proteins (13%), which represented the class of proteins most affected by PEG (31%), followed by proteins involved in binding and transport (25%) and catalytic functions (22%). Proteins involved in protein binding and

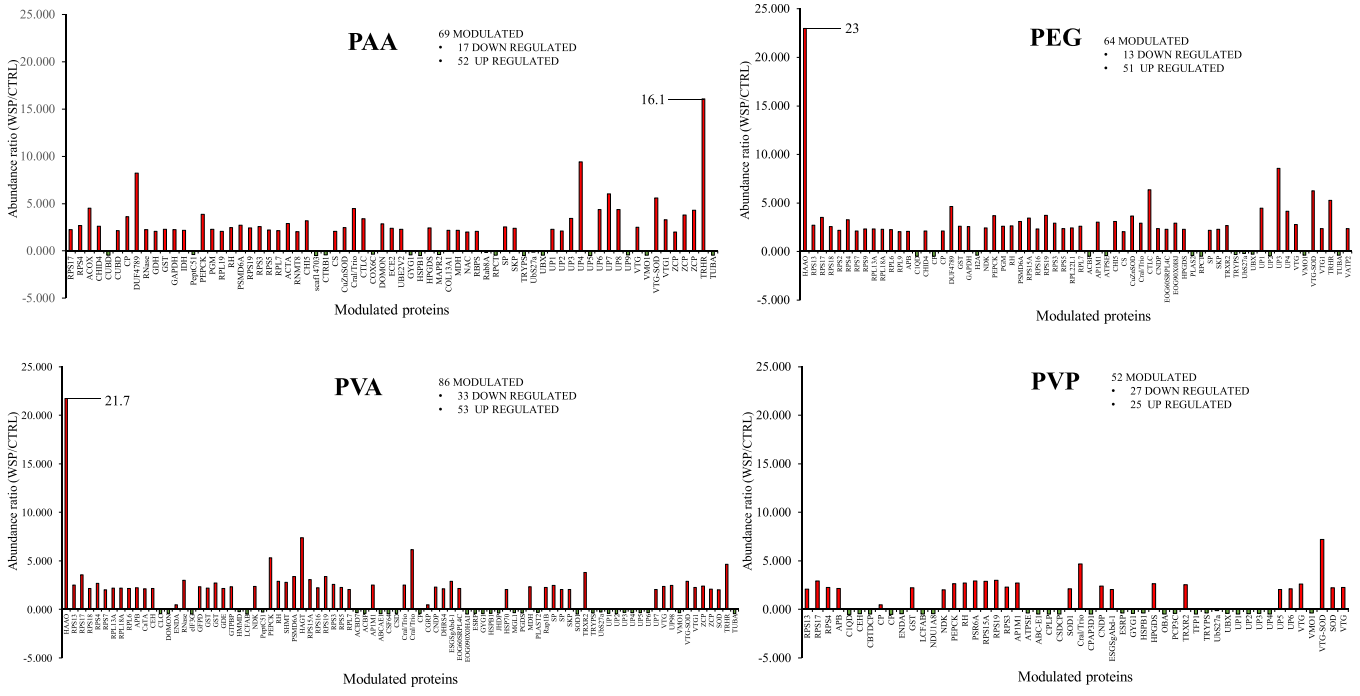


Fig. 1. Gel-free proteomics analysis results, Abundance ratio of modulated proteins by 1 mg/L of polyacrylic acid (PAA), polyethylene glycol (PEG), polyvinyl alcohol (PVA) and polyvinylpyrrolidone (PVP) compared to controls (red=up-regulation; green=down-regulation). Acronymous and the corresponding protein names are reported in the Tables S2.

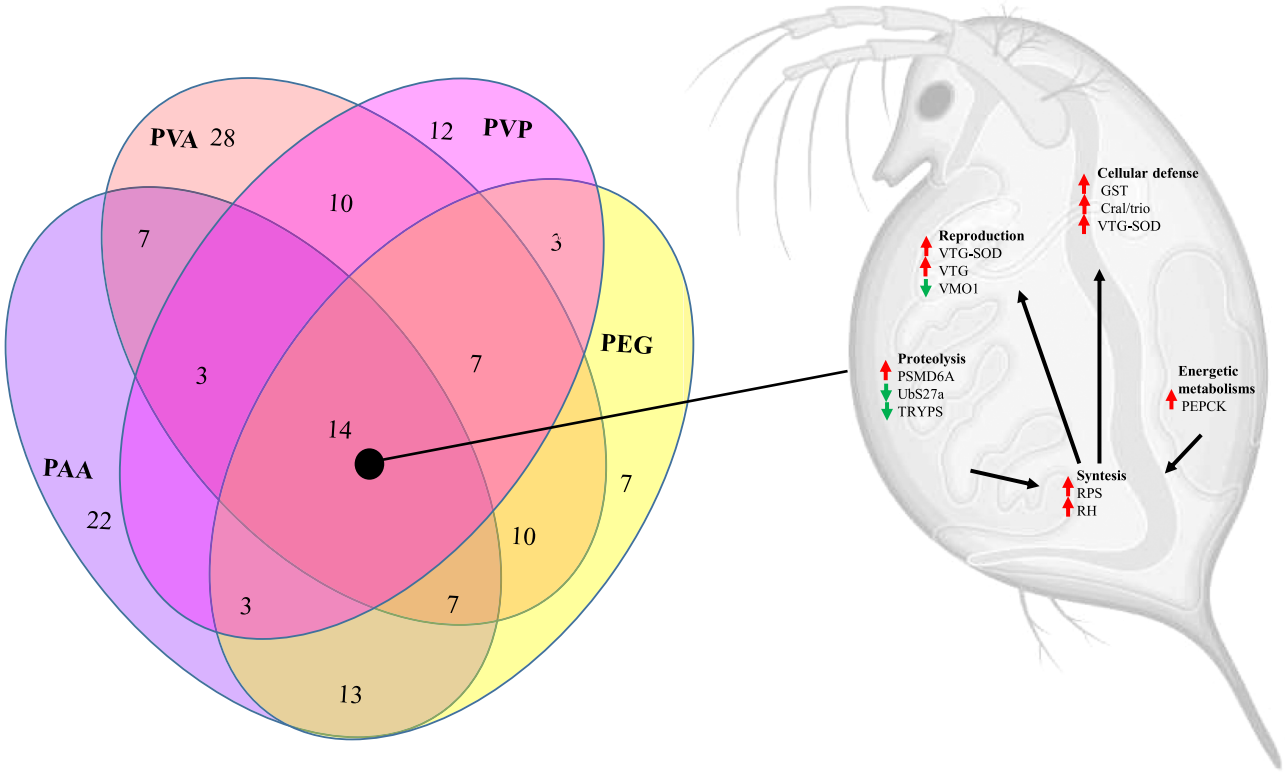


Fig. 2. Venns' diagram showing proteins modulated by individual Water-soluble polymer (WSP) at 1 mg/L of polyacrylic acid (PAA), polyethylene glycol (PEG), polyvinyl alcohol (PVA) and polyvinylpyrrolidone (PVP) in common with each other. On the right a representation of the common modulated proteins by each WSP in *D. magna* is reported (as described in Table S2). Acronymous correspond to the following protein names (compare also Tables S2): VTG-SOD –Vitellogenin fused with superoxide dismutase, VTG –Vitellogenin domain-containing protein, VMO1 –Vitelline membrane outer layer protein 1, PSMD6A –26 S protease regulatory subunit 6 A, UbS27a –Ubiquitin-40S ribosomal protein S27a, TRYPS – Trypsin serine protease, GST –, Cral/Trio – Cral/trio domain-containing protein, PEPCK–-phosphoenolpyruvate carboxykinase (GTP), RPS –ribosomal poteins, RH –RNA helicase.

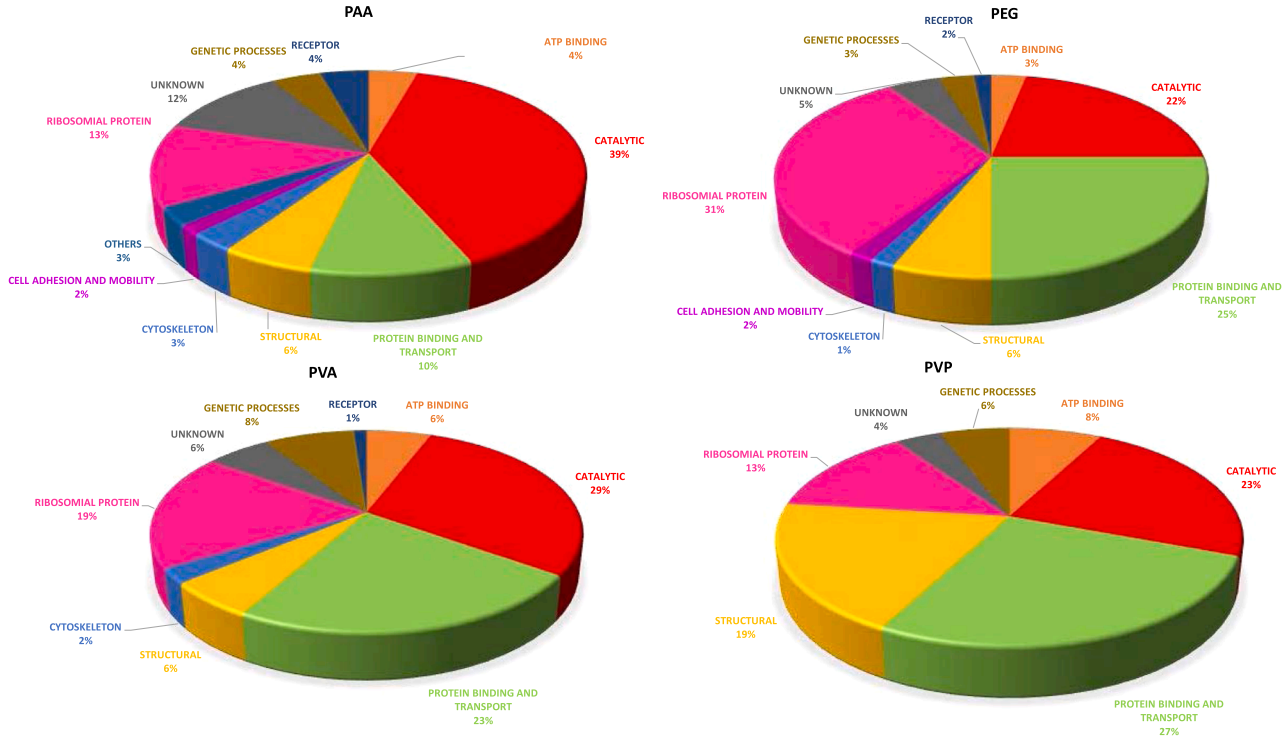


Fig. 3. Protein classes modulated by the four WSPs tested, polyacrylic acid (PAA), polyethylene glycol (PEG), polyvinyl alcohol (PVA) and polyvinylpyrrolidone (PVP), at 1 mg/L with their relative percentages.

transport accounted for the highest impacted group, at 27% after PVP exposure, followed by catalytic functions (23%), structural proteins (19%), and ribosomal proteins (13%). Similar to PAA, PVA predominantly impacted proteins involved in catalytic processes (29%), binding and transport (23%), and ribosomal proteins (19%).

3.3. Biomarkers

Regarding neurotoxicity enzymes, MAO activity remained unchanged following exposure to all concentrations of the WSPs, although a dose-dependent increase ($p < 0.05$) was observed in response to PAA exposure (Fig. 4A). Additionally, a significant increase in AChE activity was noted in specimens following exposure to all concentrations of PEG ($F_{2,5} = 13.2794$; $p < 0.05$) (Fig. 4B). As for GLY content, no differences were detected between treatment groups and the control (Fig. 4C).

3.4. Heart rate

The heart rate assay revealed significant alterations in heartbeat following exposure to all WSPs, as depicted in Fig. 5. Specifically, a notable increase in heart rate was observed after exposure to PAA ($F_{3,111} = 11.924$; $p < 0.05$) at a concentration of 0.5 mg/L, and after exposure to PVA ($F_{3,116} = 9.100$; $p < 0.05$) at concentrations of 0.5 mg/L and 1 mg/L. Similarly, a significant increase in heart rate was observed after exposure to PEG ($F_{2,80} = 6.294$; $p < 0.05$) at the highest concentration (1 mg/L), as well as after exposure to PVP ($F_{3,113} = 10.25$; $p < 0.05$) at all concentrations.

3.5. Behavioural alteration

3.5.1. Horizontal swimming

After exposure to all WSPs, the parameter of "distance moved" was affected for the average total distance moved by the specimens (Fig. 6B), while no differences were observed for the average trend of all specimens every 30 s (Table S1; Fig. 6A). Specifically, an increase in the distance travelled by *D. magna* specimens was observed during the dark phase following exposure to the highest concentration (1 mg/L) of PAA ($F_{3,99} = 10.15$; $p < 0.05$), PVA ($F_{3,106} = 3440$; $p < 0.05$), PVP ($F_{3,101} = 2.96$; $p < 0.05$), and after the highest concentrations (0.5 mg/L, 1 mg/L) of PEG ($F_{2,76} = 6.39$; $p < 0.05$) (Fig. 6B). No differences were detected during the light phases after exposure to all concentrations of the WSPs.

Similarly, concerning the parameter of "acceleration", significant differences compared to the control group were observed only when analysed as the average of the specimens during light and dark conditions (Fig. 7B). No differences were observed when analysed as the average trend of all specimens every 30 s (Table S1; Fig. 7A). Specifically, specimens were significantly affected by an increase in rapid movements during the dark condition after exposure to the highest concentration (1 mg/L) of PAA ($F_{3,92} = 11.99$; $p < 0.05$), PVA ($F_{3,100} = 3.49$; $p < 0.05$), PEG ($F_{2,73} = 12.72$; $p < 0.05$), and the middle concentration (0.5 mg/L) of PVP ($F_{3,96} = 2.87$; $p < 0.05$). However, a different pattern emerged during the light phases, with a significant decrease in rapid movement of the specimens ($F_{3,99} = 3.65$; $p < 0.05$) observed after exposure to the lower concentration of PAA (0.001 mg/L). Additionally, a significant decrease in the acceleration parameter was observed after exposure to the highest concentration (1 mg/L) of PEG ($F_{2,75} = 5.91$; $p < 0.05$), and after exposure to the lowest (0.001 mg/L) and highest (1 mg/L) concentrations of PVP ($F_{3,97} = 4.8013$; $p < 0.05$) compared to the control group (Fig. 7B). Similarly, the parameter of mobility (Fig. 8) was impacted during both light and dark conditions. During dark phases, specimens were affected after exposure to 1 mg/L PAA ($F_{3,97} = 12.74$; $p < 0.05$) and PEG ($F_{2,74} = 30.26$; $p < 0.05$), showing an increase in their mobility. Additionally, increased activity of the specimens was detected after exposure to lower concentrations (0.001 mg/L and 0.5 mg/L) of PVA ($F_{3,106} = 4.07$; $p < 0.05$) and PVP ($F_{3,99} = 13.18$; $p < 0.05$). During the light phases, this

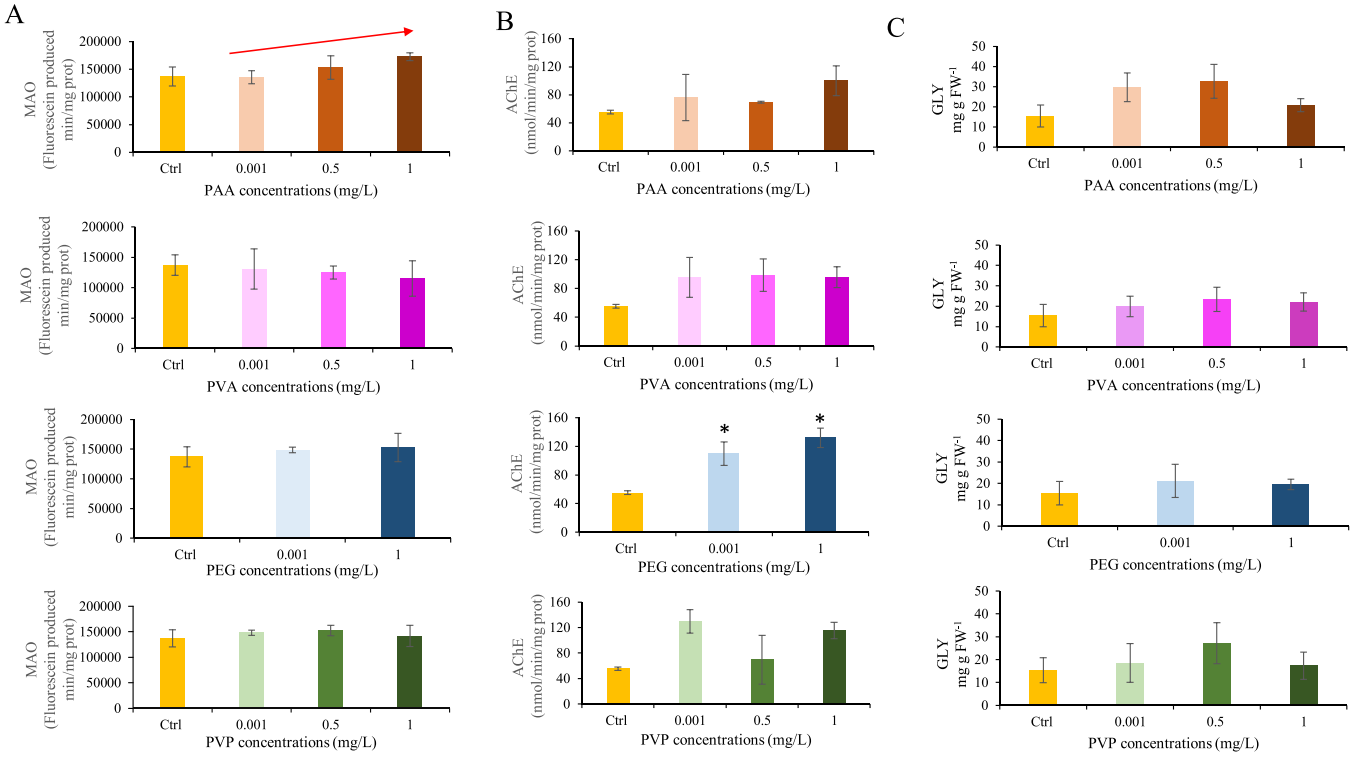


Fig. 4. Effects of different concentrations of polyacrylic acid (PAA), polyvinyl alcohol (PVA), polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP) on monoamine oxidase activity (MAO) **(A)**, acetylcholinesterase activity (AChE) **(B)**, and glycogen content (GLY) **(C)**. Exposure in triplicate N = 30 specimens *per* treatment. Data are expressed as mean ± SD. * means significant difference compared to control, one - way ANOVA ($p < 0.05$). Arrow indicates significant correlation between concentrations.

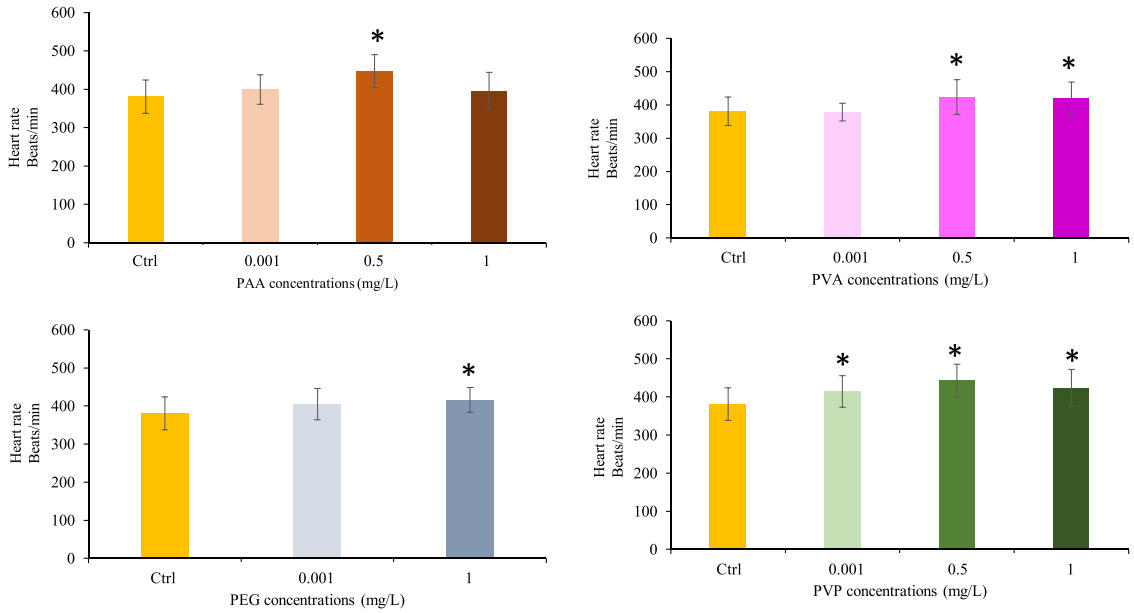


Fig. 5. Heart rate every min. *D. magna* exposed to different concentrations of polyacrylic acid (PAA), polyvinyl alcohol (PVA), polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP). Exposure in triplicate N = 30 specimens *per* treatment. Data are expressed as mean ± SD. * means significant difference compared to control, one - way ANOVA ($p < 0.05$).

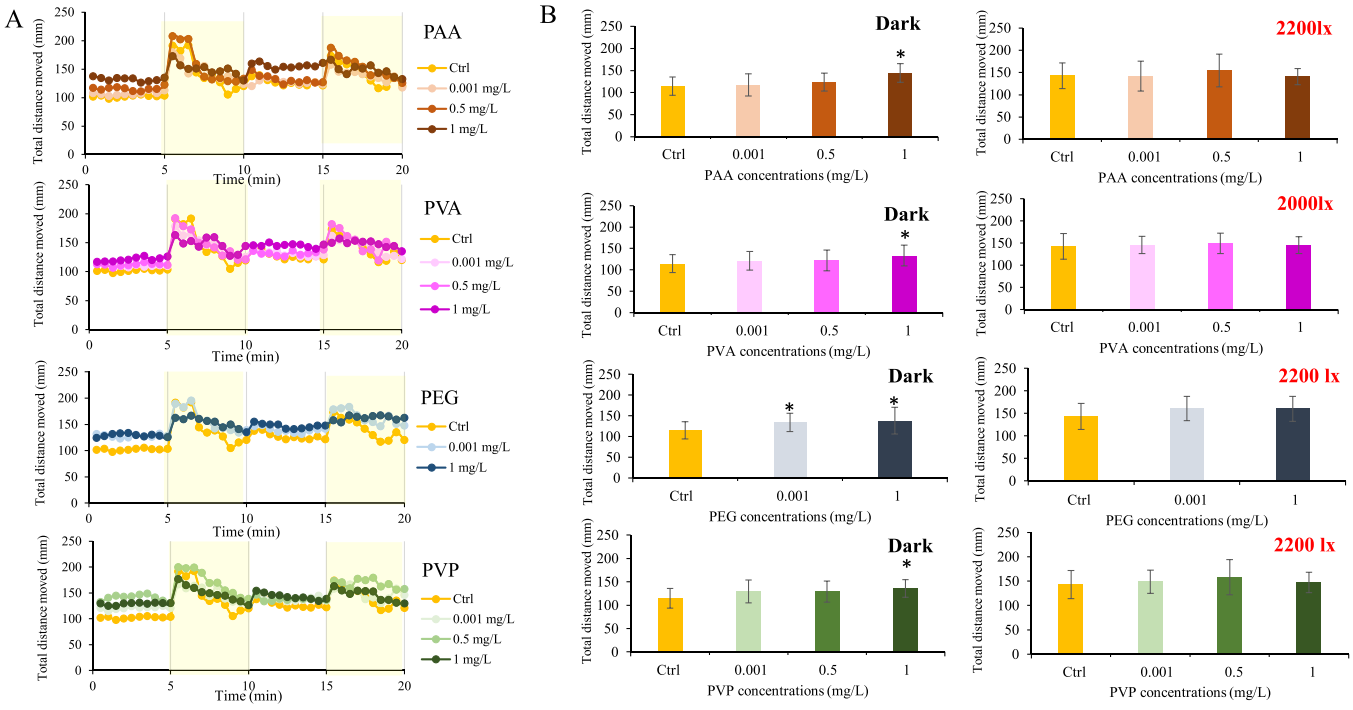


Fig. 6. Distance moved every 30 s **(A)** by *D. magna* exposed to different concentrations of polyacrylic acid (PAA), polyvinyl alcohol (PVA), polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP). Organisms undergone to two consecutive cycles of light/dark phases whose duration was 5 min each one. The light phase selected had an intensity of 2200 lx. Exposure in triplicate N = 30 specimens *per* treatment. Data are expressed as mean, two - way ANOVA ($p < 0.05$); the standard deviations (SDs) were removed from the graphs to increase the readability of presented results, see the Supplementary materials (SM). Average of the total distance moved **(B)** by *D. magna* exposed to different concentrations of PAA, PVA, PEG and PVP under both Dark and Light (2200 lx) conditions. Exposure in triplicate N = 30 specimens *per* treatment. Data are expressed as mean ± SD. * means significant difference compared to control, one - way ANOVA ($p < 0.05$).

parameter was impacted after exposure to the middle concentration of PAA (0.5 mg/L; $F_{3,96}$ =7.45; $p < 0.05$), exhibiting a decrease in this behavioural parameter. Similarly, a decrease in activity was observed after exposure to the lowest concentration of PEG (0.001 mg/L), while organisms exposed to 1 mg/L PEG showed an increase in their mobility ($F_{2,75}$ =25.13; $p < 0.05$), as in specimens exposed to the lowest concentration of PVP ($F_{3,102} = 7.49$; $p < 0.05$).

Regarding the analysis of cumulative duration, no significant differences were observed in the time spent in the centre or in the corner of the well for all tested concentrations (Fig. S3).

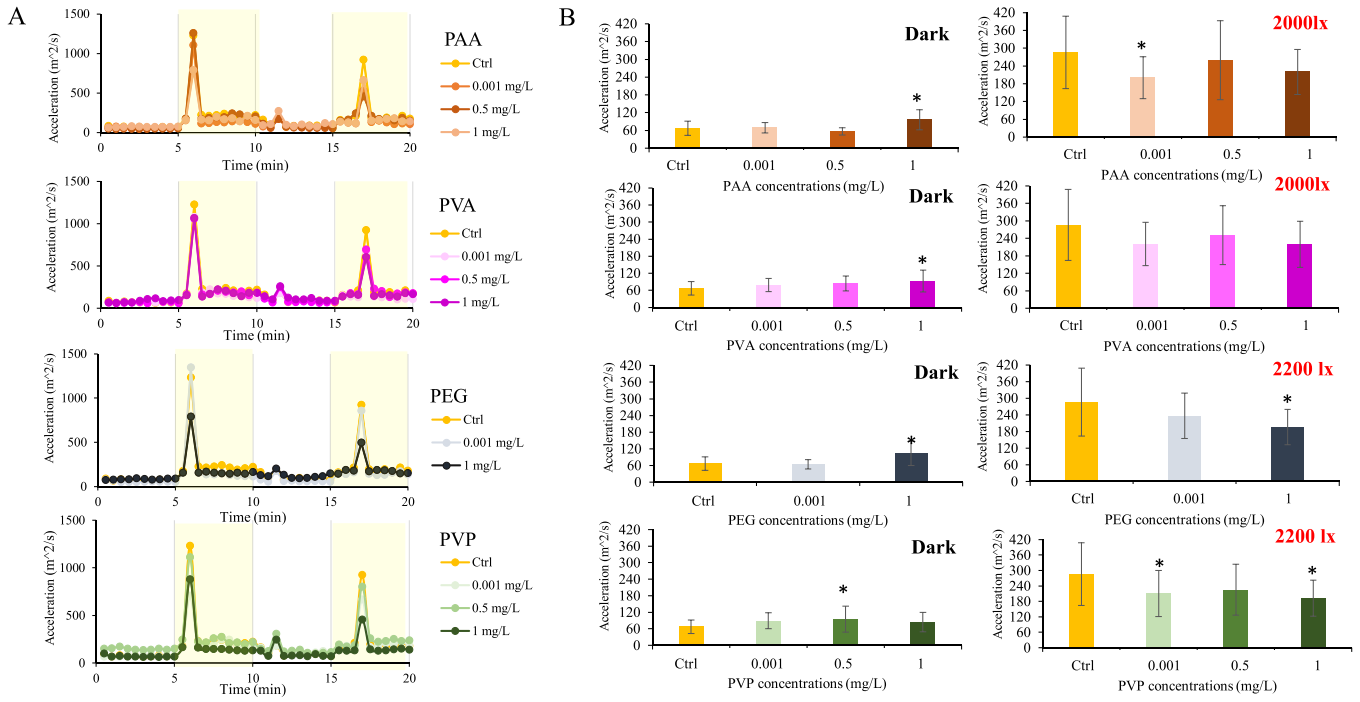


Fig. 7. Acceleration every 30 s **(A)** by *D. magna* exposed to different concentrations of polyacrylic acid (PAA), polyvinyl alcohol (PVA), polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP). Organisms undergone to two consecutive cycles of light/dark phases whose duration was 5 min each one. The light phase selected had an intensity of 2200 lx. Exposure in triplicate N = 30 specimens *per* treatment. Data are expressed as mean, two - way ANOVA ($p < 0.05$); the standard deviations (SDs) were removed from the graphs to increase the readability of presented results, see the Supplementary materials (SM). Average of the total acceleration **(B)** by *D. magna* exposed to different concentrations of PAA, PVA, PEG and PVP under both Dark and Light (2200 lx) conditions. Exposure in triplicate N = 30 specimens *per* treatment. Data are expressed as mean ± SD. * means significant difference compared to control, one - way ANOVA ($p < 0.05$).

3.5.2. *Vertical migration*

Regarding the analyses focused on the vertical migration of the specimens, a decrease in the total distance moved after exposure to all WSPs during the dark phase has been noted when analysed as the average of the total distance moved by the specimens (Fig. 9B). No differences were observed when analysed as the average trend of all specimens every 30 s (Table S1; Fig. 9A). Specifically, a decrease in the total distance travelled by specimens was observed after exposure to all concentrations of PAA ($F_{3,88}$ =10.03; $p < 0.05$), PVA ($F_{3,99}$ =12.83; $p < 0.05$), PEG ($F_{2,72}$ =28.21; $p < 0.05$), and the highest concentration (1 mg/L) of PVP ($F_{3,92}$ = 7.28; $p < 0.05$) (Fig. 9B).

Regarding the analysis of cumulative duration (representative of negative phototaxis), the time spent by the specimens in the lower zone (zone 1) of the vial was affected by exposure to PAA, PVA, and PEG, especially during the light phase. Specifically, an increase in the time spent in zone 1 during the light phase was observed after exposure to the highest concentrations (1 mg/L and 0.5 mg/L) of PAA ($F_{3,95}$ =4.55; $p < 0.05$) and after exposure to all concentrations of PVA ($F_{3,105}$ =7.30; $p < 0.05$). Conversely, focusing on exposure to PEG, an increase in specimens staying in the lower zone of the vial was observed during the dark phase after exposure to 1 mg/L ($F_{2,98}$ =8.20; $p < 0.05$) and after exposure to 0.001 mg/L during the light phase ($F_{2,99}$ =4.02; $p < 0.05$) (Fig. 10).

4. Discussion

This study is the first to investigate the effects of four commonly used WSPs at various levels of biological organization using *D. magna*, thereby providing a better understanding and refinement of their modes of action.

As a general observation, while all the WSPs affected the proteome, physiological, and behavioural parameters of *D. magna*, only PEG exhibited acute toxicity at 0.5 mg/L (Fig. S2). We lack sufficient information to explain the significant mortality induced at this intermediate concentration, which was even tested twice to confirm this apparent anomaly. It is plausible that PEG toxicity could follow a *non*-monotonic dose-response slope [41], as also observed to a *non*-significant extent for PVA and PVP (Fig. S2). Alternatively, since we observed a deposit of algae provided as food at the bottom of the beakers containing *D. magna* specimens exposed solely to this PEG concentration, without any changes in pH or oxygen parameters, we can suggest a possible decrease in food availability due to specific properties of PEG at this concentration, such as gelation [42], and its consequent effect on specimen vitality. It will certainly be very interesting to explain this specific and unexpected effect in future studies.

We will now discuss the results obtained after exposure to the four WSPs, starting from the lowest level of biological organization (molecular) and progressing through cellular and physiological effects, up to those at the organismic level.

4.1. *Proteomics*

Proteomics has emerged as a powerful tool in the field of ecotoxicology, enabling elucidation of protein expression patterns and providing a deeper understanding of how natural and human-induced stress factors can impact cellular pathways and functions [43].

If we focus on the quantitative aspect of the protein alterations caused by the four selected WSPs, it becomes evident that PVA had the most significant effect on the *D. magna* proteome. Notably, exposure to 1 mg/L of PVA resulted in changes in approximately 8% of the commonly identified proteins, followed by PAA and PEG, which exhibited a modulation of 6%, while PVP had the least impact, influencing 5% of the identified proteins in common (Table S2). This finding is interesting, as in our previous study [25], PVP emerged as the most effective WSP on *D. rerio* larvae, while PAA had the least impact [24,25]. Therefore, our current findings reveal a contrasting response between

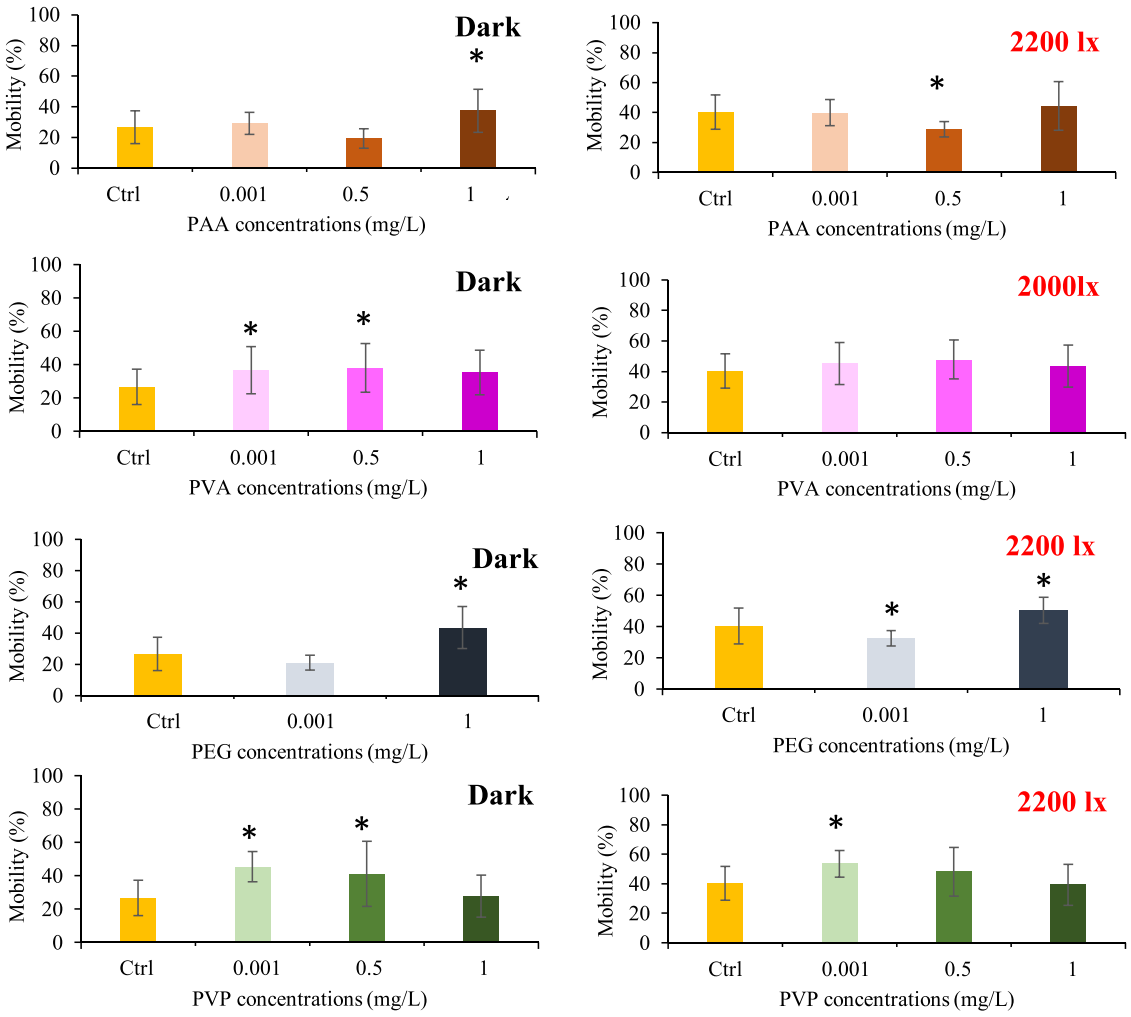


Fig. 8. Mobility of *D. magna* exposed to different concentrations of polyacrylic acid (PAA), polyvinyl alcohol (PVA), polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP) under both Dark and Light (2200 lx) conditions. Exposure in triplicate N = 30 specimens *per* treatment. Data are expressed as mean ± SD. * means significant difference compared to control, one - way ANOVA ($p < 0.05$).

these vertebrate and invertebrate models, underscoring the validity of our initial hypothesis, which involves comparing two different biological models by exposing them to the same concentrations of contaminants and measuring the same endpoints. Furthermore, Binelli et al. [25] demonstrated that after exposure to the same concentrations of PVP, PEG, and PAA, most proteins were up-regulated, suggesting an organismal response to the administration of such contaminants and the onset of the general adaptation syndrome (GAS). However, in the present study, protein modulation varies greatly, with the highest number of down-regulated proteins observed only after PVP exposure, albeit with a percentage very similar to that of up-regulated ones (27 down-regulated compared to 25 up-regulated; Fig. 1). This indicates that the invertebrate model may not be capable of counteracting the action of these WSPs but rather passively suffers their effects.

Similarly, focusing on the qualitative analysis, our previous study indicated that exposure to PEG and PVP mainly affected *D. rerio* proteins involved in genetic processes, whereas PAA exposure led to changes in transport and binding proteins [25]. In contrast, the present study revealed that many modulated proteins following exposure to PEG and PVP are linked to binding and transport processes, whereas after exposure to PAA, most of the modulated proteins are involved in catalytic activities (Fig. 3). The only common feature between the two proteomic analyses conducted with the same WSPs administered at identical concentrations to *D. magna* and *D. rerio* is that the modulated proteins do not belong to common cellular pathways, as indicated by the protein-protein interaction network functional enrichment analysis conducted using the STRING© freeware.

Moving on to the qualitative examination of the proteomic dataset obtained in the present study, it is possible to identify the categories of proteins that were modulated by the three WSPs and their respective relationships. For instance, of the 14 proteins in common among the three WSPs (Fig. 2), most belong to structural constituent of ribosome proteins (36%), followed by proteins involved in binding and transport (22%), or catalytic processes (22%) (Table S2). Notably, within this last category, it is interesting to observe that the phase II detoxifying enzyme glutathione S-transferase (GST) showed up-regulation (Fig. 2; Table S2) after exposure to all WSPs. Previous studies have observed how the up-regulation of GST plays a key role in the response to stress in various organisms, including *D. magna* [44,45]. Specifically, this enzyme is responsible for catalysing the binding of glutathione (GSH) to a wide range of exogenous compounds, serving as a crucial step in the cellular defence system against oxidative stress and toxic chemicals [46,47]. Consistent with these results, specimens exposed to 1 mg/L PAA also showed up-regulation of isocitrate dehydrogenase, an enzyme involved in NADPH synthesis, which is implicated in reducing glutathione disulfide to GSH for antioxidant purposes. Concurrently, a broader picture emerges when considering other up-regulated proteins involved in responding to stressors that are common among the WSPs. For instance, there is reported up-regulation of the cral/trio domain-containing proteins (Cral/trio) (Fig. 2; Table S2), which have been identified in

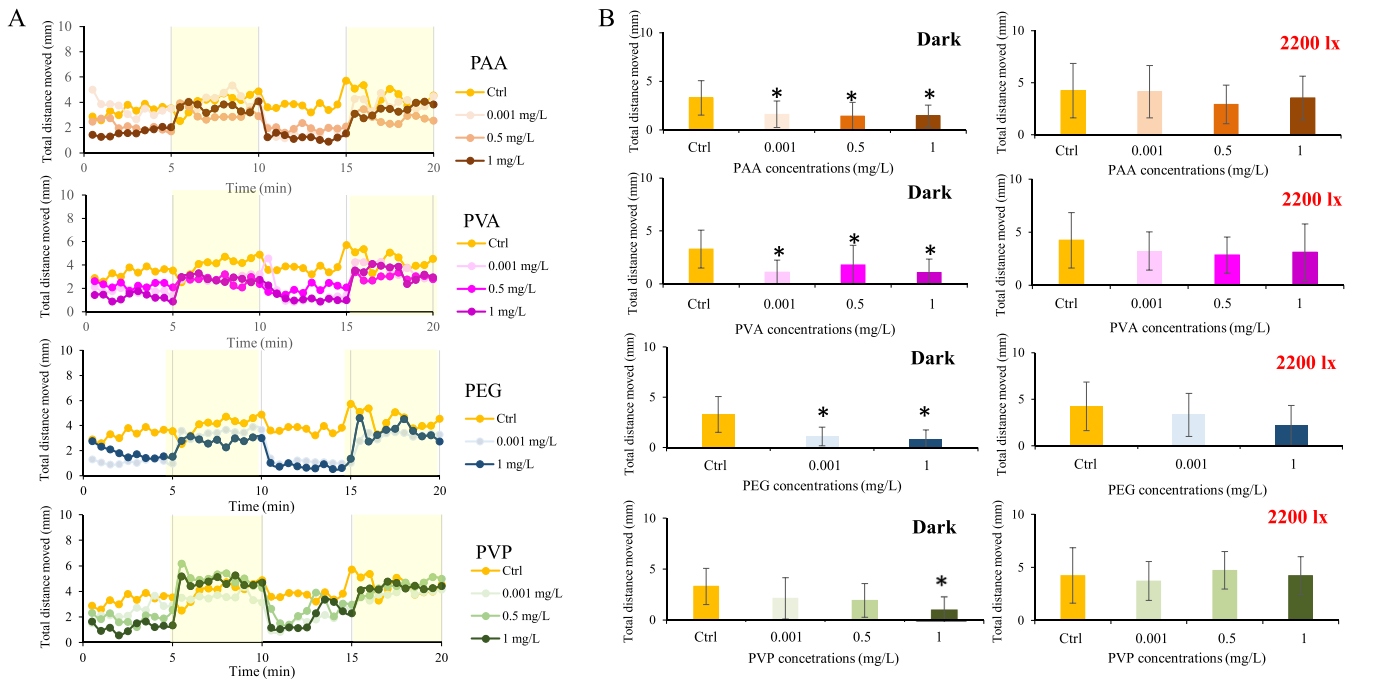


Fig. 9. Vertical migration every 30 s **(A)** by *D. magna* exposed to three different concentrations of polyacrylic acid (PAA), polyvinyl alcohol (PVA), polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP). Organisms undergone to two consecutive cycles of light/dark phases whose duration was 5 min each one. The light phase selected had an intensity of 2200 lx. Exposure in triplicate N = 30 specimens *per* treatment. Data are expressed as mean, two - way ANOVA ($p < 0.05$); the standard deviations (SDs) were removed from the graphs to increase the readability of presented results, see the Supplementary materials (SM). Average of the total vertical migration **(B)** by *D. magna* exposed to three different concentrations of PAA, PVA, PEG and PVP under both Dark and Light (2200 lx)conditions. Exposure in triplicate N = 30 specimens *per* treatment. Data are expressed as mean ± SD. * means significant difference compared to control, one - way ANOVA ($p < 0.05$).

network modules involved in early transcriptional responses to biotic stressors in *D. magna* [48]. Additionally, an increase was observed in vitellogenin fused with superoxide dismutase (VTG-SOD) (Fig. 2; Table S2), which plays a key role in stress responses and has been observed in daphnids under specific stress conditions [44, 49–51]. Specifically, the up-regulation of VTG-SOD may indicate the production of specialized structures, such as ephippia, as an emergency response to stress, which may enhance stress tolerance and ensure successful reproduction in challenging conditions [50,52]. Moreover, an up-regulation of vitellogenin domain-containing protein (VTG) was also observed (Fig. 2; Table S2). VTG, similar to VTG-SOD, plays a key role in providing energy to developing offspring in oviparous organisms [53–56]. Since vitellogenin maturation is a highly regulated process influenced by stressors and developmental stages [50,57], variations in the maturation process could be used as valuable indicators of toxic stress [51]. Indeed, VTG transcription has been used in several studies to assess the impact of chemical stressors on the reproductive system of *D. magna* [58,59]. Consistent with these findings, an impact on the reproductive system [60] or the development of *D. magna* [61] can also be suggested by the alteration in the expression of vitelline membrane outer layer protein 1 (VMO1), which appeared down-regulated after exposure to all the WSPs (Fig. 2; Table S2). In crustaceans, VMO1 proteins are indeed the primary component of the outer layer of the vitelline membrane of eggs and play a crucial role in preventing the mixing of yolk and albumen [62].

These results underscore the intricate interactions among these altered proteins in response to WSP exposure. This interplay involves a multifaceted stress response that not only protects against oxidative stress but also enhances overall resilience to this class of contaminants. Consistent with these findings, we also observed an up-regulation of the ATP-dependent RNA helicase (RH) (Fig. 2; Table S2), which is involved in multiple cellular functions such as transcription, ribosomal RNA biogenesis, and RNA export. In previous studies, RH has been linked to promoting the cell cycle in *D. magna*, leading to earlier maturation and increased offspring production in response to stressors such as the presence of predators [63]. The up-regulation of RH and vitellogenin-related proteins, as previously described, is consistent with the observed up-regulation of certain ribosomal structural proteins (40 S ribosomal protein S17, 40 S ribosomal protein S19, 40 S ribosomal protein S3, and 40 S ribosomal protein S4) (Fig. 2; Table S2). This up-regulation may be associated with increased protein synthesis, necessary for the production of yolk proteins and the allocation of more resources for reproduction in response to stressors [64]. This hypothesis finds support in the up-regulation of phosphoenolpyruvate carboxykinase (PEPCK) (Fig. 2; Table S2), presumably to generate additional ATP and GTP [65], and in the up-regulation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), observed only after exposure to PAA and PEG, indicating enhanced energy synthesis. Furthermore, the down-regulation of trypsin serine protease (TRYPS) and the consequent reduction in protein digestion, which may lead to increased proteasome degradation activity, could indirectly result in alterations in free amino acid content [66,67]. This alteration may be linked to the aforementioned need for energy biosynthesis. The induction of stress conditions mediated by exposure to WSPs is further underscored by the up-regulation of the 26 S protease regulatory subunit 6 A (PSMD6A) (Fig. 2; Table S2), a component of the 26 S proteasome, a multiprotein complex involved in the ATP-dependent degradation of ubiquitinated proteins. Fischer et al. [68] demonstrated that proteins damaged by oxidation due to ROS must either be repaired or degraded through the ubiquitin-proteasome pathway and replaced with newly synthesized proteins. Moreover, proteins associated with immune-related and proteolysis functions were down-regulated, such as Ubiquitin-40S ribosomal protein S27 (UbS27a) and TRYPS (Fig. 2; Table S2), indicating a disruption in pathways regulating immunological functions such as the cell cycle, protein digestion, and apoptosis. Indeed, these protein-degrading enzymes play diverse roles in various biological functions including digestion, immune responses, reproduction, and post-translational protein modifications [69].

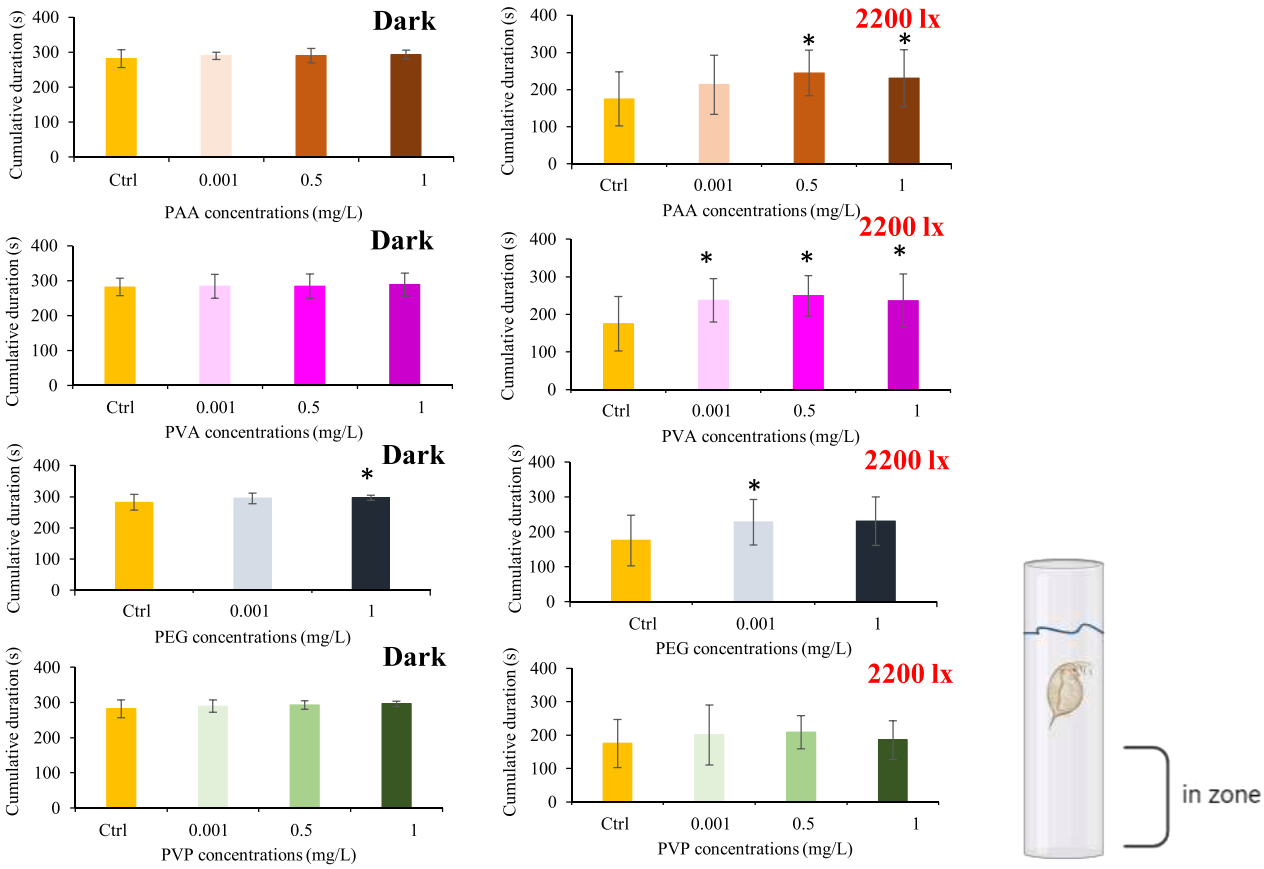


Fig. 10. Negative Phototaxis, expressed as the time spent (s) by the specimens in the lower zone of the vial, of *D.magna* exposed to three different concentrations of polyacrylic acid (PAA), polyvinyl alcohol (PVA), polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP) under both Dark and Light (2200 lx) conditions. Exposure in triplicate N = 30 specimens *per* treatment. Data are expressed as mean ± SD. * means significant difference compared to control, one - way ANOVA ($p < 0.05$).

Lastly, an increase in metabolic proteins involved in the biotransformation of xenobiotics, such as carboxypeptidase (ZCP), up-regulated after exposure to PAA and PVA, and carboxylic ester hydrolase (CEH), up-regulated after exposure to PVA and PVP, suggests that *D. magna* specimens perceive WSPs as potentially hazardous xenobiotics [70–72].

These data concerning modulated proteins suggest a specific effect of the tested WSPs on *D. magna*, evidenced by alterations in energy allocation processes and various cellular functions, including catalytic processes, digestion, immune responses, reproduction, and potential developmental variations. Comparing these results with the dataset previously obtained from *D. rerio* larvae, it becomes apparent that the effects of the tested WSPs affected a more diverse range of cellular functions in *D. magna*. Conversely, the three WSPs selectively up-regulated different proteins primarily involved in *D. rerio* embryogenesis [25]. This disparity could be also attributed to the different life cycle stages of the two biological models used, with adults for *D. magna* and larvae for *D. rerio*.

4.2. Biomarkers and heart rate

Biomarkers were found to be the least sensitive class of endpoints to exposure to the selected WSPs, at least based on our choice. For instance, our observations regarding GLY content indicated no significant changes, suggesting that the specimens likely utilized alternative energy resources (Fig. 4C). Similarly to our previous studies [23,25], exposure to PAA, PVP, and PEG did not affect the activity of MAO (Fig. 4A) and AChE. However, it is important to note that *D. magna* possesses several neurotransmitter systems that can be targeted by xenobiotics, and these systems might have been influenced by the WSPs. This includes serotonin, dopamine, epinephrine, and the GABA receptor signaling pathways [73–78]. Nevertheless, a significant increase in AChE activity was detected in specimens exposed to 0.001 mg/L and 1 mg/L of PEG (Fig. 4B). Similarly, Nascimento et al. [18] demonstrated that 5 and 10 mg/L of PEG induced an increase in the activity of this enzyme in the tadpole *Physalaemus cuvieri*, hypothesizing that the enhancements in the enzymatic activity profile could possibly result from the attachment of PEG to AChE molecular structures. Based on this evidence, future studies may consider other more sensitive endpoints, such as energy reserve biomarkers (e.g., mitochondrial electron transport activity), as well as the expression of neurotransmitters involved in both heart rate and swimming (e.g., quantification of serotonin and dopamine through Enzyme-Linked Immunosorbent Assay - ELISA; [33,79]).

Unlike the measured biochemical endpoints, heart rate proved to be a reliable measure for assessing the physiological effects of WSP exposure on *D. magna*. Indeed, we observed a significant increase ($p < 0.05$) after exposure to all WSPs (Fig. 5). Specifically, PVP emerged as the most effective interfering agent, significantly increasing heartbeats at all tested concentrations, while exposure to PVA resulted in a significant increase only for the two highest concentrations. In contrast, PEG and PAA were the two WSPs that significantly increased the heart rate of *D. magna* only for one of the three tested concentrations (Fig. 5). This effect of exposure to various toxicants has been observed in previous studies [74,80] and has been identified as an important and sensitive physiological indicator [81] reflecting pollutant levels in the blood circulation system of *D. magna* [37].

The observed variations in heart rate align with the proteomic data discussed earlier, suggesting possible alterations in metabolic pathways, such as increased oxygen demand and compensatory hyperventilation,

which require an increase in heart rate [82]. This reflects a shift in energy demand and allocation costs, as indicated by proteomics. Such an increase in heart rate can have serious ecological consequences, affecting prey/predator relationships and the ability to feed or seek shelter, as the surplus energy expended cannot be used for these fundamental survival activities of *D. magna*. It is noteworthy that Mondellini et al. [16] did not observe any variation in heart rate in *D. magna* specimens exposed to higher concentrations of the same WSPs, but for a significantly shorter time (48 h) than in our study. This suggests a crucial influence of exposure time on homeostatic mechanisms against exposure to these emerging pollutants, as expected.

4.3. Behavioural effects

The last group of endpoints analysed pertains to parameters associated with potential changes in the swimming behaviour of *D. magna*, representing apical effects that summarize the potential alterations occurring at molecular, cellular, and physiological levels described above [83, 84, 24]. The analysis of swimming activity is considered a novel methodological approach for toxicity evaluation, as it reflects motility performance and locomotor behaviour, crucial in the feeding, social, and defensive activities of this model organism throughout its entire life cycle [84,85]. The major challenge in simulating the two- or three-dimensional movements of these crustaceans in the laboratory lies in the experimental simplicity, which overlooks various environmental factors that can influence the effects of administered compounds and modify the swimming reactions of the organisms, such as microbial activity, photodegradation, thermal stratification, turbidity, and wind conditions [85].

Given the complexity of *D. magna* swimming behaviour, characterized by several parameters, we analysed numerous locomotor endpoints. Overall, the highest effects were observed after exposure to the highest concentration of all the WSPs, although PAA exhibited the greatest impact on locomotor performance (Table 1). Moving to the analysed swimming parameters, we assessed the distance travelled by the specimens, a robust indicator of potential alterations in the behavioural patterns of *D. magna* in response to the presence of contaminants [84]. This measure enables the evaluation of locomotor activity, reflecting variations in agility, vitality, and responsiveness to external stimuli. Our findings revealed an increase in the distance covered by the organisms following exposure to all the WSPs at a concentration of 1 mg/L. Changes in swimming activity are generally associated with alterations in filter-feeding activity and food uptake [86], as well as the presence of particles on the body appendages. Similarly, Lover et al. [80] hypothesized that the increased movement of *D. magna* after exposure to contaminants could be attributed to altered feeding responses, toxin sensing, interference with sensory abilities, or attempts to clean or rid themselves of perceived contaminants. These assumptions align with our hypothesis, as the shift in energy demand and allocation costs could be interpreted as an adaptive response to conserve or utilize energy more effectively in response to WSP exposure. The alteration in the distance moved might also reflect changes in mobility, which was increased under dark conditions (Fig. 8). This finding is consistent with previous explanations, as increased appendage rates might suggest an attempt to create currents for feeding activity or eliminate perceived toxins. The increased activity could also indicate an attempt to remove the perceived contaminants from their appendages [80]. Furthermore, we investigated the parameter of acceleration. Indeed, *D. magna* exhibit a distinct movement pattern known as "hops," propelled by the rhythmic beating of their second antennae pair [87], and their motion is characterized by intermittent acceleration and deceleration [85]. The main results highlighted that the specimens showed a decrease in their acceleration pattern during the light phases (Fig. 7; Table 1) and an increase under dark conditions (Fig. 7; Table 1). Since acceleration can be considered a part of the phototactic response mechanism in wildlife [88], the reduction of this parameter under light conditions due to WSP exposures could suggest an increased chance of *D. magna* predation [40].

We also observed an alteration in the distribution of the organisms in the water column and in their negative phototactic behaviour, two of the main ecotoxicological effects that can indicate possible neurotoxic

Table 1
Behavioural parameters affected by the exposure of different concentrations of polyacrylic acid (PAA), polyethylene glycol (PEG), polyvinyl alcohol (PVA) and polyvinylpyrrolidone (PVP) on *D. magna*.

			HORIZONTAL DISTANCE MOVED	VERTICAL DISTANCE MOVED	MOBILITY	ACCELERATION	THIGMOTAXIS	PHOTOTAXIS
	mg/L		mm	mm	%	m^2/s	s	s
PAA	0.001	Dark		*				
		2200 lx				*		
	0.5	Dark		*				
		2200 lx			*			*
	1	Dark	*	*	*	*		
		2200 lx						*
PVA	0.001	Dark		*	*			
		2200 lx						*
	0.5	Dark		*	*			
		2200 lx						*
	1	Dark	*	*		*		
		2200 lx						*
PEG	0.001	Dark	*	*				
		2200 lx			*			*
	0.5	Dark	-	-	-	-	-	-
		2200 lx	-	-	-	-	-	-
	1	Dark	*	*	*	*		*
		2200 lx			*	*		
PVP	0.001	Dark			*			
		2200 lx			*	*		
	0.5	Dark			*	*		
		2200 lx						
	1	Dark	*	*				
		2200 lx				*		

- = not measured for mortality
*=significative difference of the treatment in comparison to the control under the same condition of light or dark

effects potentially leading to organism disorientation [89]. The distribution of organisms in the water column is influenced by various factors, including their response to light (phototactic migration), predation, and temperature [90–92]. The analysis of vertical variations in the swimming behaviour of *D. magna* is certainly an endpoint that more closely reflects an ecological situation, as an exclusively two-dimensional approach tends to limit organism movement to the horizontal plane, given that observation dishes contain shallow water levels [85].

In this study, specimens exposed to all the WSPs exhibited a significant ($p < 0.05$) decrease in vertical migration only under dark conditions (Fig. 9; Table 1), but a significant increase in the horizontal distance moved during the same phases (Fig. 6; Table 1), indicating an elevation in lateral movement with a corresponding reduction in vertical migration of *D. magna* exposed to WSPs. Bownik et al. [85] demonstrated that a decrease in the ratio between vertical and horizontal swimming of *D. magna* exposed to toxicants may imply energy depletion and/or metabolic disorders, as detected by the proteomic approach described previously. This identified relationship is crucial, as it confirms how a molecular effect of the administered contaminants, which modulated the use of *D. magna* energy budget, then influences swimming behaviour at the apical level in the tested biological model.

However, one of the most ecologically relevant swimming behaviours in *D. magna* is phototaxis, which can be positive, negative, or intermediate, depending on habitat characteristics, predation pressure, and the specific clone [93,94]. Negative phototaxis is directly associated with daily vertical migration along the water column, which helps prevent predation during daylight [93,94]. While negative phototaxis is a natural response to changing light conditions, it may be altered by toxicants [40, 93, 94]. In this study, we also observed an alteration in phototaxis, noting how the organisms remained in the lower part of the vial, farther away from the light (Fig. 10; Table 1). This alteration in phototaxis may be attributed to neurotoxic effects resulting from WSP exposure or could also be influenced by depleted energy reserves and the consequent impaired swimming ability [95]. Our dataset appears to exclude the possibility that this effect is solely due to the neurotoxic activity of WSPs, as we did not detect alterations in either MAO or AChE activities, except for an increase in the latter only for PEG, as previously described. Conversely, proteomics data seems to support the latter hypothesis, as we precisely detected changes in energy allocation processes (see paragraph 4.1).

In conclusion, we suggest that all the observed changes in the swimming behaviour of *D. magna* following exposure to WSPs appear to result from alterations in the utilization of the energy budget, which could be attributed to cellular metabolic modifications or the necessity for organisms to allocate energy to counteract the entry of such contaminants, as indicated by the increase in heart rate.

In this context, through the factorial analysis (Fig. 11), the endpoints with the factorial weight > 70% were mobility (in both light and dark conditions), negatively correlated with each other, and vertical migration (only in dark conditions), positively correlated. Therefore, the 70% of the data was explained by 3 of the 15 different endpoints (considering as separated endpoints the dark and light conditions), suggesting that the behavioural parameters are the pivotal impacted endpoints by the WSP treatments.

4.4. Final remarks

This study employed an integrated approach to delineate the impact of four WSPs across different biological levels of *D. magna*. Through the selected endpoints, this study successfully correlated molecular effects to observable behavioural alterations, thereby revealing the underlying modes of action of the four WSPs. The findings indicate that WSP exposure, while resulting in minimal acute toxicity, leads to significant sub-lethal effects that could potentially affect the ecological fitness and survival of this freshwater model organism. Indeed, our findings underscore a significant impact of WSPs on *D. magna* across proteomic, physiological, and behavioural parameters.

The modulation of specific proteins related to stress responses, oxidative defence mechanisms, and energy allocation processes highlights a multifaceted effect due to these contaminants. Moreover,

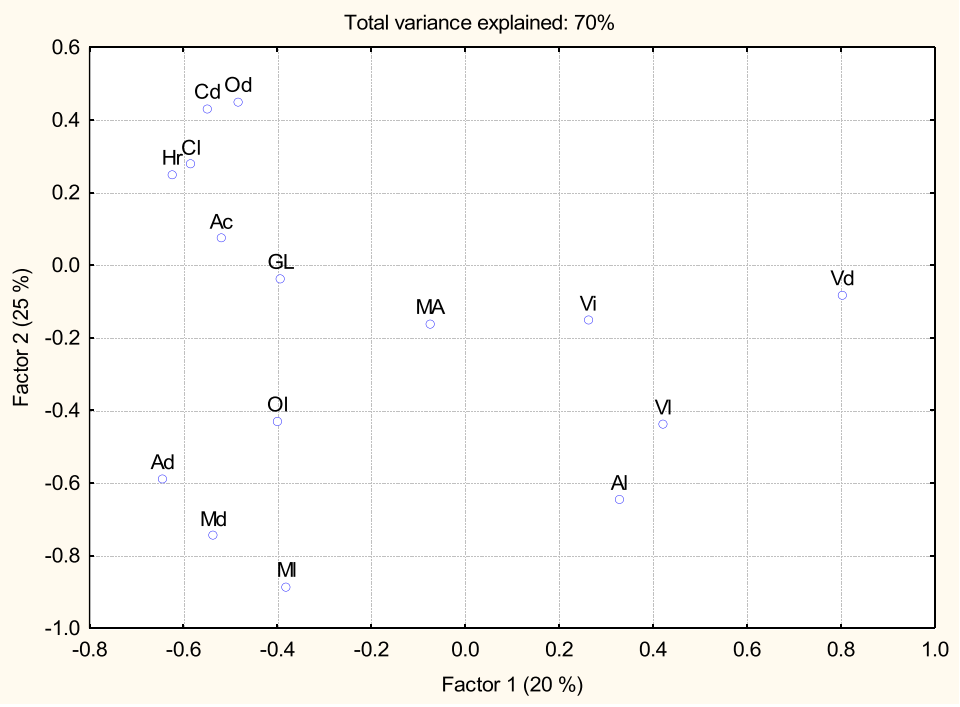


Fig. 11. Factorial analysis using all biomarkers, behavioural endpoints and vitality: AChE (Ac), MAO (MA), GLY (GL), Heart rate (Hr), Mobility (dark; Md), Mobility (light; Ml), Orizontal distance moved (dark; Od), Orizontal distance moved (light; Ol), Acceleration (dark; Ad), Acceleration (light; Al), Vertical distance moved (dark; Vd), Vertical distance moved (light; Vl), Cumulative duration (dark; Cd), Cumulative duration (light; Cl), Vitality (Vi).

changes in heart rate aligned with proteomic data, indicating potential shifts in metabolic pathways and energy allocation, further reinforcing the relationship between molecular responses and physiological outcomes. These changes could signify an increase in stress on the organism metabolic systems, requiring higher energy expenditures to maintain homeostasis or counteract the effects of these contaminants. Along the same lines, behavioural assessments highlighted alterations in swimming patterns, suggesting adaptive changes due to energy depletion, corroborating findings from proteomic and physiological analyses. The observed behaviours, such as increased horizontal movement, decreased vertical migration under dark conditions, and altered phototaxis, could have substantial ecological implications due to their roles in feeding, mating, and predator avoidance.

Table 2 shows the comparison between the results obtained in *D. magna* and *D. rerio* for the common measured endpoints. It can be observed that the responses are quite similar between the two biological models, with a greater effect of PEG on the invertebrate compared to the *D. rerio* larvae, where instead this WSP was the one that determined the lesser effects. However, if we consider the results obtained through the proteomic approach, it is possible to observe numerous differences, as PVP was the compound that modulated the majority of proteins in *D. rerio*, but the least number in *D. magna* (Table 2). Furthermore, despite no specific cellular pathway being detected as predominantly impacted by the tested WSPs in either of the two biological models (see paragraph 4.1), the majority of modulated proteins in *D. rerio* were up-regulated, suggesting a sort of organism response and subsequent appearance of the GAS. Conversely, the proteins up- or down-regulated after exposure to WSPs by *D. magna* specimens were more or less the same in numerical terms, indicating how this invertebrate model appears unable to respond to the entry of WSPs, but simply to undergo their effect on the proteome. Finally, we also observed a different effect on diverse protein classes, as the tested WSPs modulated proteins involved specifically in alterations in energy allocation processes and various cellular functions (catalytic processes, digestion, immune responses, reproduction, development) in *D. magna*, while *D. rerio* appears to be more impacted by these WSPs in terms of proteins involved in embryonic development. From the comparison of the different measured endpoints, it thus appears evident how proteomics seems to be the most sensitive approach, being capable of highlighting some differences in the mode of action of WSPs between the two biological models employed, and how it is necessary to use the measurement of numerous different endpoints to obtain the most realistic picture possible of the effects of environmental contaminants.

5. Conclusions

The outcomes suggest that WSPs, despite their prevalent use and perceived harmlessness, can have significant and complex impacts on aquatic invertebrates that should not be overlooked. These findings highlight the necessity for a re-assessment of the environmental risks posed by WSPs and call for further investigation into their long-term effects, potential bioaccumulation, and impact on food webs. Additionally, these results emphasize the importance of using a range of biological models and endpoints to gain a comprehensive understanding of the effects of WSPs to better inform conservation strategies and environmental management practices. Indeed, WSPs exhibit a complex interplay of effects on *D. magna* from molecular to behavioural levels, underscoring the importance of considering a wide spectrum of biological parameters in ecotoxicological assessments. Therefore, while in *D. rerio* a proposal of toxicity scales was provided based on obtained results [23,24], in *D. magna*, due to the heterogeneous responses obtained during the exposures to the different WSPs, it is not possible to do the same comparison.

Table 2
Effects at different levels of biological organization on both *Daphnia magna* and *Danio rerio* after the exposure to three concentrations of polyacrylic acid (PAA), polyethylene glycol (PEG), polyvinyl alcohol (PVA) and polyvinylpyrrolidone (PVP). The red tick indicates the significant differences (*$p < 0.05$) compared to control (one-way ANOVA, Bonferroni post-hoc test). Acronymous correspond to the following endpoint: AChE- acetylcholinesterase, MAO- monoamine oxidase. To do this comparison we considered only the endpoints performed in both species.

Daphnia magna	Concentrations mg/L	Vitality	AChE	MAO	Heart rate	Distance moved	Mobility	Thigmotaxis	Modulated proteins
PVA	0.001						✓		
	0.5				✓		✓		
	1				✓	✓			86
PAA	0.001								
	0.5				✓		✓		
	1					✓	✓		69
PEG	0.001		✓			✓	✓		
	0.5	☠	-	-	-	-	-	-	
	1		✓		✓	✓	✓		64
PVP	0.001				✓		✓		
	0.5				✓		✓		
	1				✓	✓			52
Danio rerio	Concentrations mg/L	Vitality	AChE	MAO	Heart rate	Distance moved	Mobility	Thigmotaxis	Modulated proteins
PAA	0.001				✓				
	0.5				✓			✓	
	1		✓		✓				20
PEG	0.001							✓	
	0.5								
	1					✓	✓		64
PVP	0.001				✓	✓	✓		
	0.5		✓		✓	✓	✓		
	1				✓	✓	✓		74

Environmental implications

Water-soluble polymers (WSPs) constitute a distinct class of synthetic polymers capable of dissolving in water. Despite their widespread use across various industrial and consumer applications, there remains a significant knowledge gap concerning their presence and impacts on ecosystems. Hence, this study focuses on evaluating the toxicity of four different WSPs -polyvinyl alcohol, polyvinylpyrrolidone, polyacrylic acid, and polyethylene glycol- at environmentally relevant concentrations of 0.001, 0.5, and 1 mg/L on *Daphnia magna*. The toxicity of WSPs was assessed at different levels of biological organization using an integrated approach encompassing biomarkers, behavioural parameters, and proteomics.

CRediT authorship contribution statement

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

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.

Acknowledgements

We thank the UNITECH Omics facility of the University of Milan for the quantification/characterization step of gel free proteomics. This study was supported by Line 2 grant of the Research Support Plan (PSR) 2021(PSR2021_DIP_002) assigned to Dr. Stefano Magni by Department of Biosciences of the University of Milan.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2024.134000.

References

[1] Kibria, M.G., Masuk, N.I., Safayet, R., Nguyen, H.Q., Mourshed, M., 2023. Plastic waste: challenges and opportunities to mitigate pollution and effective management. Int J Environ Res 17, 20.
[2] Casillas, G., Hubbard, B.C., Telfer, J., Zarate-Bermudez, M., Muianga, C., Zarus, G. M., Carroll, Y., Ellis, A., Hunter, C.M., 2023. Microplastics scoping review of environmental and human exposure data. Microplastics 2, 78–92.
[3] Hartmann, N., Hüffer, T., Thompson, R.C., Hassellöv, M., Verschoor, A., Daugaard, A.E., Rist, S., Karlsson, T.M., Brennholt, N., Cole, M., Herrling, M.P., Heß, M., Ivleva, N.P., Lusher, A.L., Wagner, M., 2019. Are we speaking the same language? Recommendations for a definition and categorization framework for plastic debris. Environ Sci Technol 53, 1039–1047.
[4] ARC Industry, 2019. Polymers Market – Forecast (2023 –2028). Market Reports. Furion analytics Research & Consulting LLP,.
[5] Wang, Z., Saadé, N.K., Ariya, P.A., 2021. Advances in ultra-trace analytical capability for micro/nanoplastics and water-soluble polymers in the environment: fresh falling urban snow. Environ Pollut 276, 116698.
[6] Duis, K., Junker, T., Coors, A., 2021. Environmental fate and effects of water-soluble synthetic organic polymers used in cosmetic products. Environ. Sci. Eur. 33, 1–20Alonso-López, O., López-Ibáñez, S., Beiras, R., 2021 Assessment of toxicity and biodegradability of poly (vinyl alcohol)-based materials in marine water. Polymers 13, 3742.
[7] Huppertsberg, S., Zahn, D., Pauelsen, F., Reemtsma, T., Knepper, T.P., 2020. Making waves: water-soluble polymers in the aquatic environment: an overlooked class of synthetic polymers? Water Res 181, 115931.
[8] Pauelsen, F., Huppertsberg, S., Knepper, T.P., Zahn, D., 2023. Narrowing the analytical gap for water-soluble polymers: a novel trace-analytical method and first quantitative occurrence data for polyethylene oxide in surface and wastewater. Sci Total Environ 882, 163563.
[9] Wang, D., Zheng, Y., Deng, Q., Liu, X., 2023. Water-soluble synthetic polymers: their environmental emission relevant usage, transport and transformation, persistence, and toxicity. Environ Sci Technol 57, 6387–6402.
[10] Antić, V.V., Antić, M.P., Kronimus, A., Oing, K., Schwarzbauer, J., 2011. Quantitative determination of Poly(vinylpyrrolidone) by continuous-flow off-line pyrolysis-GC/MS. J Anal Appl Pyrolysis 90, 93–99.
[11] Vidovic, N., Krauskopf, L.M., Jovancicevic, I., Antic, V., Schwarzbauer, J., 2022. Determination of the water-soluble polymer poly (N-vinylcaprolactam) in wastewater effluents by continuous-flow off-line pyrolysis-GC/MS. Discov Water 2, 1–10.
[12] Vidovic, N., Antic, V., Schwarzbauer, J., 2023. Determination of the water-soluble polymer poly(ethyleneimine) (PEI) in wastewater by continuous-flow off-line pyrolysis GC/MS. J Anal Appl Pyrolysis 172, 106001.
[13] Sainju, D., Lucas, R., Le Gresley, A., 2023. Evaluation of nuclear magnetic resonance spectroscopy for characterisation and quantitation of water-soluble polymers in river water. Wat Res 245, 120650.
[14] Rozman, U., Kalčíková, G., 2021. The first comprehensive study evaluating the ecotoxicity and biodegradability of water-soluble polymers used in personal care products and cosmetics. Ecotoxicol Environ Saf 228, 113016.
[15] Weston, D.P., Lentz, R.D., Cahn, M.D., Ogle, R.S., Rothert, A.K., Lydy, M.J., 2009. Toxicity of anionic polyacrylamide compounds when used for erosion control in agriculture. J Environ Qual 38, 238–247.
[16] Mondellini, S., Schott, M., Löder, M.G.J., Agarwal, S., Greiner, A., Laforsch, C., 2022. Beyond microplastics: water soluble synthetic polymers exert sublethal adverse effects in the freshwater cladoceran *Daphnia magna*. Sci Total Environ 847, 157608.
[17] Hatami, M., Banaee, M., Haghi, B.N., 2019. Sub-lethal toxicity of chlorpyrifos alone and in combination with polyethylene glycol to common carp (*Cyprinus carpio*). Chemosphere 219, 981–988.
[18] Nascimento, I.F., Guimaraes, A.T.B., Ribeiro, F., De, Lima, Rodrigues, A.S., Estrela, F.N., da Luz, T.M., Malafaia, G., 2021. Polyethylene glycol acute and sub-lethal toxicity in neotropical *Physalaemus cuvieri* tadpoles (Anura, Leptodactylidae). Environ Pollut 283, 117054.
[19] Siniakova, T.S., Raikov, A.V., Kudryasheva, N.S., 2023. Water-solublepolymer polyethylene glycol: effect on the bioluminescent reaction of the marine coelenterate obelia and coelenteramide-containing fluorescent protein. Int J Mol Sci 24, 6345.
[20] Arfsten, D.P., Burton, D.T., Fisher, D.J., Callahan, J., Wilson, C.L., Still, K.R., and, Spargo, B.J., 2004. Assessment of the aquatic and terrestrial toxicity of five biodegradable polymers. Environ Res 94, 198–210.
[21] Magni, S., 2024. Polyvinyl alcohol. In: Encyclopedia of Toxicology 4th Edition. Academic Press, 7. Elsevier,, pp. 859–863.
[22] Alonso-López, O., López-Ibáñez, S., Beiras, R., 2021. Assessment of toxicity and biodegradability of poly(vinyl alcohol)-based materials in marine water. Polymers 13, 3742.
[23] Nigro, L., Magni, S., Ortenzi, M.A., Gazzotti, S., Torre, C.D., Binelli, A., 2022. Are "liquid plastics" a new environmental threat? The case of polyvinyl alcohol. Aquat Toxicol 248, 106200.
[24] Nigro, L., Magni, S., Ortenzi, M.A., Gazzotti, S., Signorini, S.G., Sbarberi, R., Della Torre, C., Binelli, A., 2023. Assessment of behavioural effects of three water-soluble polymers in zebrafish embryos. Sci Total Environ 893, 164843.
[25] Binelli, A., Nigro, L., Sbarberi, R., Della Torre, C., Magni, S., 2024. To be or not to be plastics? Protein modulation and biochemical effects in zebrafish embryos exposed to three water-soluble polymers. Sci Total Environ 906, 167699.
[26] OECD, 2012. Test No. 211: *Daphnia magna reproduction test*.
[27] De Felice, B., Sugni, M., Casati, L., Parolini, M., 2022. Molecular, biochemical and behavioral responses of *Daphnia magna* under long-term exposure to polystyrene nanoplastics. Environ Int 164, 107264.
[28] Magni, S., Della Torre, C., Garrone, G., D'Amato, A., Parenti, C.C., Binelli, A., 2019. First evidence of protein modulation by polystyrene microplastics in a freshwater biological model. Environ Pollut 250, 407–415.
[29] Magni, S., Nigro, L., Della Torre, C., Binelli, A., 2021. Characterization of plastics and their ecotoxicological effects in the Lambro River (N. Italy). J Hazard Mater 412, 125204.
[30] Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 7, 248–254.
[31] Parolini, M., De Felice, B., Ferrario, C., Salgueiro-González, N., Castiglioni, S., Finizio, A., Tremolada, P., 2018. Benzoylecgonine exposure induced oxidative stress and altered swimming behavior and reproduction in *Daphnia magna*. Environ Pollut 232, 236–244.
[32] Nigro, L., Freitas, R., Maggioni, D., Hamza, H., Coppola, F., Protano, G., Della Torre, C., 2021. Coating with polysaccharides influences the surface charge of cerium oxide nanoparticles and their effects to *Mytilus galloprovincialis*. NanoImpact 24, 100362.

[33] Magni, S., Gagné, F., André, C., Della Torre, C., Auclair, J., Hanana, H., Parenti, C. C., Bonasoro, F., Binelli, A., 2018. Evaluation of uptake and chronic toxicity of virgin polystyrene microbeads in freshwater zebra mussel *Dreissena polymorpha* (Mollusca: Bivalvia). Sci Total Environ 631-632, 778–788.

[34] Ellman, G.L., Courtney, K.D., Andres Jr., V., Stone, R.M.F., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7, 88–95.

[35] DuBois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.T., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal Chem 28, 350–356.

[36] Fekete-Kertész, I., Stirling, T., Ullmann, O., Farkas, É., Kirchkeszner, C., Feigl, V., Molnár, M., 2018. How does experimental design modify the result of *Daphnia magna* heartbeat rate test? Analyses of factors affecting the sensitivity of the test system. Period Polytech Chem Eng 62, 257–264.

[37] Liang, R., He, J., Shi, Y., Li, Z., Sarvajayakesavalu, S., Baninla, Y., Guo, F., Chen, J., Xu, X., Lu, Y., 2017. Effects of Perfluorooctane sulfonate on immobilization, heartbeat, reproductive and biochemical performance of *Daphnia magna*. Chemosphere 168, 1613–1618.

[38] Xu, E.G., Cheong, R.S., Liu, L., Hernandez, L.M., Azimzada, A., Bayen, S., Tufenkji, N., 2020. Primary and secondary plastic particles exhibit limited acute toxicity but chronic effects on *Daphnia magna*. Environ Sci Technol 54 (11), 6859–6868.

[39] Tilzer, M.M., Stambler, N., Lovengreen, C., 1995. The role of phytoplankton in determining the underwater light climate in Lake Constance. Hydrobiologia 316, 161–172.

[40] Rivetti, C., Campos, B., Barata, C., 2016. Low environmental levels of neuro-active pharmaceuticals alter phototactic behaviour and reproduction in *Daphnia magna*. Aquat Toxicol 170, 289–296.

[41] Hill, C.E., Myers, J.P., Vandenberg, L.N., 2018. Nonmonotonic dose–response curves occur in dose ranges that are relevant to regulatory decision-making. Dose-Response 16, 1559325818798282.

[42] Kadajji, V.G., Betageri, G.V., 2011. Water soluble polymers for pharmaceutical applications. Polymers 3, 1972–2009.

[43] Raposo de Magalhães, C., Schrama, D., Farinha, A.P., Revets, D., Kuehn, A., Planchon, S., Rodrigues, P.M., Cerqueira, M., 2020. Protein changes as robust signatures of fish chronic stress: a proteomics approach to fish welfare research. BMC Genom 21, 1–16.

[44] Vandegehuchte, M.B., Vandenbrouck, T., Coninck, D.D., Coen, W.M.D., Janssen, C. R., 2010. Can metal stress induce transferable changes in gene transcription in *Daphnia magna*? Aquat Toxicol 97, 188–195.

[45] Lin, L., Xu, M., Mu, H., Wang, W., Sun, J., He, J., Qui, J.W., Luan, T., 2019. Quantitative proteomic analysis to understand the mechanisms of zinc oxide nanoparticle toxicity to *Daphnia pulex* (crustacea: Daphniidae): comparing with bulk Zinc oxide and Zinc salt. Environ Sci Technol 53, 5436–5444.

[46] Donahue, A.N., Aschner, M., Lash, L.H., Syversen, T., Sonntag, W.E., 2006. Growth hormone administration to aged animals reduces disulfide glutathione levels in hippocampus. Mech Ageing Dev 127, 57–63.

[47] Cai, M., Liu, Z., Chen, M., Zhang, M., Jiao, Y., Chen, Q., Zhao, Y., 2020. Comparative proteomic analysis of senescence in the freshwater cladoceran *Daphnia pulex*. Comp Biochem Physiol B, Biochem Mol Biol 239, 110352.

[48] Asselman, J., Semmouri, I., Jackson, C.E., Keith, N., Van Nieuwerburgh, F., Deforce, D., Shaw, J.R., De Schamphelaere, K.A., 2019. Genome-wide stress responses to copper and arsenic in a field population of Daphnia. Environ Sci Technol 53, 3850–3859.

[49] Tokishita, S.I., Kato, Y., Kobayashi, T., Nakamura, S., Ohta, T., Yamagata, H., 2006. Organization and repression by juvenile hormone of a vitellogenin gene cluster in the crustacean, *Daphnia magna*. Biochem Biophys Res Commun 345, 362–370.

[50] Chen, S., Chen, D.F., Yang, F., Nagasawa, H., Yang, W.J., 2011. Characterization and processing of superoxide dismutase-fused vitellogenin in the diapause embryo formation: a special developmental pathway in the brine shrimp, *Artemia parthenogenetica*. Biol Reprod 85, 31–41.

[51] Rainville, L.C., Carolan, D., Varela, A.C., Doyle, H., Sheehan, D., 2014. Proteomic evaluation of citrate-coated silver nanoparticles toxicity in *Daphnia magna*. Analyst 139, 1678–1686.

[52] Becker, D., Reydelet, Y., Lopez, J.A., Jackson, C., Colbourne, J.K., Hawat, S., Hippler, M., Zeis, B., Paul, R.J., 2018. The transcriptomic and proteomic responses of *Daphnia pulex* to changes in temperature and food supply comprise environment-specific and clone-specific elements. BMC Genom 19, 1–30.

[53] Kato, Y., Tokishita, S.I., Ohta, T., Yamagata, H., 2004. A vitellogenin chain containing a superoxide dismutase-like domain is the major component of yolk proteins in cladoceran crustacean *Daphnia magna*. Gene 334, 157–165.

[54] Subramoniam, T., 2011. Mechanisms and control of vitellogenesis incrustaceans. Fish Sci 77, 1–21.

[55] Le, T.H., Lim, E.S., Hong, N.H., Lee, S.K., Shim, Y.S., Hwang, J.R., Kim, Y.H., Min, J., 2013. Proteomic analysis in *Daphnia magna* exposed to As (III), As (V) and Cd heavy metals and their binary mixtures for screening potential biomarkers. Chemosphere 93, 2341–2348.

[56] Grzesiuk, M., Mielecki, D., Pilżys, T., Garbicz, D., Marcinkowski, M., Grzesiuk, E., 2018. How cyclophosphamide at environmentally relevant concentration influences *Daphnia magna* life history and its proteome. PLoS One 13, e0195366.

[57] Gündel, U., Benndorf, D., von Bergen, M., Altenburger, R., Küster, E., 2007. Vitellogenin cleavage products as indicators for toxic stress in zebra fish embryos: a proteomic approach. Proteomics 7, 4541–4554.

[58] Hannas, B.R., Wang, Y.H., Thomson, S., Kwon, G., Li, H., LeBlanc, G.A., 2011. Regulation and dysregulation of vitellogenin mRNA accumulation in daphnids (*Daphnia magna*). Aquat Toxicol 101, 351–357.

[59] Jeong, S.W., Lee, S.M., Yum, S.S., Iguchi, T., Seo, Y.R., 2013. Genomic expression 503 responses toward bisphenol-A toxicity in *Daphnia magna* in terms of reproductive 504 activity. Mol Cell Toxicol 9, 149–158.

[60] Houde, M., Douville, M., Gagnon, P., Sproull, J., Cloutier, F., 2015. Exposure of *Daphnia magna* to trichloroethylene (TCE) and vinyl chloride (VC): evaluation of gene transcription, cellular activity, and life-history parameters. Ecotoxicol Environ Saf 116, 10–18.

[61] Heckmann, L.H., Sibly, R.M., Connon, R., Hooper, H.L., Hutchinson, T.H., Maund, S.J., Hill, C.J., Bouetard, A., Callaghan, A., 2008. Systems biology meets stress ecology: linking molecular and organismal stress responses in *Daphnia magna*. Genome Biol 9, R40.

[62] Sricharoena, S., Kima, J.J., Tunkijjanukijb, S., Söderhälla, I., 2005. Exocytosisand proteomica nalysis of the vesicle content of granular hemocytes from a crayfish. Dev Comp Immunol (12), 1017–1031.

[63] Zhang, X., Wolinska, J., Blair, D., Hu, W., Yin, M., 2023. Responses to predation pressure involve similar sets of genes in two divergent species of Daphnia. J Anim Ecol 92, 1743–1758.

[64] Effertz, C., Müller, S., Elert, E.V., 2015. Differential peptide labeling (iTRAQ) in LC–MS/MS based proteomics in Daphnia reveal mechanisms of an antipredator response. J Proteome Res 14, 888–896.

[65] Grieshaber, M.K., Hardewig, I., Kreutzer, U., Portner, H.O., 1994. Physiological and metabolic responses to hypoxia in invertebrates. Rev Physiol Biochem Pharmacol 125, 43–147.

[66] Von Elert, E., Agrawal, M.K., Gebauer, C., Jaensch, H., Bauer, U., Zitt, A., 2004. Protease activity in guts of *Daphnia magna*: evidence for trypsin and chymotrypsin enzymes. Comp Biochem Physiol B Biochem Mol Biol 137, 287–296.

[67] Borgatta, M., Hernandez, C., Decosterd, L.A., Chevre, N., Waridel, P., 2015. Shotgun ecotoxicoproteomics of *Daphnia pulex*: biochemical effects of the anticancer drug tamoxifen. J Proteome Res 14, 279–291.

[68] Fischer, J.M., Fields, P.A., Pryzbylkowski, P.G., Nicolai, J.L., Neale, P.J., 2006. Sublethal exposure to UV radiation affects respiration rates of the freshwater cladoceran *Daphnia cafawba.* Photochem Photobiol 82, 547–550.

[69] Hedstrom, L., 2002. Serine protease mechanism and specificity. Chem Rev 102, 4501–4524.

[70] Parkinson, A., Ogilvie, B.W., 2001. Biotransformation of Xenobiotics. McGraw-Hill,, New York.

[71] Jeong, T.Y., Asselman, J., De Schamphelaere, K.A., Van Nieuwerburgh, F., Deforce, D., Kim, S.D., 2018. Effect of β-adrenergic receptor agents on cardiac structure and function and whole-body gene expression in *Daphnia magna*. Environ Pollut 241, 869–878.

[72] Choi, Y., Jeon, J., Kim, S.D., 2021. Identification of biotransformation products of organophosphate ester from various aquatic species by suspect and non-target screening approach. Water Res 200, 117201.

[73] Ehrenstrom, F., Berglind, R., 1988. Determination of biogenic amines in the water flea, *Daphnia magna* (Cladocera, Crustacea) and their diurnal variations using ion-pair reversed phase hplc with electrochemical detection. Comp Biochem Physiol C Comp Pharmacol 90, 123–132.

[74] Campbell, A.K., Wann, K.T., Matthews, S.B., 2004. Lactose causes heart arrhythmia in the water flea *Daphnia pulex*. Comp Biochem Physiol B 139, 225–234.

[75] McCoole, M.D., D'Andrea, B.T., Baer, K.N., Christie, A.E., 2012. Genomic analyses of gas (nitric oxide and carbon monoxide) and small molecule transmitter (acetylcholine, glutamate and GABA) signaling systems in *Daphnia pulex*. Comp Biochem Physiol Genom Prote 7, 124–160.

[76] McCoole, M.D., Atkinson, N.J., Graham, D.I., Grasser, E.B., Joselow, A.L., McCall, N.M., Welker, A.M., Wilsterman, E.J., Baer, K.N., Tilden, A.R., Christie, A. E., 2012. Genomic analyses of aminergic signaling systems (dopamine, octopamine and serotonin) in *Daphnia pulex*. Comp Biochem Physiol Genom Prote 7, 35–58.

[77] Weiss, L.C., Tollrian, R., Herbert, Z., Laforsch, C., 2012. Morphology of the Daphnia nervous system: a comparative study on Daphnia pulex, Daphnia lumholtzi, and Daphnia longicephala. J Morphol.

[78] Campos, B., Garcia-Reyero, N., Rivetti, C., Escalon, L., Habib, T., Tauler, R., Tsakovski, S., Pina, B., Barata, C., 2013. Identification of metabolic pathways in *Daphnia magna* explaining hormetic effects of selective serotonin reuptake inhibitors and 4-nonylphenol using transcriptomic and phenotypic responses. Environ Sci Technol 47, 9434–9443.

[79] Gagné, F., 2014. Biochemical Ecotoxicology: Principles and Methods, 1st edition.,. Elsevier,, London.

[80] Lovern, S.B., Strickler, J.R., Klaper, R., 2007. Behavioral and physiological changes in *Daphnia magna* when exposed to nanoparticle suspensions (titanium dioxide, nano-C60, and C60HxC70Hx). Environ Sci Technol 41, 4465–4470.

[81] Duong, T.B., Odhiambo, B.K., Oldham, D., Hoffman, M., Frankel, T.E., 2021. Acute exposure to an organochlorine pesticide alters locomotor behavior and cardiac function in the freshwater invertebrate *Daphnia magna*. Mar Freshw Behav Physiol 54, 51–64.

[82] Mercy, C., Prabha, P.S., 2022. Stress tolerance of ornamental fishes. Int J Innov Res Technol 9, 156182.

[83] Ankley, G.T., Bennett, R.S., Erickson, R.J., Hoff, D.J., Hornung, M.W., Johnson, R. D., Mount, D.R., Nichols, J.W., Russom, C.L., Schmieder, P.K., 2010. Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. Environ Toxicol Chem Int J 29, 730–741.

[84] Simao, C.P.F., Jeronimo, F.M., Blasco, V., Moreno, F., Porta, J.M., Pestana, J.L.T., Soares, A.M.V.M., Raldúa, D., Barata, C., 2019. Using a new high-throughput videotracking platform to assess behavioural changes in *Daphnia magna* exposed to neuroactive drugs. Sci Total Environ 662, 160–16.

[85] Bownik, A., 2017. Daphnia swimming behaviour as a biomarker in toxicity assessment: a review. Sci Total Environ 601-602, 194–205.

[86] Enserink, L., De La Haye, M., Maas, H., 1993. Reproductive strategy of *Daphnia magna*: implications for chronic toxicity tests. Aquat Toxicol 25, 111–123.
[87] Dodson, S., Ramcharan, C., 1991. Size-specific swimming behavior of *Daphnia pulex*. J Plankton Res 13, 1367–1379.
[88] Van Gool, E., Ringelberg, J., 1997. The effect of accelerations in light increase on the phototactic downward swimming of Daphnia and the relevance to diel vertical migration. J Plankton Res 19, 2041–2050.
[89] Gutierrez, M.F., Gagneten, A.M., Paggi, J.C., 2012. Exposure to sublethal chromium and endosulfan alter the diel vertical migration (DVM) in freshwater zooplankton crustaceans. Ecotoxicology 21, 37–47.
[90] Geritsen, J., 1982. Behavioral response of Daphnia to rate of temperature change: possible enhancement of vertical migration. Limnol Oceanogr 27, 254–261.
[91] Ringelberg, J., 1995. An account of a preliminary mechanistic model of swimming behaviour in Daphnia: its use in understanding diel vertical migration. Hydrobiologia 307, 161–165.
[92] Rinke, K., Petzold, T., 2008. Individual-based simulation of diel vertical migration of Daphnia: a synthesis of proximate and ultimate factors. Limnologica 3/4, 269–285.
[93] De Meester, L., 1993. Genotype, fish-mediated chemicals, and phototactic behavior in *Daphnia magna*. Ecology 74, 1467–1474.
[94] Cousyn, C., De Meester, L., Colbourne, J.K., Brendonck, L., Verschuren, D., Volckaert, F., 2001. Rapid, local adaptation of zooplankton behavior to changes in predation pressure in the absence of neutral genetic changes. Proc Natl Acad Sci U S A 98, 6256–6260.
[95] Johnson, P.T., Stanton, D.E., Forshay, K.J., Calhoun, D.M., 2018. Vertically challenged: how disease suppresses Daphnia vertical migration behavior. Limnol Oceanogr 63, 886–896.