



From polymer to commercial product: Unravelling the ecotoxicological effects of PVA-based dishwasher pods

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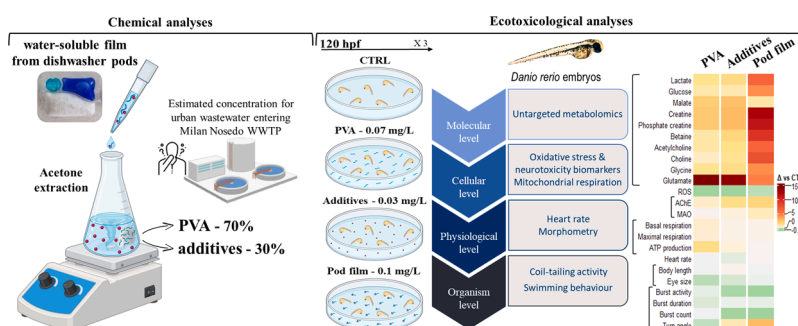
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HIGHLIGHTS

- Chemical characterization revealed 30% additives in commercial PVA-based film.
- Multitier toxicological assessment of commercial PVA-based products using zebrafish.
- Additives substantially contribute to the overall toxicity profile.
- Pod film toxicity emerged from mixture interactions, even with synergistic effects.
- Need to consider commercial formulations for environmental risk assessment.

GRAPHICAL ABSTRACT



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ABSTRACT

Water-soluble polymers (WSPs) are emerging as a largely overlooked class of contaminants. Despite their widespread use, the lack of specific regulation contributes to the uncontrolled release, especially into aquatic ecosystems, raising concerns for environmental and human health. Among WSP-based consumer products, dishwasher pods are widely used and are typically enclosed in a water-soluble film made of polyvinyl alcohol (PVA) and formulation additives. This study provided an integrated ecotoxicological assessment of such films in zebrafish (*Danio rerio*), combining physicochemical characterization and multitier approach spanning molecular to organismal levels, including metabolomics, oxidative stress and neurotoxicity biomarkers, energetic metabolism, cardiac activity, morphology, and behaviour. PVA was first separated from additives, and the resulting fractions were chemically characterized. Embryos were then exposed to the whole film (0.1 mg/L), reflecting environmentally-relevant concentrations, and the corresponding concentrations of PVA (0.07 mg/L) and additives (0.03 mg/L). Chemical analyses indicated that the film consisted of 70% PVA and 30% additives, mainly polyhydric alcohols and polyethylene glycol oligomers. Exposures induced subtle yet biologically meaningful alterations across all levels of the biological organization: PVA selectively affected mitochondrial respiration and eye development, while additives and pod film induced broader metabolic reprogramming, neurotoxicity, and decreased tail-coiling activity. Additives also induced significant bradycardia and increased body length. Notably, these findings revealed a substantial contribution of additives to the observed toxicity patterns, despite

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being present at lower concentrations. Overall, pod film toxicity could not be predicted from individual components, indicating mixture-specific effects and challenging the assumption that PVA-based products are environmentally safe, highlighting the need to assess commercial formulations as complex mixtures.

1. Introduction

Water-soluble polymers (WSPs) are gaining increasing attention as a largely overlooked class of emerging contaminants in aquatic ecosystems. These synthetic polymers are produced and used in large volume, raising concern about their continuous environmental release [1]. Frequently used WSPs include polyvinyl alcohol (PVA), polyethylene glycol (PEG), polypropylene glycol (PPG), polyacrylamide (PAM), polyvinylpyrrolidone (PVP), polyacrylic acid (PAA), polyethyleneimine (PEI), and polyallylamine (PAH) [2]. Their versatile physicochemical properties underpin their widespread use in personal care products and cosmetics [3], detergents [4], food additives [5], pharmaceuticals [6], pesticides and fertilizers [7], paints [8], and as flocculants in wastewater treatment plants (WWTPs) [9].

Despite their widespread occurrence, WSPs are generally considered environmentally safe due to their water solubility and relatively high molecular weight, which is assumed to limit their passage through cell membranes [10]. This is also largely based on conventional acute and chronic (eco)toxicity assays, which indicate high toxicity threshold and limited lethality in several model organisms. For instance, PVP showed relatively high lethal concentration (LC₅₀) or effect concentration (EC₅₀) in rats, *Daphnia magna* and *Oncorhynchus mykiss* [11]. Recently, Pastorino et al. obtained high EC₅₀ values in two species of marine diatoms (*Coscinodiscus tenuissimus* and *Phaeodactylum tricornutum*) exposed to PEG [12]. Similarly, PVA showed high values of acute and sub-lethal thresholds in rats [13,14] and *Paracentrotus lividus* larvae [15].

However, such assessments primarily focus on apical endpoints, potentially overlooking more subtle yet ecologically relevant biological alterations. In fact, recent evidence challenges this assumption of safety since many sub-lethal and sub-organismal studies have demonstrated that several WSPs can induce molecular, biochemical, and behavioural changes in aquatic organisms [16–22]. The observed effects can impair fitness, development, reproduction, and predator-prey dynamics [23]. Furthermore, the recent evidence of PVP internalization in *Daphnia magna* [24] highlighted that WSPs may cross biological barriers, contradicting the long-standing assumption that their molecular size prevents cellular uptake.

An additional and critical factor influencing WSPs (eco)toxicity, largely neglected in previous assessments, is the distinction between pure polymers and their commercial formulations. In fact, most WSP-containing products include plasticizers, stabilizers, and other additives that may significantly alter both physicochemical properties and toxicity profiles. Our previous findings showed that *Danio rerio* larvae exposed to a commercial PVA-based product exhibited significant ($p < 0.05$) behavioural impairments not observed with analytical-grade PVA [25]. Gas chromatographic analysis identified three ethylene glycol derivatives (tri-, tetra-, and penta-ethylene glycol) used as plasticizers [26–28] and associated with various toxicological effects [29–32], suggesting that additives may contribute to observed biological effects.

Among consumer products containing WSPs, dishwasher pods enclosed in a water-soluble PVA-based film represent a particularly relevant case study. Market analyses indicate that the global dishwasher tablet market was valued at USD 0.512 billion in 2023 and is projected to grow at an annual rate of 7.2% through 2030 [33]. Their increasing market demand implies growing environmental input of PVA through wastewater systems. Yet the environmental hazard associated with these products remains unclear, as it is unknown whether potential toxicity is driven by the polymer itself, by formulation additives, and/or by their combined effects.

To address this knowledge gap, the present study aimed to (i) extract

and (ii) chemically characterize additives from the PVA-based film of a commercial dishwasher pod, and (iii) evaluate the sub-lethal effects on *Danio rerio* larvae of the intact pod film (excluding detergents), the separated PVA matrix after extraction, and the extracted additives.

The selected exposure concentration for the pod film (0.1 mg/L) reflects the predicted environmental concentration (PEC) estimated for the discharge waters entering the Milan main WWTP (influent scenario), while PVA (0.07 mg/L) and additive (0.03 mg/L) concentrations were determined proportionally based on gravimetric analyses after the extraction.

Danio rerio embryos were exposed for 120 h post-fertilization (hpf) to the three treatments (pod film, PVA and additives), and sub-lethal effects were evaluated across molecular, cellular, physiological, and behavioural endpoints. These included metabolomic profiling, reactive oxygen species (ROS) production, superoxide dismutase (SOD), acetylcholinesterase (AChE), and monoamine oxidase (MAO) activities, mitochondrial respiration, cardiac activity, morphometric analyses, spontaneous tail-coiling activity, and swimming behaviour.

The emerging evidence of sub-lethal and additives-dependent effects highlights a critical gap in the current risk assessment approaches, which largely rely on tests performed on pure polymers and apical endpoints. By disentangling the respective roles of the polymer matrix and its associated additives at environmentally relevant concentrations, this study aims to provide a mechanistic and ecologically relevant evaluation of one of the most commonly used WSP-based consumer products. Such knowledge is essential to refine environmental risk frameworks and support the development of safer and more sustainable household products, which frequently enter aquatic systems without dedicated treatment or recovery processes.

2. Materials and methods

2.1. Chemical analyses: characterization of the PVA-based film

Due to the limited technical information available on the chemical composition of commercially available PVA-based films used to wrap liquid dishwasher pods, a comprehensive physicochemical characterization was performed to identify the components constituting the PVA-based film and their relative molecular weight. To this aim, an integrated analytical approach was employed, combining Proton Nuclear Magnetic Resonance (¹H NMR), gel permeation chromatography (GPC), and Gas Chromatography-Mass Spectrometry (GC-MS). First, a ¹H NMR analysis was performed to investigate the chemical structure of the PVA-based film and to detect the eventual presence of formulation additives. Spectra were acquired at 400 MHz using a Bruker Ultrashield 400 spectrometer at 298 K. A commercially available PVA powder (CAS n.: 9002–89–5, molecular weight (Mw) 89,000–98,000 Da, viscosity in water (4% at 20°C) 11.6–15.4 cps, molar degree of hydrolysis 99.0–99.8%, Sigma-Aldrich) was used as reference standard.

As commercial-grade PVA is commonly compounded with additives and other plasticizers, the PVA-based films was purified by means of a selective precipitation procedure aimed at investigating the effects of both PVA and additives employed for the processing. Preliminary tests were performed using different solvents (acetone, ethanol, methanol, and dichloromethane, Fig. S1A); however, after several trials, three sequential extractions with acetone were found to provide the most effective separation between PVA and additives (Fig. S1B). Briefly, 1 g of PVA-based film fragments, collected exclusively from the pods border to avoid any detergent contamination, was dissolved in 6 mL of Milli-Q water to obtain a viscous solution. This solution was then slowly

dropped into 500 mL of acetone under vigorous magnetic stirring to induce selective precipitation of PVA. Under these conditions, PVA aggregated as a white block, while additives remained dissolved in the organic solvent.

The precipitated PVA fraction was recovered by vacuum filtration, rinsed thoroughly with acetone, and dried overnight at 60 °C under nitrogen flux. These solubilization-precipitation steps were repeated three times to ensure the complete extraction of additives from the PVA matrix. The acetone phase containing the additives was then dried by rotary evaporation to yield the extracted additives fraction.

¹H NMR and GPC analyses were performed on the PVA-based film as it is, the purified PVA matrix, and the extracted additives to confirm the effectiveness of separation procedure and to determine their relative molecular weight. The relative contributions of PVA and additives in the pod film were estimated by gravimetric analysis using an analytical balance, based on the mass of the recovered fractions.

Finally, GC-MS analyses were performed to characterize the additives using an ISQ™ QD single quadrupole GC-MS (Thermo Fisher) equipped with a VF-5ms capillary column (Agilent technology, 30 m x 0.25 mm i.d. x 0.25 µm). Parameters used in the GC oven were as follows: 60 °C held for 2 min, 50–300 °C at 10 °C/min, and 300 °C held for 5 min. Carrier gas helium (He; purity ≥ 99.999%) at a flow rate 1.2 mL/min, injection temperature 250 °C, injection volume 1 µL, and split flow 6.0 mL/min. The MS transfer line and ion source temperatures were set at 270 °C, with a delay time of 5 min, and mass spectra were acquired in the *m/z* range 45–1000. For GC-MS analysis, 100 mg of additives were dispersed in 2 mL of dichloromethane (CH₂Cl₂; HPLC purity) or methanol (MeOH) and stirred overnight at room temperature. An aliquot of 1 mL of the resulting solutions was then filtered and analysed.

2.2. Preparation and selection of the testing solutions

All testing solutions were prepared in reconstituted zebrafish water, composed by MilliQ water supplemented with 0.2 g/L calcium sulphate (CaSO₄), 0.1 g/L sodium bicarbonate (NaHCO₃), 0.1 g/L Instant Ocean®, and 0.1% methylene blue (C₁₆H₁₈ClN₃S, used as antifungal agent [34,35]), according to standardized zebrafish embryo exposure protocols.

Stock solutions (50 mg/L) of separated PVA matrix, extracted additives, and complete pod film (hereafter referred to as PVA, additives, and pod film, respectively) were prepared and diluted the day prior to exposure to achieve working concentrations of 0.07 mg/L for PVA, 0.03 mg/L for additives, and 0.1 mg/L for pod film. The pod film concentration was selected to reflect the PEC calculated for discharge waters entering the Milano-Nosedo WWTP (influent scenario, see S.I.), while PVA and additives concentrations were calculated based on their relative contribution to the pod film as determined by the previous gravimetric analyses. This value is further supported by the PEC of PVA in surface waters (PEC_{sw} = 3–19 µg/L) recently reported by Brunning et al. [36], remaining within approximately one order of magnitude. Importantly, due to dilution effect, the PEC_{sw} value is expected to be lower than those estimated for WWTP influent.

Control and treatment solutions were aerated overnight to achieve oxygen saturation and equilibrated at 27.5 °C in a thermostat, in accordance with test guideline (TG) 236 of the Organization for Economic Cooperation and Development [37].

For energetic metabolism assay, the same stock and testing solutions were prepared using E3 medium instead of reconstituted zebrafish water. E3 medium was prepared by dissolving 17.4 g NaCl, 0.8 g KCl, 2.9 g CaCl₂·2 H₂O and 4.9 g MgCl₂·6 H₂O, and 100 µL of 1% methylene blue (used as antifungal agent [34,35]) in 1 L of deionized water. If needed, pH was adjusted to 7.2 using NaOH.

2.3. Zebrafish larvae exposure

Zebrafish (*Danio rerio*) fertilized eggs were provided by the breeding

facility of the Department of Earth and Environmental Sciences of the University of Milano-Bicocca, according to the Italian regulations (Legislative Decree no. 116/92; authorization n. 0020984–12/02/2018) from adult AB wild-type pairs maintained at 28 °C under a 14:10 h light: dark photoperiod.

Embryos were exposed to controls, PVA (0.07 mg/L), additives (0.03 mg/L), and pod film (0.1 mg/L) from eggs fertilization until 120 hpf under static exposure conditions at 27.5 °C. Exposures were conducted in 50 mL Petri dish, or 24-well plates (1.5 mL per well) for behavioural analyses. To consider biological variability and ensure sufficient biological material for all ecotoxicological endpoints, three independent exposures were performed, each including approximately 100 embryos per treatment, yielding a total of around 300 specimens per experimental group.

Embryos were daily inspected under a stereo-microscope to assess the eventual occurrence of lethal effects (e.g. coagulated embryos, lack of somite formation, non-detachment of the tail, and absence of heart-beat), and incidence of morphological abnormalities (reduced pigmentation, scoliosis, development delay, edemas, and malformations) [34, 37–39]. Dead embryos were removed to prevent water contamination.

Burst activity and energetic metabolism assays were conducted at 48 hpf, while heart rate, morphometric measurements and swimming behaviour analyses at 120 hpf. At the end of the exposures, specimens were either immediately processed or stored at –80 °C for subsequent evaluation of remaining endpoints.

2.4. Metabolomic analyses

For metabolomic analyses, as molecular endpoint, polar intracellular metabolites were isolated using a biphasic extraction protocol based on methanol, chloroform, and water, adapted from established metabolomic workflows [40]. Frozen larvae (n = 3 pools of 20 specimens per treatment, from 3 independent exposures) were suspended in pre-cooled methanol:water (8:2.5 mL/g tissue) and homogenized using a probe sonicator (Sonic Dismembrator, Thermo Fisher Scientific) set at 25% amplitude for 3 × 30 s on ice, with 30 s pause between bursts to prevent overheating.

For the following homogenization, chloroform (8 mL/g) and water (4 mL/g) were added to induce phase separation. The mixture was vortexed briefly and incubated on ice for 10 min before centrifugation at 2000 g for 5 min at 4 °C. The upper aqueous phase, containing polar metabolites, was carefully collected, dried under vacuum using a centrifugal concentrator (Eppendorf 5301), and stored at –80 °C until analysis.

Dried polar extracts were reconstituted in 600 µL of 0.1 M sodium phosphate buffer in 100% D₂O (pH 7.0), supplemented with 1 mM of 2,2-dimethyl–2-silapentane–5-sulfonate (DSS; Sigma-Aldrich) as internal standard for chemical shift referencing and quantification. Samples were transferred to standard 5-mm NMR tubes.

Spectra were acquired on a Varian 500 MHz NMR spectrometer (Agilent Technologies, USA) at 298 K. One-dimensional ¹H NMR spectra were obtained using a PRESAT water suppression pulse sequence with a spectral width of 6009 Hz, relaxation delay of 2 s, and 256 scans collected into 16,384 data points. Free induction decays (FIDs) were zero-filled to 32,768 points and subjected to exponential line broadening (0.5 Hz) before Fourier transformation. Spectra were manually phased, baseline-corrected, and referenced to DSS at 0.00 ppm using Chenomx NMR Suite (v12.0, Chenomx Inc., Edmonton, Canada). Metabolites were identified and quantified using the Chenomx 500 MHz reference library, supported by publicly available spectral databases (e.g., HMDB, BMRB). Relative metabolite concentrations were normalized to total spectral area and subjected to multivariate and univariate statistical analyses in MetaboAnalyst 6.0 platform [41].

To explore the biological relevance of the metabolomic alterations observed in zebrafish larvae, functional metabolic pathway enrichment and topological network analyses were performed using the

MetaboAnalyst 6.0. A list of statistically significant metabolites (ANOVA, $p < 0.05$, FDR-corrected) was used as input for the enrichment analysis based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, with *Danio rerio* selected as reference organism. Overrepresentation analysis (ORA) was combined with pathway topology analysis to identify metabolite sets that were significantly enriched in the exposed groups. Enrichment scores were calculated using hypergeometric testing, and pathway impact values were derived from node centrality measures (betweenness centrality). The top 25 enriched pathways were visualized using a bubble plot, with enrichment ratio represented by circle size and p -value by colour intensity. A complementary network analysis of the enriched pathways integrated shared metabolite nodes and their interactions across multiple pathways, enabling the identification of functionally interconnected metabolic modules. Edges represent shared metabolites, while node size and colour indicate pathway relevance (impact and p -value, respectively). Network layout was generated using a force-directed algorithm and visualized directly within MetaboAnalyst [42].

In order to define interactions between treatments (additive, synergistic or antagonistic effects), metabolite effects of each treatment were quantified as $\log_2$ fold-change relative to controls. Expected additive effect in pod film was estimated as the sum of $\log_2$ fold-changes of PVA and additives, consistent with classical models of combination effects, as Bliss independence and Loewe additivity frameworks [43]. Interaction type was determined by quantifying the delta (Δ) between observed and expected effects: deviations of the observed pod film effect from the expected (additive) value were interpreted as synergistic ($\Delta > 0.25$) or antagonistic ($\Delta < -0.25$) interactions. The selected threshold of $\pm 0.25 \log_2$ units (approximately 19% change) is consistent with common practice in metabolomics and mixture toxicology and corresponds to a biologically meaningful deviation exceeding typical analytical and biological variability [43–45].

2.5. Biomarkers of oxidative stress and neurotoxicity

Biomarkers of oxidative stress (ROS, SOD) and neurotoxicity (AChE, MAO) were evaluated following standardized protocols [46–48]. The specimens ($n = 3$ pools of 25 specimens per treatment, from 3 independent exposures) were potted in 100 mM phosphate buffer (pH 7.4), containing 100 mM potassium chloride (KCl), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), and protease inhibitors (1:100 v/v, Complete Tablets mini, Roche). The homogenates were then centrifuged at 15000 g for 30 min at 4 °C to obtain the S15 fraction.

The homogenates for MAO activity were obtained by potting the specimens ($n = 3$ pools of 10 specimens per treatment, from 3 independent exposures) in 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-sodium hydroxide (HEPES-NaOH) buffer (pH 7.4), at a 1:10 W/V ratio, containing 100 mM sodium chloride (NaCl), 0.1 mM DTT, and protease inhibitors. The homogenates were then centrifuged at 1000g for 20 min at 4 °C to obtain the S1 fraction.

Protein content in all supernatants was quantified using the Bradford reagent (Merck) according to Bradford protocol [49], and used for normalization of all biomarkers activities.

Oxidative stress was evaluated through the measuring SOD activity and ROS levels. The kinetic of SOD was evaluated spectrophotometrically (6715 UV-Vis spectrophotometer, Jenway) at $\lambda = 550$ nm for 2 min through the reduction inhibition of 10 μ M cytochrome C induced by the superoxide anion generated by the complex xanthine oxidase–50 μ M hypoxanthine. ROS levels were quantified fluorometrically using the EnSight™ multimode plate reader (PerkinElmer) at $\lambda_{ex} = 485$ nm, $\lambda_{em} = 530$ nm using 10 mg/mL dichlorofluorescein diacetate (DCFH-DA in dimethyl sulfoxide, DMSO) as substrate, with 5% H_2O_2 as positive control.

As neurotoxicity endpoints, AChE was measured using the EnSight™ multimode plate reader (PerkinElmer) at $\lambda = 412$ nm for 15 min (1 min

read interval) at 30 °C using the Ellman reagent, containing 1 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in 100 mM Tris-acetate (pH 7.4), and 1 mM acetylthiocholine in 50 mM Tris-HCl as substrate. The kinetic of MAO was determined fluorometrically using the EnSight™ multimode plate reader (PerkinElmer) at $\lambda_{ex} = 485$ nm, $\lambda_{em} = 528$ nm for 10 min (42 s read interval) using 1 mM tyramine as substrate. The reaction buffer contained 140 mM NaCl, 10 mM HEPES, and NaOH buffer (pH 7.4), 1 mg/mL peroxidase and 10 mM 3-amino-1,2,4-triazole, enriched with 10 μ M DCFH-DA.

2.6. Biomarkers of cellular energetic metabolism

Mitochondrial respiration and metabolic partitioning were assessed using the Seahorse XFe24 Extracellular Flux Analyzer (Agilent Technologies, USA), following established protocols for zebrafish embryos [50–53]. OCR was measured at 48 hpf due to technical constraints of the Seahorse assay, which requires embryos prior to hatching to ensure stable positioning and reproducible measurements. About 24 h before the test, the sensor cartridge was hydrated overnight in XF Calibrant fluid and incubated at 28 °C in a non-CO₂ incubator. On the day of the assay, 48 hpf embryos, prior to hatching, were randomly selected and transferred one embryo per well ($n = 15$ per treatment, from 3 independent exposures) to Seahorse XF islet microplates containing 525 μ L of E3 medium. An islet capture screen was positioned over each embryo, carefully avoiding the formation of air bubbles, to ensure immobilization and stable positioning during the assay. Four wells per plate were used as temperature control wells and filled with assay medium only. Plates were equilibrated for up to 30 min in a non-CO₂ incubator at 28 °C prior to analysis.

Oxygen consumption rate (OCR, pmol O₂/min) was measured using repeated cycles consisting of a mixing phase (2 min), pause (1 min), and measurement of oxygen level (2 min). To dissect the different components of mitochondrial respiration, a sequential injection of pharmacological inhibitors was performed. Basal respiration was recorded for 10 measurement cycles prior to oligomycin injection. Oligomycin inhibits ATP synthase (complex V), thereby impairing electron flow through the Electron Transport Chain (ETC) and allowing the estimation of respiration coupled to ATP production. Following oligomycin injection, OCR was recorded for 18 cycles, after which carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) was injected and OCR recorded for 8 cycles. FCCP is a mitochondrial uncoupler that collapses the proton gradient and disrupts the mitochondrial membrane potential, resulting in uninhibited electron flow through the ETC and maximal OCR by complex IV, therefore FCCP was used to determine maximal respiratory capacity. Finally, sodium azide, an inhibitor of cytochrome C oxidase (complex IV of the ETC) was injected to completely inhibit mitochondrial respiration, enabling the determination of non-mitochondrial respiration. Following sodium azide injection, OCR was measured for 24 cycles.

The total assay duration was approximately 6 h. Each pharmaceutical working solution was loaded into the drug injection ports of the sensor cartridge to achieve final well concentrations of 20 μ M oligomycin, 8 μ M FCCP, and 20 mM sodium azide. Respiratory parameters were calculated from OCR profiles according to manufacturer guidelines and previously published studies [51,54,55], as described in Tab. S1.

2.7. Heart rate assay

Cardiac activity was evaluated by measuring the heart rate in 120 hpf zebrafish ($n = 15$ per treatment, from 3 independent exposures). At the end of the exposure period, samples were randomly selected and gently placed on a glass slide, allowing them to acclimate for 2 min to the light condition of an optical microscope (Leica Microsystems CMS GmbH). Video recordings (15 s, .mp4) were acquired at a constant frame rate of 25 frames per seconds (fps) for each specimen using a video camera (Basler acA1300–60gm Gige) mounted on the microscope

eyepiece, and connected to EthoVision XT 11.5 software (Noldus IT, NL). Heart rate was then determined using the Tracker software by counting the number of cardiac contractions over a 10 s interval and expressed as beats per minute (bpm).

2.8. Morphometric analyses

Morphometric parameters, including total body length and eye size, were assessed in 120 hpf zebrafish using DanioScope™ software (Noldus IT, NL).

At the end of the exposure period, samples ($n = 15$ per treatment, from 3 independent exposures) were randomly selected and gently placed on self-prepared 2% low-melting agarose (LMA) blocks cast in 50 mL Petri dishes. Larvae were positioned laterally and oriented uniformly to ensure consistent anatomical alignment and to minimize variability during image acquisition. Video recordings (.mp4) were acquired at a constant frame rate of 25 fps using a video camera (Basler acA1300–60gm GigE) mounted on the eyepiece of a stereomicroscope, and connected to EthoVision XT 11.5 software. Each video was then uploaded on DanioScope™ software, and a representative still frame was extracted for each zebrafish and used for morphometric analysis. Prior to measurements, spatial calibration was performed by setting a fixed reference distance, ensuring accurate conversion from pixels to metric units. Total body length was measured as the distance from the anterior tip of the head (snout) to the posterior end of the caudal fin, while eye size was quantified by manually delimitating the eye perimeter and calculating eye area. Regions of interest were defined using the dedicated DanioScope™ morphometric tools, and all measurements were subsequently calculated automatically by the software.

2.9. Behavioural analyses

Behavioural analyses were conducted at two developmental stages: at 48 hpf on chorionated embryos to evaluate spontaneous burst activity, and at 120 hpf on free-swimming zebrafish to assess locomotor performances.

At 48 hpf, samples ($n = 30$ per treatment, from 3 independent exposures) were randomly selected and gently placed in Petri dishes. Video recordings (3 min, .mp4) were acquired at a constant frame rate of 25 fps. Video recording started immediately after the embryos acclimatized for 2 min to the lighting condition. Regions of interest were defined selecting the “Chorion” setting of DanioScope™ software, allowing automated detection of tail-coiling events occurring inside the chorion. The following endpoints were analysed: burst count/min, burst activity (%), and mean burst duration (s).

Swimming performances were evaluated using DanioVision™ instrument (Noldus IT, NL). At the end of the exposures, samples ($n = 36$ per treatment, from 3 independent exposures), individually placed into 24-wellplate, were inserted into the DanioVision™ observation chamber under constant temperature of 27.5 °C. Following a 10 min-acclimation period in the dark, locomotor activity was recorded during two consecutive cycles of alternating dark (10 min) and light (5 min, 2200 lux) conditions, for a total test duration of 30 min. Video recordings (.mp4) were acquired at 30 fps using a video camera (Basler acA1300–60gm GigE), and EthoVision XT software was used to automatically track the zebrafish. The distance moved by each individual zebrafish was calculated and binned into 1 min intervals. The following behavioural endpoints were then evaluated: distance moved (mm/min), acceleration (mm/s), thigmotaxis expressed as cumulative duration (%) of time spent by the specimens in the outer zone of the arena, and turn angle (deg/s).

2.10. Statistical analysis and data elaboration

Shapiro-Wilk and Levene's tests were used to test the normality and homogeneity of variance of data, respectively. For endpoints based on

pooled larvae from the 3 independent exposures (metabolomics analyses, biomarkers of oxidative stress and neurotoxicity), significant differences among treatments were assessed using one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) *post-hoc* test ($p < 0.05$). Data were expressed as mean $\pm$ standard deviation (SD). For metabolomics analyses, all metabolite concentrations were normalized to total spectral area prior to statistical evaluation. To visualize global metabolomic alterations across treatments, a heatmap with hierarchical clustering (Euclidean distance, complete linkage) was generated using MetaboAnalyst 6.0. Metabolite intensities were auto-scaled (mean-centred and divided by SD) before clustering. The resulting heatmap provided an overview of metabolite pattern similarities and group separation across treatments. Principal Component Analysis (PCA) was also performed in MetaboAnalyst 6.0 after log-transformation and Pareto scaling of metabolomic data to evaluate global variance structure and clustering patterns across exposure groups.

For endpoints assessed on individual zebrafish larvae (energetic metabolism, heart rate, morphometric parameters and behavioural analyses), linear mixed models (LMM) were applied. To meet model assumptions data were log-transformed, when necessary. Treatment was included as fixed factor, while exposure as a random factor to account for inter-exposure variability (lmer function, lme4 R package). For swimming performances, differences among treatments were assessed separately for dark and light conditions using one-way ANOVA, followed by Fisher's LSD *post-hoc* test ($p < 0.05$). Data were expressed as estimated marginal means (EMM) and *post-hoc* comparisons were obtained using the emmeans package on R. For behavioural endpoints acquired over time, individual larvae were additionally included as a random factor to account for repeated measures and avoid pseudo-replication.

Given the large amount and heterogeneity of collected data, a heatmap was generated to provide an integrated visualization of the overall responses among treatments. Prior to visualization, endpoints were expressed as deviations from their respective controls. The heatmap allowed the simultaneous visualization of both magnitude and direction (up- or down-regulation) of significant alterations observed in this study, facilitating the identification of response trends across the different biological levels. To explore clustering among treatments, a factorial analysis through the principal component analysis (PCA) was performed using all the measured endpoints (excluding embryotoxicity).

Finally, to compare the toxicity among treatments based on the biological trend of each response, all endpoints were integrated into the Integrated Endpoint Response Index (IERI), adapted from the Biomarker Response Index (BRI) [35,56,57]. For each endpoint, normalized to an equivalent concentration of 0.1 mg/L, the percentage of alteration level (AL) relative to controls was calculated, and a score was assigned based on predefined thresholds (AL < 20% score = 1; 20% < AL < 50% score = 2; 50% < AL < 100% score = 3; AL > 100% score = 4). Each AL score was then multiplied by a fixed biological weight reflecting the level of biological organisation of the corresponding endpoint: molecular endpoints (metabolomics analyses) = 1; cellular (ROS, SOD, AChE, MAO, and energetic metabolism) = 2; physiological endpoint (heart rate) = 3; organismic endpoints (morphometric and behavioural analyses) = 4. For each biological category (metabolomics, oxidative stress, neurotoxicity, energetic metabolism, heart rate, morphology, and behaviour), category-level responses were calculated as the mean of the weighted scores of all endpoints included within that category. Lastly, to obtain the IERI value for each treatment, the mean of category-level responses values were normalized by the corresponding fixed biological weight assigned to each level of biological organisation. This approach allows the integration of multilevel biological responses while accounting for differences in biological complexity among categories, preventing overrepresentation of higher-order biological endpoints and ensuring their proportional contribution to the final index.

Moreover, a radar plot was used to provide an integrated visualization of the observed responses. Each endpoint, normalized to an

equivalent concentration of 0.1 mg/L, was expressed as the deviation from the respective control, allowing direct comparison and visualization across heterogeneous biological responses.

Both the responses for IERI and radar plot were normalized to 0.1 mg/L to enable a final comparison across treatments tested at different concentrations. This approach assumes local linearity within the central part of the curve and therefore should not be interpreted as providing precise dose-response characterization.

All statistical analyses in this study were conducted using R version 4.5.2, and data were graphically represented using GraphPad Prism software, unless otherwise specified.

3. Results

3.1. Physicochemical separation and characterization

^1H NMR spectra of standard PVA and pod film were recorded, both were identified by a peak centred at 1.96 ppm, which can be attributed to the methyl group of the acetyl group of the non-hydrolysed repeating units of PVA (Fig. S2A). However, the comparison between the two spectra highlighted the presence of additional peaks in pod film that were absent in standard PVA (4.38, 4.31, 4.25, 4.13, 3.69–3.37, 3.18, 1.63, 1.02, 1.00 ppm), likely due to additives formulation (Fig. S2B). To further investigate this, pod film was subjected to acetone-induced separation to disentangle the two components, PVA and additives. ^1H NMR analyses performed on PVA, additives and pod film confirmed the successful extraction of additives from PVA. Peaks corresponding to additives, present in the pod film spectrum (Fig. 1A), were absent in that of PVA (Fig. 1B) but clearly present in the additives fraction (Fig. 1C). GPC analyses revealed the molecular weight distribution of the three components (Table 1, Fig. S3). The discrepancy between the starting pod film and the separated PVA may arise from a partial loss of the high molecular weight fractions during the purification procedures.

Gravimetric analysis of the separated fractions indicated that pod film consisted of approximately 70% PVA and 30% additives, which guided the selection of exposure concentrations for PVA and additives chosen in this study (0.07, and 0.03 mg/L, 70% and 30% of 0.1 mg/L pod film, respectively).

Finally, GC-MS analyses provided a detailed chemical characterization of the extracted additives. The chromatographic profiles (Fig. S2C–D, methanol and dichloromethane extracts, respectively) revealed several peaks, reflecting the presence of a complex mixture of organic compounds.

Methanol extraction identified two predominant peaks at 14.314 and 16.606 min, which were assigned by NIST2017 database to glycerine, the most abundant compound based on the chromatographic area, followed by 2-ethyl–2-(hydroxymethyl)–1,3-propanediol. Various PEG oligomers were also detected, including tetraethylene, pentaethylene, and hexaethylene glycols (4, 5, 6-EG). In addition to several polyols, methanol extraction also revealed the presence of long-chain aliphatic alcohols (1-hexadecanol, 1-octadecanol, octacosanol), and unsaturated

Table 1

molecular weight distribution of pod film, PVA, additives determined through GPC analyses.

Sample	$\overline{M}_n$ (g/mol) ^a	$\overline{M}_w$ (g/mol) ^a
Pod film	34200	106400
PVA	24900	72400
Additives	20400	63100

^aDetermined as PEG standards.

hydrocarbons (1-tridecene, 1-octadecene). Nitrogen-containing compounds, including N,N-dimethyl–1-tetradecanamine, N-methyl–1-hexadecanamine, and dimethyl palmitamine, were also detected, while trace levels of other compounds, such as dimantine were observed (Table 2A).

Dichloromethane extraction provided a partially overlapping but compositionally complementary profile, characterized by a higher relative abundance of moderately polar and non-polar compounds.

Table 2

characterization of additives after extraction in (A) methanol, and (B) dichloromethane.

(A)		
R.T. (min)	Area	Compound Name
14,314	29362904	Glycerin
16,606	12028962	2-ethyl–2-(hydroxymethyl)–1,3-propanediol
17,525	1028180	1-Tridecene
22,212	950773	Pentaethylene glycol
19,059	904971	Tetraethylene glycol
25,326	790638	1-Octadecanol
23,564	559961	Dimethyl palmitamine
25,076	551889	Hexaethylene glycol
25,54	442853	Dimantine
21,392	371729	1-Tetradecanamine, N,N-dimethyl-
21,47	210984	1-Hexadecanamine, N-methyl-
24,447	153539	1-Hexadecanol
22,372	135040	1-Octadecene
26,34	119829	1-Hexadecanol
20,084	99949	Cetene
28,078	56697	Octacosanol
(B)		
R.T. (min)	Area	Compound Name
15,98	2676286	2-ethyl–2-(hydroxymethyl)–1,3-propanediol
11,642	972671	Glycerin
18,901	795203	Tetraethylene glycol
22,178	575984	Pentaethylene glycol
4384	403987	R-(–)-1,2-propanediol
25,326	347823	1-Hexadecanol
17,526	248305	Dodecanol
21,409	211666	1-Tetradecanamine, N,N-dimethyl-
7426	205475	2-Pentanone, 4-hydroxy–4-methyl-
15,063	165851	Triethylene glycol
25,06	159454	Hexaethylene glycol
23,572	58534	Dimethyl palmitamine
26,341	49800	n-Heptadecanol–1
24,45	40946	1-Octadecene

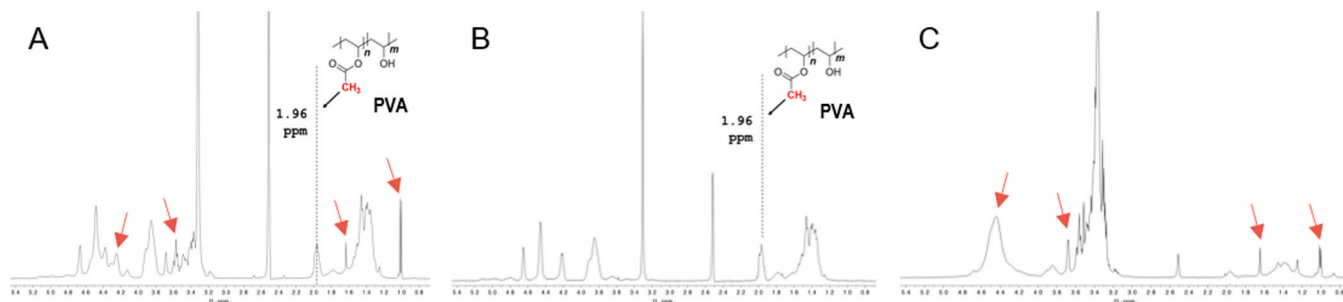


Fig. 1. ^1H NMR spectra of (A) pod film, (B) PVA, (C) additives. The peak at 1.96 ppm refers to methyl group of the acetyl group of the non-hydrolysed repeating units of PVA, while additives (4.38, 4.31, 4.25, 4.13, 3.69–3.37, 3.18, 1.63, 1.02, 1.00 ppm) were highlighted by red arrows.

Alongside glycerin and 2-ethyl-2-(hydroxymethyl)-1,3-propanediol, PEG oligomers were again predominant, including triethylene glycol (3-EG) through 6-EG. Notably, 4-EG and 5-EG were among the most abundant PEG species identified in dichloromethane extracts. Additionally, low-molecular-weight alcohols (dodecanol and n-heptadecanol-1), and ketonic compounds (4-hydroxy-4-methyl-2-pentanone) were detected, which were not prominent in methanol extraction (Table 2B). Overall, despite not being fully quantitative, the applied analytical approach provided a comprehensive overview of the species present in the material, including an estimation of their relative abundances.

The presence of these compounds was consistent with signals previously detected in the ^1H NMR spectra (Figs. 1, S2B).

The combination of ^1H NMR, GPC, and GC-MS with a precipitation-based purification approach provided a comprehensive, although partial, characterization of the chemical composition of the PVA-based film.

While ^1H NMR successfully demonstrated the efficient removal of the extractable additives fraction from the PVA matrix, GPC analysis revealed that the extract contains low- to medium-molecular-weight species. These likely include both low-molecular-weight additives and a fraction of shorter PVA chains that are soluble in the extraction medium. Finally, GC-MS analysis further enabled the identification of the

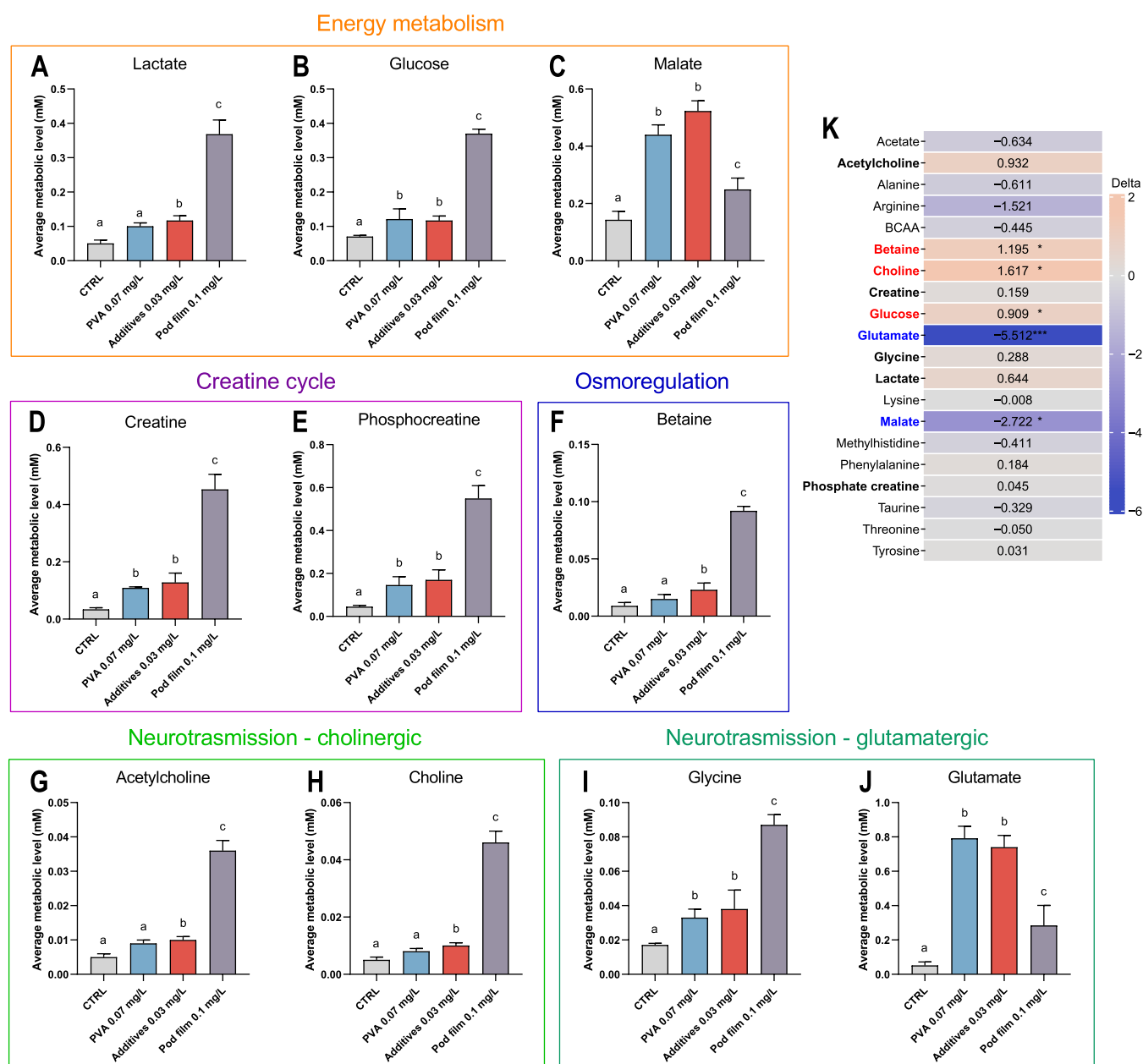


Fig. 2. ^1H NMR analysis of key metabolites affected in zebrafish larvae (120 hpf) exposed to PVA (0.07 mg/L), additives (0.03 mg/L), and pod film (0.1 mg/L). Key metabolites are associated with energy metabolism (A. lactate, B. glucose, C. malate), creatine cycle (D. creatine, E. phosphocreatine), osmoregulation (F. betaine), cholinergic neurotransmission (G. acetylcholine, H. choline), and glutamatergic neurotransmission (I. glycine, J. glutamate). ^1H NMR measurements (mM) are expressed as mean $\pm$ standard deviation (SD), statistical differences ($p < 0.05$) were determined by one-way ANOVA, followed by Fisher LSD *post-hoc* test ($n = 3$ pools of 20 specimens per treatment, from 3 independent exposures). Different letters indicate significant differences between groups; (K) Interaction effects between treatments based on metabolomic responses. Values represent Δ ($\log_2$ fold-change deviation) between pod film effects (combined exposure) and expected additive effects derived summing PVA and additives effects. Red or blue labels indicate synergistic and antagonistic interactions, respectively. The colour scale indicates direction and magnitude of deviation (red: synergistic; grey: additive; blue: antagonistic).

low-molecular weight additives, providing insight into the chemical nature of a substantial portion of the additives present.

Overall, these results demonstrate that pod film is composed not only of the PVA matrix alone, but also contains a heterogeneous mixture of additives that can influence the ecotoxicological effects.

3.2. Evaluation of possible acute effects

No significant lethal effects were observed in any of the experimental groups, with normal larvae consistently exceeding 93% in all treatments, and reaching 97% in controls; only a slight non-significant decreasing trend was observed in additives and pod film (Fig. S4). Similarly, the incidence of developmental abnormalities (*i.e.* edema, pigmentation defects, malformations, scoliosis, and developmental delay) did not differ significantly between exposed groups and controls ($p > 0.05$), although a slight, non-significant increase of some of these abnormalities was observed in larvae exposed to additives and pod film (Fig. S4). After confirming the absence of acute effects, the possible sub-lethal effects were assessed through a multi-level approach.

3.3. Metabolic response in zebrafish

To gain insight into the metabolic responses of zebrafish larvae to the treatments, a comprehensive profiling of endogenous metabolites was conducted (Fig. S5). PVA, additives, and pod film elicited significant alterations for key metabolites associated with energy metabolism (glucose, lactate, malate), osmoregulation (betaine), creatine-phosphocreatine energy shuttle, cholinergic neurotransmission (choline, acetylcholine), and the glutamatergic system (glycine, glutamate) (Fig. 2).

Lactate levels were significantly higher in larvae exposed to additives ($p < 0.05$), and even more to pod film ($p < 0.001$) compared to controls and PVA (Fig. 2A). Similarly, significant increases in additives and pod film compared to controls ($p < 0.05$) were observed for betaine, acetylcholine, and choline, with pod film consistently inducing the highest concentrations across all treatments ($p < 0.01$, Fig. 2F-H). Glucose levels rose significantly in all experimental groups compared to controls ($p < 0.05$), with the strongest effect in pod film ($p < 0.001$), which also showed significantly higher levels than the single components (Fig. 2B). Similarly, creatine, phosphocreatine, and glycine levels

were all significantly increased ($p < 0.05$) in treated larvae, with pod film showing again the most pronounced increase ($p < 0.01$ vs other treatments, Fig. 2D-E, I). In contrast, while malate and glutamate significantly rose across all treatments ($p < 0.05$), the most substantial increases were observed for PVA and additives, rather than for pod film (Fig. 2C, J). Other compounds (*i.e.* acetate, alanine, arginine, BCAA, and various amino acids) showed no significant differences with controls (Fig. S6).

Analysis on the interactions of metabolites between treatments revealed synergistic effects for betaine, choline, and glucose, while glutamate and malate showed antagonistic effects (Fig. 2K). Together, these results highlight that pod film induced the most pronounced modulations of energy metabolism compared to the single components tested at their respective concentrations.

3.4. Biomarkers

The treatments induced selective alterations in oxidative stress- and neurotoxicity-related biomarkers, as well as disruption in the mitochondrial bioenergetics. Regarding oxidative stress, ROS levels were significantly affected by the treatments. Specifically, a highly significant ($p < 0.01$) decrease in ROS production for both PVA and additives was observed in treated larvae compared to controls (Fig. 4A). In contrast, SOD activity was not significantly altered by any treatment ($p > 0.05$) (Fig. 4B).

Neurotoxic effects seemed to emerge from the analysis of cholinergic and monoaminergic biomarkers. AChE activity revealed a significant increase due to additives and pod film ($p < 0.001$), both inducing more than a two-fold rise compared to controls (Fig. 4C). No significant difference in AChE activity was detected between PVA and controls, whereas the increase observed in additives and pod film was also significantly ($p < 0.01$) higher than that measured in PVA. MAO activity showed a more modest response, as only exposure to the pod film was significantly ($p < 0.01$) different from controls and PVA (Fig. 4D).

Analysis of mitochondrial bioenergetics (Fig. S7) revealed a treatment-specific response. Both basal and maximal respiration showed a significant increase ($p < 0.01$) only in larvae exposed to PVA compared to controls and the other treatments (Fig. 4E-F). Consistently, only PVA induced a significant increase ($p < 0.005$) in ATP production compared to controls (Fig. 4G).

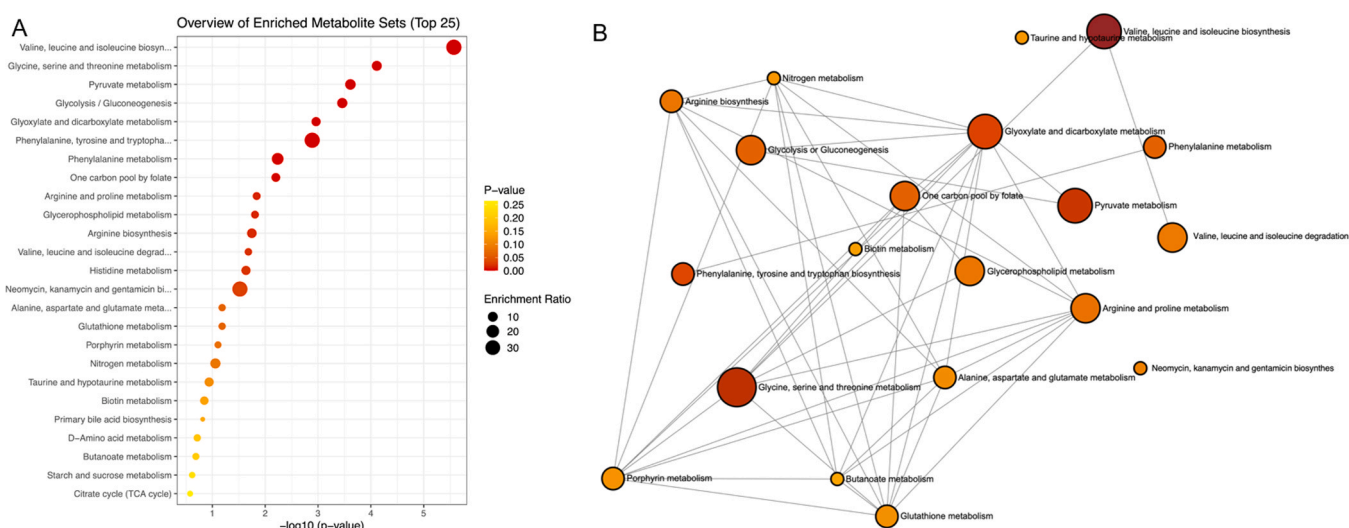
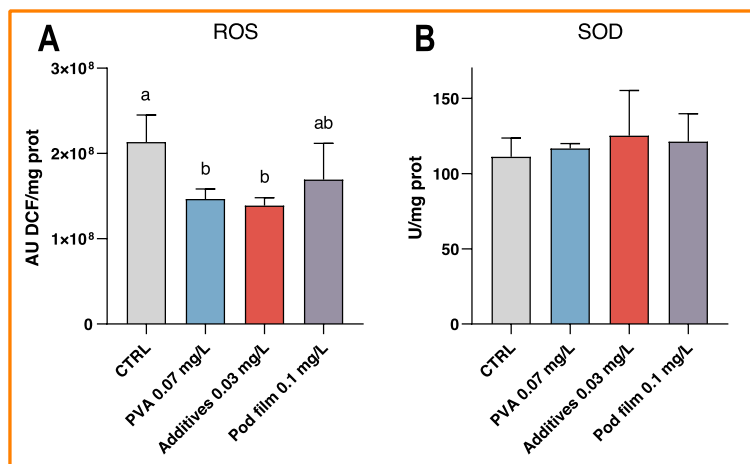
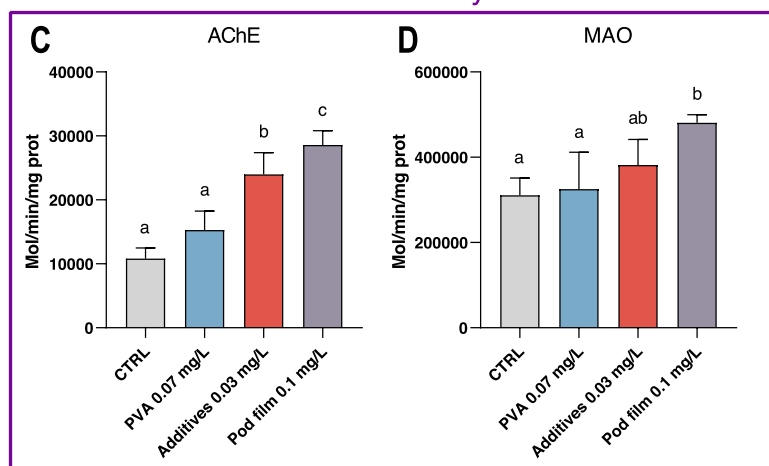


Fig. 3. (A) Metabolic pathway enrichment analysis of significantly altered metabolites in zebrafish embryos exposed to PVA, additives, and pod film. Bubble plot showing the top 25 significantly enriched KEGG pathways based on ^1H NMR metabolomic data. Circle size represents the enrichment ratio, and color intensity indicates statistical significance (from yellow: $p > 0.2$ to red: $p < 0.01$). Pathways are ranked by $-\log_{10}(p\text{-value})$; (B) Metabolic interaction network based on shared metabolites among significantly enriched pathways. Nodes represent individual KEGG pathways, and edges indicate shared metabolites between pathways. Node size reflects pathway impact (topological importance), and color corresponds to $p\text{-value}$.

Oxidative stress



Neurotoxicity



Energetic metabolism

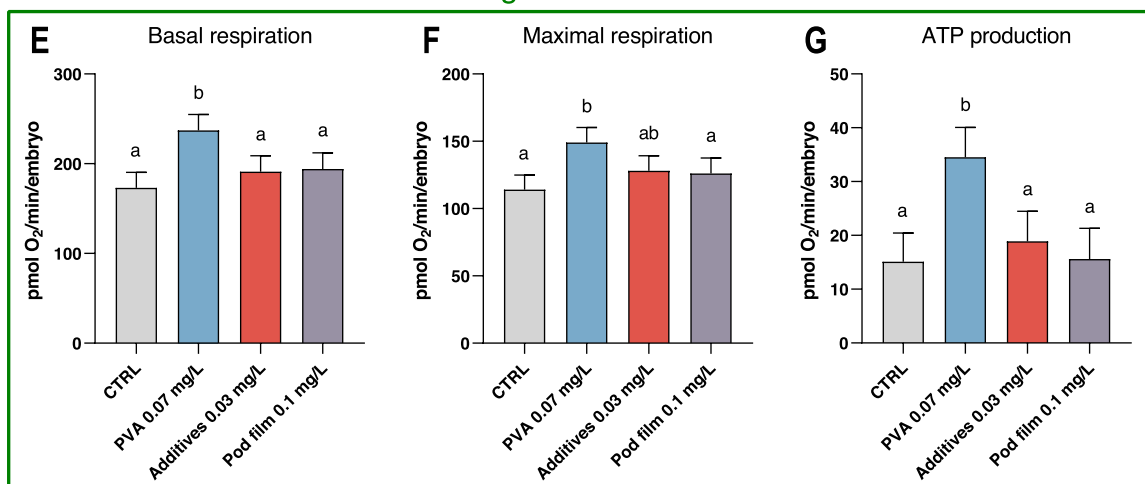


Fig. 4. Biomarkers of (i) oxidative stress: (A) reactive oxygen species (ROS) quantification, and (B) superoxide dismutase (SOD) activity; (ii) neurotoxicity: (C) acetylcholinesterase (AChE) activity, and (D) monoamine oxidase (MAO) activity measured in zebrafish larvae (120 hpf) exposed to PVA (0.07 mg/L), additives (0.03 mg/L), and pod film (0.1 mg/L). Data expressed as mean $\pm$ standard deviation (SD), statistical differences ($p < 0.05$) were determined by one-way ANOVA, followed by Fisher LSD *post-hoc* test ($n = 3$ pools of 25 specimens per treatment, from 3 independent exposures); (iii) energetic metabolism: (E) basal respiration, (F) maximal respiration, and (G) ATP production measured in zebrafish embryos (48 hpf) exposed to PVA (0.07 mg/L), additives (0.03 mg/L), and pod film (0.1 mg/L). Data are expressed as estimated marginal means (EMM) $\pm$ standard error (SE) derived from linear mixed models (LMM), statistical differences ($p < 0.05$) were determined by one-way ANOVA, followed by Fisher LSD *post-hoc* test ($n = 15$ embryos per treatment, from 3 independent exposures). Different letters indicate significant differences between groups.

3.5. Heart rate

Heart rate was significantly affected by the treatments, with a significant ($p < 0.05$) decrease only in larvae exposed to additives compared to controls and the other treatments, while a slight non-significant increase was induced by pod film (Fig. 5A).

3.6. Morphometry

Body length was significantly increased ($p < 0.01$) in larvae exposed to additives compared to controls and other treatments (Fig. 5B). Conversely, eye size was significantly ($p < 0.005$) decreased in larvae exposed to PVA, while a non-significant decreasing trend was observed for additives and pod film (Fig. 5C).

3.7. Behavioural assays: tail-coiling and locomotor activity

Spontaneous tail-coiling activity occurring inside the chorion at 48 hpf was significantly affected by additives and pod film. Specifically, both treatments caused a marked decrease ($p < 0.005$) in both burst count/min and burst activity compared to controls and PVA (Fig. 6A–B), while burst duration was not significantly affected by any treatment (Fig. 6C).

Behavioural analyses at 120 hpf revealed a robust light-dark response among all treatments, with a significant decrease in locomotor activity under light conditions compared to dark (Fig. 6D–E), as expected. Locomotor performance was not significantly altered by the treatments, although pod film induced a slight non-significant decrease in both total distance moved (Fig. 6D, S8) and mean swimming velocity under dark and light conditions (Fig. 6E). Thigmotactic behaviour was not significantly affected by any treatment (Fig. 6F), while the swimming pattern was altered by pod film under light condition, with a significant ($p < 0.05$) increase in turn angle compared to controls and PVA (Fig. 6G).

4. Discussion

This study represents the first integrated ecotoxicological assessment of a commercially available PVA-based dishwasher pod, combining chemical characterization with a multitier endpoints approach on zebrafish larvae, ranging from molecular to organism level. By separating the PVA matrix from the embedded additives and testing both isolated components individually and as complete pod film, we aimed to unravel both the single contribution of PVA and additives, and the potential mixture interactions. This goal provided a better understanding of the ecotoxicological effects of these commonly used household products, which can freely enter aquatic environments.

For clarity and ease of presentation, results for each endpoint group will be first discussed individually and subsequently integrated in the final remarks to provide a comprehensive overview of the overall effects.

4.1. Chemical separation and characterization

Overall, chemical analyses clearly demonstrated that the PVA-based film wrapping liquid dishwasher pods is not composed of PVA alone, but contains a complex mixture of additives, including polyols, PEG oligomers, alcohols, nitrogen-containing compounds, and hydrocarbon (Table 2). These compounds are commonly employed in polymer formulations as plasticizers, humectants, surfactants, antistatic agents, and processing aids [26–28]. Importantly, considering the sum of the different ethylene glycol derivatives (*i.e.* 3–6 EG), PEG oligomers represented one of the most abundant additives across both extraction methods, highlighting their central role in the formulation of PVA-based pod film. PEG oligomers are widely used as plasticizers and humectants due to their hygroscopic properties and compatibility with PVA matrices, increasing film flexibility and reducing moisture absorption by

PVA, thus improving product stability, conservation, and quality [28, 58]. Similarly, glycerin and 2-ethyl-2-(hydroxymethyl)-1,3-propanediol are commonly employed as secondary plasticizers and moisture-retention agents, enhancing mechanical resistance, preventing cracking during handling and storage, and regulating moisture uptake and dissolution behaviour of the film wrapping [59]. Alcohols and nitrogen-containing compounds are typically incorporated as surfactants, antistatic agents, or processing aids, facilitating film processing, reducing electrostatic charge accumulation during manufacturing, and influencing surface wettability and dissolution dynamics [27,58].

Overall, the identified compounds are predominantly low- to medium-molecular-weight species, as expected for additives designed to be water-soluble and compatible with PVA-based formulations. However, it should be noted that the adopted analytical approach targets the extractable and low-molecular-weight compounds and does not allow full characterization of additives fraction. Therefore, the obtained chemical profile should be considered as a partial but representative characterization of the additives mixture.

Despite this limitation, the results clearly demonstrate that the commercial product contains a complex mixture of multiple additives, most of which are expected to be low- to medium-molecular-weight compounds compatible with water solubility. This complex additives mixture is highly relevant from an ecotoxicological perspective, as exposure to commercial products inevitably involves complex mixtures rather than single compounds, thereby highlighting the need to address the issue of potential additive, synergistic, and antagonistic effects. Indeed, numerous studies demonstrated that combined exposures often differ from simple additivity, resulting in synergistic or antagonistic effects that cannot be predicted from individual components alone [60, 61]. Based on this evidence, the separation of PVA matrix from embedded additives and the characterization of this complex additives mixture provided essential information for interpreting the observed ecotoxicological results.

4.2. Metabolomics

Metabolomics enabled the identification of compound-specific metabolic fingerprints and offered mechanistic insights into cellular responses at sub-lethal concentrations [62–64]. In zebrafish larvae, metabolomic profiling allowed the detection of early alterations in key metabolic pathways, including energy metabolism, amino acid turnover, oxidative stress response, and neurotransmitter pathways such as glutamatergic and cholinergic signalling [65–67]. Moreover, integration of metabolomics with cellular, physiological and organismic endpoints, such as cardiac activity, immune modulation, neuromuscular activity, and locomotor performance, can enhance the predictive power of developmental toxicity assessments [40,66,68,69]. This multitier approach has proven particularly effective in identifying latent or delayed toxicological effects, especially when assessing environmentally relevant concentrations of contaminants [20,57].

Untargeted ^1H NMR metabolomics revealed a consistent trend among treatments with significant increases in key metabolites, particularly those involved in energy metabolism, creatine cycle, osmoregulation, and neurotransmission (Fig. 2). In detail, all treatments induced significant increases in key energy-related metabolites (glucose, creatine/phosphocreatine, lactate), a pattern commonly reported in zebrafish larvae exposed to chemical stressors and interpreted as an adaptive response to maintain cellular energy homeostasis [70,71].

Specifically, exposure to PVA alone was characterized by high glutamate and malate increases. These profiles are consistent with enhanced mitochondrial oxidative metabolism, as supported by the observed stimulation of respiratory parameters, with significant increases in basal and maximal respiration, as well as in ATP production (Fig. 4). In larvae exposed to additives, malate showed the strongest increase among treatments (Fig. 2), suggesting a preferential engagement of anaplerotic inputs supporting the tricarboxylic acid (TCA) cycle

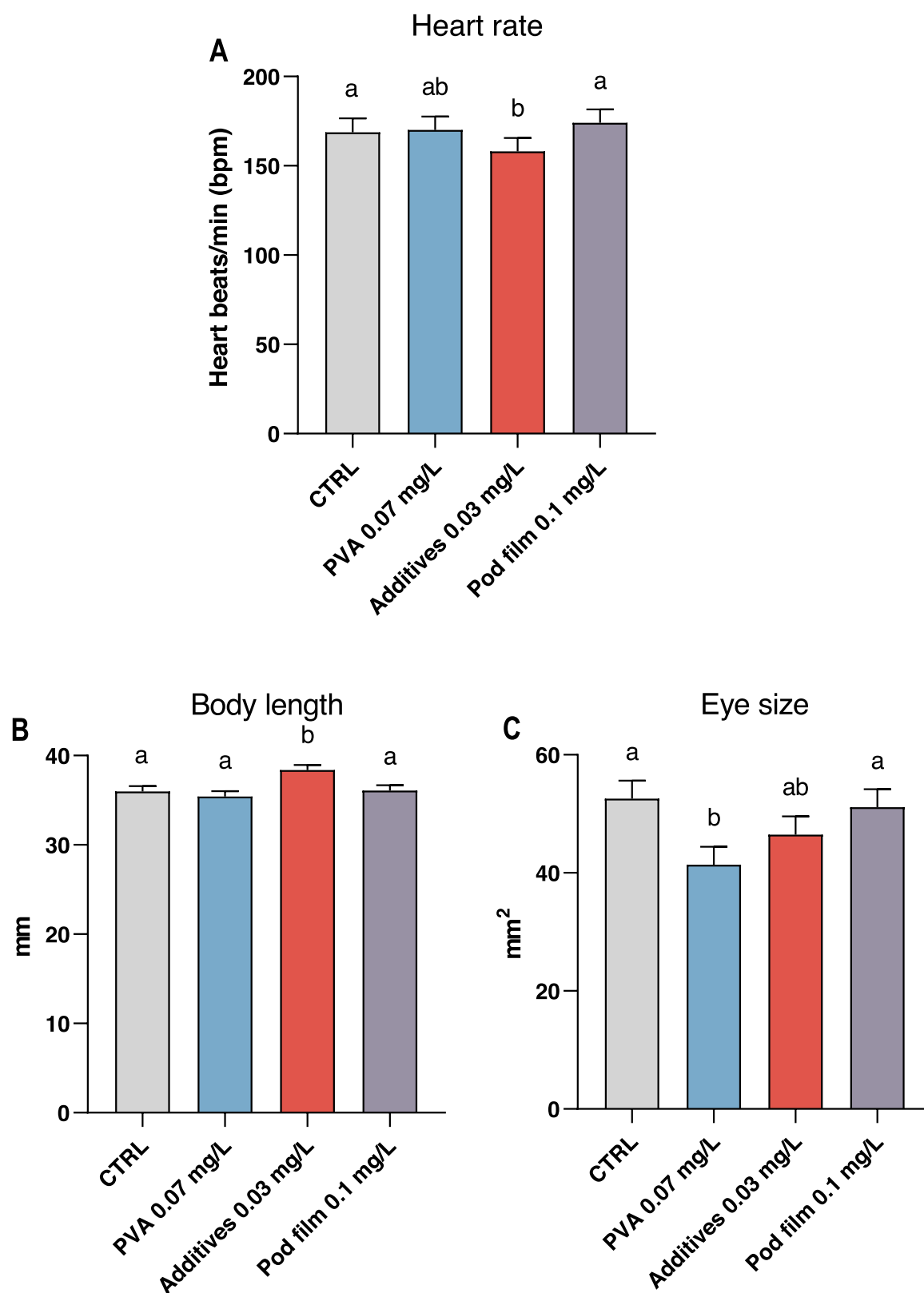


Fig. 5. (A) Cardiac activity, and morphometric parameters: (B) body length, and (C) eye size of zebrafish larvae (120 hpf) exposed to PVA (0.07 mg/L), additives (0.03 mg/L), and pod film (0.1 mg/L). Data are expressed as estimated marginal means (EMM) $\pm$ standard error derived from linear mixed models (LMM), statistical differences ($p < 0.05$) were determined by one-way ANOVA, followed by Fisher LSD *post-hoc* test ($n = 15$ larvae per treatment, from 3 independent exposures). Different letters indicate significant differences between groups.

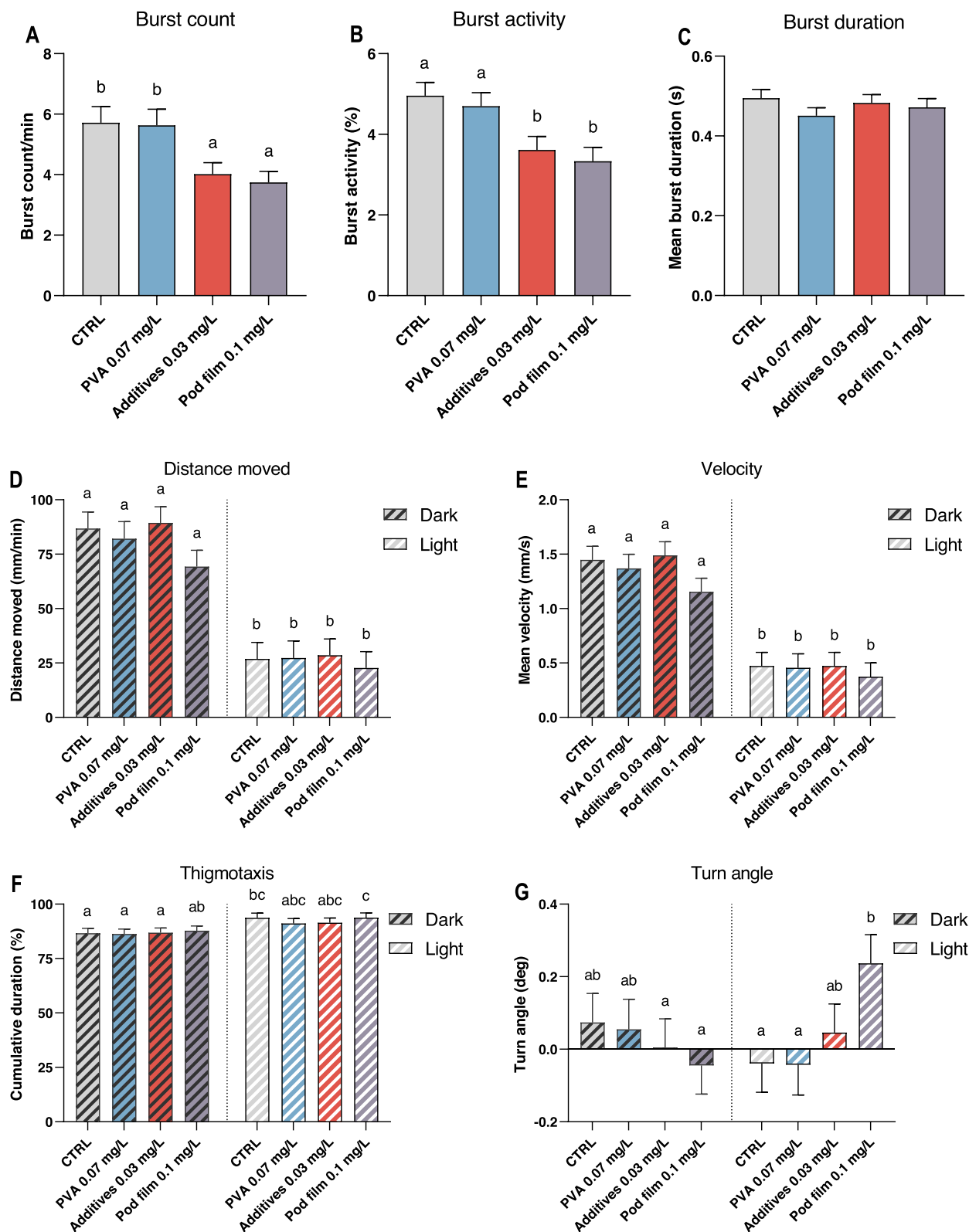


Fig. 6. Behavioural parameters of zebrafish exposed to PVA (0.07 mg/L), additives (0.03 mg/L), and pod film (0.1 mg/L) (i) after 48 hpf: (A) burst count per min, (B) burst activity, and (C) mean burst duration ($n = 30$ embryos per treatment, from 3 independent exposures); and (ii) after 120 hpf: (D) total distance moved per min, (E) velocity, (F) thigmotaxis, (G) turn angle ($n = 36$ larvae per treatment, from 3 independent exposures). For panels D-G larvae were subjected to two consecutive cycles of alternating dark (10 min, black striped pattern) and light (5 min, 2200 lux, white striped pattern) conditions, for a total test duration of 30 min. Data are expressed as estimated marginal means (EMM) $\pm$ standard error derived from linear mixed models (LMM), statistical differences ($p < 0.05$) were determined by one-way ANOVA, followed by Fisher LSD *post-hoc* test. Different letters indicate significant differences between groups and/or condition (light-dark).

to contribute to energy production [72]. This metabolic signature may reflect an increased involvement of the TCA cycle-related pathways and a parallel activation of glycolytic support mechanisms, but in the absence of a corresponding increase in mitochondrial respiration and ATP production (Fig. 4). Such partial metabolic activation without respiratory upregulation has been reported in zebrafish exposed to PFAS, where cells rely on mixed metabolic strategies to cope with chemical stress [73].

Finally, exposure to pod film produced the most pronounced accumulation of glycolytic and phosphagen metabolites, without OCR stimulation (Figs. 2, 4). This response is consistent with a shift toward glycolysis rather than enhanced oxidative phosphorylation. Similar rise of glycolytic intermediates and high-energy phosphate reservoirs have been described in zebrafish larvae following exposure to plastic-associated contaminants [74,75] and other environmental toxicants [76], reflecting increased energetic demand under stress conditions, and early compensatory metabolic stress responses.

To contextualize metabolite-level changes, a global pathway enrichment analysis was carried out (Fig. 3) enabling the identification of metabolic pathways consistently disrupted by the treatments during early zebrafish development. Specifically, pathway enrichment analysis revealed that pod film induced a coordinated reprogramming of many core metabolic functions. The most significantly affected pathways included glycolysis/gluconeogenesis, pyruvate metabolism, and glycine, serine and threonine metabolism, reflecting a substantial disturbance in cellular energy homeostasis and mitochondrial function. These pathway-level changes agree with the observed elevated glucose and lactate levels, as well as increased creatine and phosphocreatine (Fig. 2), indicating a shift toward aerobic glycolysis and altered energy buffering systems. Additional enrichment was observed in glutathione metabolism, arginine and proline metabolism, as well as in glycerophospholipid metabolism, suggesting emerging redox imbalance, membrane remodelling, and activation of osmotic stress responses. Notably, the involvement of one-carbon pool by folate and betaine metabolism points to perturbed methylation cycles and osmoregulatory mechanisms, particularly in additives and pod film. The pathway enrichment bubble plot (Fig. 3A) ranked these pathways by statistical significance and enrichment ratio, highlighting clusters related to amino acid turnover, energy metabolism, and redox balance as the most impacted.

Complementary network analysis (Fig. 3B) further supported this hypothesis, identifying glycolysis, glyoxylate and dicarboxylate metabolism, glutathione metabolism, and glycine/serine/threonine metabolism as central hubs within the metabolic interaction network. These hubs bridge critical functional domains involved in bioenergetics, oxidative stress regulation, and amino acid metabolism, underlining the systemic nature of the metabolic disruption. Together, these results indicate that exposure to pod film and their chemical components induces an integrated remodelling of zebrafish larvae metabolism, characterized by enhanced glycolytic flux, mitochondrial compensation, redox pathway activation, and adaptive responses in amino acid and osmoregulatory circuits, likely contributing to the functional phenotypes observed in exposed larvae. Interestingly, these findings indicate a clear treatment-specific response in key metabolic pathways, rather than a simple generalized metabolic alteration, with the most pronounced increases typically observed in larvae exposed to the pod film.

Multivariate analysis of the ^1H NMR metabolomic profiles supported the clear treatment-specific metabolomic response observed among treatments. The first two principal components, PC1 and PC2, accounted for 48.8% and 20.6% of the total variance, respectively (Fig. S8). PCA revealed tight clustering of biological replicates within each treatment, indicating high within-group consistency, and a clear separation among treatments, forming distinct clusters in the PCA space. Control samples were clearly separated from all other treatments, while PVA and additives displayed intermediate profiles with partially overlapping regions of the plot, distinct from both controls and pod film. In contrast, larvae

exposed to the pod film occupied the furthest distance from controls along PC1. This pattern indicates robust divergence suggesting that pod film induces stronger metabolic alterations than the component individually, consistent with the observed synergistic effects (Fig. 2K). However, the limited number of biological replicates ($n = 3$) reduces the statistical robustness of the observed clustering between treatments.

4.3. Biomarkers

Despite the clear metabolic disruption, ROS levels were significantly decreased in larvae exposed to PVA and additives, while pod film induced only a non-significant decreasing trend (Fig. 4). The lack of SOD activation further suggests that larvae experienced a metabolic disruption characterized by redox remodelling, rather than experiencing evident oxidative stress. This interpretation is consistent with the metabolomic evidence indicating a shift toward glycolytic pathways, as discussed above, and is further supported by the perturbation of glutathione-related metabolism. Indeed, glutathione plays a central role in cellular redox regulation, acting as a key antioxidant buffer and modulator of ROS signalling.

Nevertheless, these findings are in contrast with the classical oxidative stress response observed in zebrafish exposed to micro- and nanoplastics, which typically determines increased ROS production and activation of antioxidant enzymes linked to oxidative stress [77–79]. Similarly, oxidative activation was also reported in zebrafish larvae exposed to other WSPs, such as PVP and PAA [21], or in tadpoles exposed to PEG [17].

Notably, ROS should not be considered only as toxic byproducts of cellular metabolism. At physiological levels, ROS also act as essential signalling molecules regulating multiple biological processes, including innate immune maturation, cell differentiation, cardiac development and organogenesis [80,81]. Therefore, suppression of basal ROS levels can impair immune activation and alter developmental pathways in zebrafish larvae. Thus, the observed ROS decrease after PVA and additives exposures suggests the emergence of a non-classical redox response, characterized by suppression of basal ROS signalling, which may potentially compromise redox-dependent developmental pathways.

Moving to neurotoxicity biomarkers, additives and pod film elicited clear neurotoxic alterations, with increased AChE and MAO activities, consistent with metabolomics. This response is supported by previous studies reporting neurotoxic alterations in zebrafish and other organisms following exposure to polymers, including micro- and nanoplastics, as well as WSPs [21,82]. For instance, increased AChE activity was reported in adult zebrafish exposed to PEG-400 and organic solvent mixtures [83], in *Daphnia magna* following PEG exposure [20], and in amphibian tadpoles exposed to PEG [17]. Conversely, some WSPs, such as PVP, have shown concentration-dependent or even opposite responses [21], highlighting the complexity of polymer-specific neurotoxic mechanisms, although the underlying MoA remains poorly understood.

A possible hypothesis is that the observed enhanced AChE activity may reflect a compensatory regulation of cholinergic signalling in response to neurochemical imbalance, potentially aimed to limit excess of acetylcholine in synaptic clefts, as similarly reported in fish exposed to sub-lethal neurotoxicants [84]. Indeed, increased AChE activity enhances the hydrolysis of acetylcholine, resulting in accumulation of free choline in the synaptic cleft of motoneurons and consequent reduced cholinergic signalling, potentially impairing muscle contraction and neurodevelopmental processes [84]. Contemporarily, the increased MAO activity only under pod film exposure enhanced the monoamine catabolism and turnover, suggesting neurotoxic stress response, with disruption of monoaminergic pathways [85].

Notably, these neurotoxicity biomarkers align closely with the metabolomic findings exhibiting increase in choline, betaine, and acetylcholine metabolites following additives and pod film exposure.

The stronger neurotoxic effects observed for pod film compared to individual components further demonstrate the occurrence of mixture-specific toxicity, consistent with the synergistic metabolic alterations previously discussed.

The two major cellular pathways engaged in energy production, namely the mitochondrial oxidative phosphorylation and glycolysis, involve cellular consumption of oxygen and protons efflux, respectively. Accordingly, OCR provide a direct representation of mitochondrial respiration, while changes in OCR could indicate metabolic switching from oxidative phosphorylation to glycolysis [51].

In the present study, OCR measurements were performed at 48 hpf, a developmental stage that captures early metabolic responses prior to hatching. The energetic metabolism results revealed that PVA significantly altered mitochondrial activity in zebrafish embryos, as indicated by increased basal and maximal respiration as well as ATP production (Fig. 4). These findings closely align with the metabolomic signatures observed for PVA, suggesting an upregulation of mitochondrial oxidative phosphorylation to sustain an increased energetic demand. Such responses are typically interpreted as compensatory mitochondrial activation aimed at maintaining ATP homeostasis under stress conditions or during increased energy requirements [51,55].

In contrast, despite the stronger metabolomic signatures indicative of energetic alterations, additives and pod film did not induce significant changes in OCR. This suggests a possible metabolic rearrangement with a greater contribution of glycolysis to energy production, although glycolytic flux was not directly measured through extracellular acidification rate (ECAR) [51,55]. Similar responses have been reported in zebrafish embryos exposed to complex contaminant mixtures, where mitochondrial respiration did not meet the energetic demand, leading to increased reliance on glycolytic metabolism to sustain developmental processes [50]. Similarly, the β -methylamino-L-alanine (BMAA) induced metabolic reprogramming characterized by lactate accumulation and altered amino acid metabolism, indicating a shift toward glycolytic energy production to compensate for mitochondrial dysfunction or energetic stress [70]. Together, these findings suggest that PVA primarily stimulates mitochondrial respiration to sustain increased energy demand, while additives and pod film induce a metabolic shift toward glycolysis. This divergence further supports the hypothesis that the different treatments exert distinct and complex MoAs, once again suggesting the need to assess the (eco)toxicological effects of commercial products rather than pure polymers.

4.4. Heart rate

Cardiac function represents a particularly sensitive physiological endpoint during early zebrafish development, tightly coupled to metabolism and neurochemical regulation [86]. Indeed, several studies have demonstrated that emerging polymeric contaminants, such as micro- and nanoplastics, can cause cardiotoxicity and altered cardiac system development in zebrafish larvae [75,77,79], and even WSPs [20,21].

In the present study, the heart rate of zebrafish larvae was significantly decreased only by additives (Fig. 5 A), suggesting a treatment-specific response likely due to several factors. First, the chemical composition of additives could directly contribute to the observed bradycardia. Indeed, several compounds identified by ^1H NMR, including polyhydric alcohols and PEG oligomers, have been associated with developmental cardiotoxicity in aquatic organisms [87,88]. A significant heart rate reduction was reported in zebrafish larvae exposed to high-molecular-weight PEGs (8000 and 20000 Da) already at 0.1 mg/L [87]. Similarly, exposure to electronic cigarette liquids, mainly composed of propylene glycol and glycerin, induced a significantly decreased heart rate, suggesting a potential role of these polyols in early cardiotoxicity [88].

The decreased heart rate could be also due to the metabolic and neurochemical alterations identified for additives (Fig. 5 A). Since catecholaminergic signalling plays a central role in cardiac regulation,

the observed alterations in cholinergic-related metabolites and increased MAO activity in larvae exposed to additives could reduce adrenergic stimulation of the heart, caused by increased monoamine degradation, and therefore contribute to the observed bradycardia [89]. In contrast, pod film did not induce significant bradycardia despite exhibiting stronger metabolomic alterations. This apparent discrepancy may reflect compensatory mechanisms activated in response to combined complex exposure that may cover early cardio-depressive effects.

Together, these findings indicate that the observed cardiac responses could result from several factors, including metabolic stress, neurochemical alterations, redox imbalance, and compensatory mechanisms. These findings, namely additives inducing a clearer bradycardic response and pod film eliciting a more complex buffered response, suggest that single components and pod film can modulate different toxicity profiling.

4.5. Morphometric analyses

At the organismic level, the increased body length induced by additives and the decreased eye size induced by PVA (Fig. 5B-C) revealed clear treatment-specific effects. Notably, these findings deviated from the expected classical stress response, characterized by the simultaneous increase of metabolic rate, heart rate, and body size. Instead, our data indicate a different and more complex response for additives exposure, with evidence of energetic metabolism activation with increased body length, however accompanied by decreased ROS level and heart rate, likely reflecting a metabolic reprogramming. Similar findings, with significantly decreased heart rate, but increased body weight, were recently reported by Hua et al. in zebrafish larvae exposed to microplastics of polypropylene and explained as developmental toxicity [75]. They further hypothesised that the increase in body weight may be linked to metabolic disruption leading to glucose accumulation. Consistent with this interpretation, the present study also reported increased energy-related metabolites (Fig. 2), supporting metabolic disruption as a plausible explanation for the observed increased body size.

In contrast, PVA selectively reduced eye size, highlighting this organ as a potential target of this WSP. A previous study already reported significant alterations of some proteins involved in retinal development and visual system maturation of zebrafish larvae exposed to some WSPs [21]. Furthermore, also exposure to nanoplastics of polystyrene was reported to impair eye development in zebrafish larvae [90]. These findings suggest that PVA, as other synthetic polymers, could target ocular tissues, possibly due to direct interaction with mucosal surfaces or due to the transport from the blood system.

4.6. Behaviour

Behavioural changes following exposure to micro- and nanoplastics, as well as WSPs, have been increasingly documented in zebrafish and other aquatic organisms, frequently linked to neurotransmission alterations and metabolic disruptions [20,75]. In more detail, the spontaneous tail-coiling activity and locomotor behaviour of zebrafish are sensitive indicators of neurodevelopment alterations induced by some environmental contaminants [75,79,91]. Specifically, tail-coiling activity represents one of the earliest functional outputs of the developing motor coordination and is commonly used to detect subtle neurodevelopmental alterations [75,79,91].

In the present study, a significant decrease in spontaneous tail-coiling activity was observed following exposure to additives and pod film (Fig. 6). Similar suppression of spontaneous tail-coiling activity were reported for zebrafish larvae exposed to nanoplastics of polystyrene and polypropylene, supporting the sensitivity of this endpoint to polymer contaminants [75,79]. This decreased tail-coiling activity aligns closely with the significant alterations in metabolomics (Fig. 2) and biomarkers related to cholinergic and monoaminergic pathways

described above (Fig. 4), all affecting neuromuscular control and playing a critical role in shaping early locomotor networks [78,92]. Notably, PVA alone did not significantly affect early tail-coiling activity, consistent with its limited effects on neurotoxicity-related endpoints, further supporting distinct treatment-specific MoA.

On the contrary, zebrafish larvae after 120 hpf exhibited no considerable alterations among treatments in swimming patterns and stress-related behaviour. Only minor responses were observed, including a significant increase in turn angle under light conditions for PVA, and a slight non-significant decrease under both dark and light conditions induced by pod film (Fig. 6). Importantly, larvae exhibited a robust light-dark response among all treatments, with significant decrease in locomotor activity under light conditions compared to dark. This response, consistent with the expected freezing behaviour adopted by zebrafish larvae to reduce predation [93], confirms both the validity of the behavioural assay and the robustness of the experimental setup.

These results indicate that larvae preserved overall swimming behaviour at later developmental stages, despite earlier alterations discussed above. This apparent recovery may reflect compensatory mechanisms, suppressing earlier behavioural responses, and neurodevelopmental plasticity that allow larvae to maintain locomotor performance despite underlying neurotoxic stress. Supporting this, is well documented that zebrafish larvae can buffer early functional disturbances during rapid embryonic development [94,95].

Alternatively, alterations observed at the lower levels of biological organisation may preferentially affect behavioural domains not highlighted by the measured basal locomotor endpoints. For instance, previous studies have shown that subtle neurotoxic stress could impair stimulus-response behaviours, such as touch-escape response, prey capture efficiency, startle response, or predator avoidance [92,96], all endpoints that could be investigated in the future.

4.7. Final remarks

The extensive dataset, encompassing multiple endpoints across different levels of biological organization, was very challenging to manage. In an attempt to correlate the obtained results, to investigate both the MoAs and the effects induced by the individual pod components (PVA and additives), as well as by the complete pod, metabolomic findings were integrated with biomarkers of oxidative stress and neurotoxicity, energetic metabolism, and cardiac, morphometric, and behavioural responses. Such integrative frameworks are increasingly recognized as essential for capturing early responses to emerging contaminants and improving environmental risk assessment [20,57], particularly in the case of commercial products, whose highly heterogeneous composition may lead to different biological effects across biota.

Notably, alterations at higher levels of biological organisation integrated coherently with molecular and cellular responses, providing mechanistic insight into treatment-specific MoA. This consistency among different levels of biological organization highlights the importance of multitier approaches, as reliance on single endpoints could fail to explain the complexity of responses. Indeed, this multilevel approach has proven particularly effective in identifying subtle or early toxicological effects, especially when assessing environmentally relevant concentrations [20,57].

Despite the use of environmentally relevant concentrations (0.1 mg/L for pod film and the corresponding concentration for individual components: 0.07 and 0.03 mg/L for PVA and additives, respectively) and the relatively short exposure time (120 hpf), multiple biologically relevant alterations were observed. Notably, treatments affected the different levels of biological organization in distinct and sometimes partially independent ways, highlighting the complexity of the toxicological responses.

In this regard, the application of integrative statistical approaches can help in the interpretation of such a complex dataset and of the effects

induced by the three treatments to zebrafish larvae, while simultaneously highlighting the most affected metabolic pathways. In particular, the heatmap provided a comprehensive visualization of the direction and magnitude of responses among measured endpoints, highlighting distinct response patterns among treatments (Fig. 7A). PVA mainly targeted mitochondrial respiration and eye development, suggesting a MoA centred on energetic dysregulation and tissue-specific sensitivity. In contrast, additives exerted widespread metabolomic disruption, stronger neurotoxic responses, bradycardia, and increased body length, consistent with exposure to a heterogeneous mixture of additives and plasticizers. Pod film, representing the combined matrix, generally elicited the most pronounced metabolic reprogramming, also through synergistic interactions, together with marked neurotoxic activation, and behavioural suppression, while simultaneously showing possible compensatory mechanism that may partially preserve cardiac function and swimming behaviour. Furthermore, the heatmap also evidenced a general trend of stronger alterations at lower levels of biological organization, particularly metabolomics, with progressively attenuated responses at higher levels such as physiological and behavioural endpoints. This pattern is consistent with the concept that molecular responses often represent early indicators of stress that may not always translate directly into organism-level effects.

To further explore treatment-related divergence, PCA was used to confirm the divergence in ecotoxicological responses observed among the three components. It clearly showed controls and PVA clustering in the same quadrant, additives occupying the opposite side along PC1, and pod film occupying the furthest distance from other treatments along PC2 (Fig. 7B). This confirms that the three treatments elicited different biological pathways, reinforcing the hypothesis that PVA, additives, and pod film exerted distinct MoA.

A key finding of this study is that biological responses from pod film exposure cannot be predicted by simply summing the effects of their individual components (PVA and additives). Indeed, pod film yielded synergistic emergent effects that differed both qualitatively and quantitatively from exposures to individual components. This likely reflect different bioavailability and release kinetics when additives are embedded in the PVA matrix compared to isolated additives. PVA matrix can modulate the effective concentration and exposure dynamic of additives, leading to toxicological outcomes that are not predictable from single components exposures [60,90,97]. Therefore, these findings highlight the importance of mixture effects, and underscore the need to evaluate commercial products rather than extrapolating from individual components alone.

It is important to stress out once again that exposure concentrations differed markedly among treatments, with PVA and additives representing 70 and 30% of the pod film concentration, respectively. Therefore, to better compare the relative contribution of each specific treatment to overall toxicity, responses were concentration-normalized to 0.1 mg/L, assuming a local linear dose-response. Since toxicological responses often follow sigmoidal dose-response slope [98,99], the assumption of local linearity provides a reasonable approximation in the central part of the curve, allowing an overall realistic normalization, while not providing precise dose-response characterization.

After normalization, to further integrate the observed toxicity across biological levels and allow direct comparison across heterogeneous biological responses, each endpoint was transformed into a normalized index (IERI) which confirmed that additives exerted the highest toxicity (Fig. 7C). Finally, the radar plot revealed that additives always exerted the most pronounced alterations across endpoints, particularly for metabolomics, neurotoxicity, energetic metabolism, and behaviour (Fig. 7D), suggesting their substantial contribution to the observed toxicity. Accordingly, our previous study on another commercial PVA-based product suggested that additives could substantially contribute to the toxicity of these products [25]. However, so far, we lack sufficient information to explain the specific contribution of single additives, and will certainly be very interesting to explain this specific effect in future

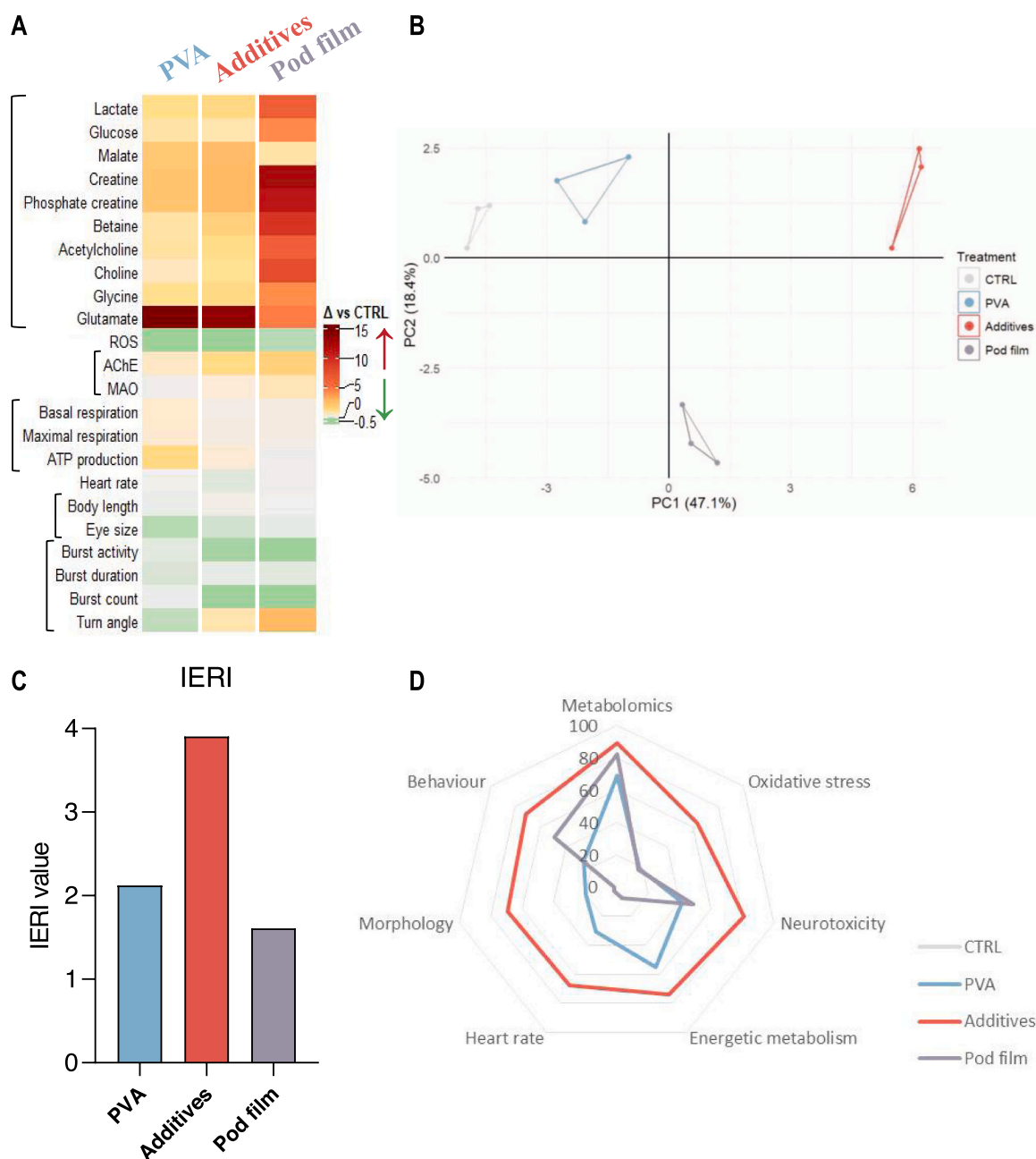


Fig. 7. Integrated comparison of ecotoxicological responses across treatments (CTRL, PVA, additives, and pod film). (A) heatmap of endpoint-specific deviations from controls (Δ vs CTRL) among treatments, showing magnitude and direction of responses using a colour scale (red: up-regulation; grey: no-effects; green: down-regulation); (B) Principal Component Analysis (PCA) performed on all measured endpoints (excluding embryotoxicity) showing clustering patterns among treatments; (C) Integrated Endpoints Response Index (IERI), summarizing the effects (normalized to 0.1 mg/L equivalent concentration) of all endpoints into a single weighted score, with higher IERI values indicating stronger overall biological impact; and (D) Radar plot showing normalized responses of significant endpoints grouped by categories (0.1 mg/L equivalent concentration) expressed as deviations from controls, enabling cross-level comparison of biological effects.

studies.

The study highlights the role of additives in the observed toxicity, while emphasizing the importance of mixture-specific effects arising from the interaction between polymer matrix and associated compounds.

Overall, although acute lethality and evident malformations were absent, subtle yet biologically relevant alterations were clearly detected. This highlights that conventional apical endpoints may considerably underestimate the ecological impact of pods exposure. Indeed, metabolic reprogramming, neurodevelopmental disruption, and physiological alterations, such as those reported in this study, could have long-term consequences for the fitness, including altered predator

avoidance and reproductive success, even in the absence of mortality [92,96]. Therefore, these results underscore the importance of integrating sensitive sub-lethal and multitier endpoints when evaluating WSPs.

Importantly, in realistic environmental scenarios, the continuous environmental release of these household products likely results in long-term and persistent exposure of aquatic organisms. Thus, the alterations observed in this study at environmentally relevant concentrations may represent early warning signals of potential environmental risks to aquatic species.

Taken together, these findings underscore the need to move beyond exclusively single-compound approaches by considering real household

products, typically composed of complex mixture of chemicals, in environmental risk assessment.

5. Conclusions

Despite this study provides the first integrated ecotoxicological assessment of commercial PVA-based dishwasher pods, several questions remain open. For instance, future studies should aim to identify the specific additives responsible for the observed biological responses and to determine their environmental occurrence in aquatic ecosystems. Furthermore, extending exposure scenarios to longer developmental stages would help to clarify whether the early alterations detected in zebrafish embryos translate into long-term ecological consequences. Understanding the environmental fate, bioavailability, and interaction dynamics of WSP formulations will therefore be essential to fully assess their environmental impact. In this perspective, the development of safer polymer formulations and alternative additives with reduced ecotoxicological effects could represent an important step toward more sustainable household products.

Environmental implication

This study provides evidence that water-soluble PVA-based films from widely used dishwasher pods can induce biologically relevant sublethal effects in aquatic organisms at environmentally relevant concentrations. Considering the extensive and continuous release of these products into wastewater systems, the potential chronic exposure to aquatic organisms should not be overlooked. Importantly, the observed toxicity could not be predicted from the sum of individual components alone, highlighting the critical role of mixture interactions and the limitations of current risk assessment approaches based on single substances. The substantial contribution of additives to the observed toxicity further highlights the need to evaluate commercial products as complex chemical mixtures rather than isolated polymers. Overall, these findings suggest that WSP-based formulations, often claimed as environmentally safe, may pose underestimated ecological risks and should be therefore more carefully considered in environmental monitoring and regulatory frameworks.

CRedit authorship contribution statement

Gazzotti Stefano: Writing – review & editing, Investigation. **Cremonesi Cristina:** Writing – review & editing, Investigation. **Ortenzi Marco:** Writing – review & editing. **Binelli Andrea:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization. **Caorsi Giada:** Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Galati Mariachiara:** Software, Investigation, Formal analysis, Data curation. **Billè Barbara:** Writing – review & editing, Writing – original draft, Visualization, Software, Formal analysis. **Magni Stefano:** Writing – review & editing, Supervision, Methodology. **Cappello Tiziana:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Data curation.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jhazmat.2026.143192](https://doi.org/10.1016/j.jhazmat.2026.143192).

Data availability

Data will be made available on request.

References

- [1] Lefay, C., Guillauneuf, Y., 2023. Recyclable/degradable materials via the insertion of labile/cleavable bonds using a comonomer approach. *Prog Polym Sci* 147, 101764. <https://doi.org/10.1016/j.progpolymsci.2023.101764>.
- [2] 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. <https://doi.org/10.1016/j.watres.2020.115931>.
- [3] Duis, K., Junker, T., Coors, A., 2021. Environmental fate and effects of water-soluble synthetic organic polymers used in cosmetic products. *Environ Sci Eur*. <https://doi.org/10.1186/s12302-021-00466-2>.
- [4] DeLeo, P.C., Summers, H., Stanton, K., Lam, M.W., 2020. Environmental risk assessment of polycarboxylate polymers used in cleaning products in the United States. *Chemosphere* 258, 127242. <https://doi.org/10.1016/j.chemosphere.2020.127242>.
- [5] EFSA, 2020. FAF Panel (EFSA Panel on Food Additives and Flavourings (FAF)), Younes, M., Aquilina, G., Castle, L., Engel, K.H., et al., 2020. Scientific opinion on the re-evaluation of polyvinylpyrrolidone (E 1201) and polyvinylpyrrolidone (E 1202) as food additives and extension of use of polyvinylpyrrolidone (E 1201). *Sci Total Environ* 6215. <https://doi.org/10.2903/j.efsa.2020.6215>.
- [6] Hisar, O., Oehlmann, J., 2023. Individual and combined ecotoxic effects of water-soluble polymers. *PeerJ* 11, e16475. <https://doi.org/10.7717/peerj.16475/supp-1>.
- [7] Kleemann, K., Sander, M., 2023. Water-soluble and water-dispersible polymers used in commercial agricultural formulations: inventory of polymers and perspective on their environmental fate. *Chimia (Aarau)* 77, 764–772. <https://doi.org/10.2533/chimia.2023.764>.
- [8] Halake, K., Birajdar, M., Kim, B.S., Bae, H., Lee, C.C., Kim, Y.J., et al., 2014. Recent application developments of water-soluble synthetic polymers. *J Ind Eng Chem* 20, 3913–3918. <https://doi.org/10.1016/j.jiec.2014.01.006>.
- [9] Vajihinejad, V., Gumfekar, S.P., Bazoubandi, B., Rostami Najafabadi, Z., Soares, J. B.P., 2019. Water soluble polymer flocculants: synthesis, characterization, and performance assessment. *Macromol Mater Eng* 304, 1800526. <https://doi.org/10.1002/mame.201800526>.
- [10] 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. *Water Res* 245, 120650. <https://doi.org/10.1016/j.watres.2023.120650>.
- [11] ECHA, povidone. EC number: 658-049-4, CAS number: 9003-39-8. <https://echa.europa.eu/it/substance-information/-/substanceinfo/100.184.844>.
- [12] Pastorino, P., Broccoli, A., Anselmi, S., Bagolin, E., Prearo, M., Barceló, D., et al., 2022. The microalgae *Chaetoceros tenuissimus* exposed to contaminants of emerging concern: a potential alternative to standardized species for marine quality assessment. *Ecol Indic* 141, 109075. <https://doi.org/10.1016/j.ecolind.2022.109075>.
- [13] PubChem, 2026. Compound summary for CID 11199, vinyl alcohol; National Center for Biotechnology Information, U.S. National Library of Medicine. <https://pubchem.ncbi.nlm.nih.gov/compound/11199>.
- [14] DeMerlis, C.C., Schoneker, D.R., 2003. Review of the oral toxicity of polyvinyl alcohol (PVA). *Food Chem Toxicol* 41, 319–326. [https://doi.org/10.1016/s0278-6915\(02\)00258-2](https://doi.org/10.1016/s0278-6915(02)00258-2).
- [15] Olalla, A.L., Sara, L.I., Ricardo, B., 2021. Assessment of toxicity and biodegradability of poly(vinyl alcohol)-based materials in marine water. *Polym (Basel)* 13. <https://doi.org/10.3390/polym13213742>.
- [16] Hatami, M., Banaee, M., Nematdoost Haghi, B., 2019. Sub-lethal toxicity of chlorpyrifos alone and in combination with polyethylene glycol to common carp (*Cyprinus carpio*). *Chemosphere* 219, 981–988. <https://doi.org/10.1016/j.chemosphere.2018.12.077>.
- [17] Nascimento, Í.F., Guimarães, A.T.B., Ribeiro, F., Rodrigues de Lima, A.S., Estrela, F. N., da Luz, T.M., et al., 2021. Polyethylene glycol acute and sub-lethal toxicity in neotropical *Physalaemus cuvieri* tadpoles (Anura, Leptodactylidae). *Environ Pollut* 283, 117054. <https://doi.org/10.1016/j.envpol.2021.117054>.
- [18] 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. <https://doi.org/10.1016/j.scitotenv.2022.157608>.
- [19] Nigro, L., Magni, S., Ortenzi, M.A., Gazzotti, S., Signorini, S.G., Sbarberi, R., et al., 2023. Assessment of behavioural effects of three water-soluble polymers in zebrafish embryos. *Sci Total Environ* 893, 164843. <https://doi.org/10.1016/j.scitotenv.2023.164843>.

- [20] Nigro, L., Magni, S., Ortenzi, M.A., Gazzotti, S., Della Torre, C., Signorini, S.G., et al., 2024. Unveiling the multilevel impact of four water-soluble polymers on *Daphnia magna*: from proteome to behaviour (a case study). *J Hazard Mater* 469, 134000. <https://doi.org/10.1016/j.jhazmat.2024.134000>.
- [21] 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. <https://doi.org/10.1016/j.scitotenv.2023.167699>.
- [22] Robison-Smith, C., Masud, N., Tarring, E.C., Ward, B.D., Cable, J., 2024. A class of their own? Water-soluble polymer pollution impacting a freshwater host-pathogen system. *Sci Total Environ* 907, 168086. <https://doi.org/10.1016/j.scitotenv.2023.168086>.
- [23] Sanderson, H., Solomon, K., 2009. Contaminants of emerging concern challenge ecotoxicology. *Environ Toxicol Chem* 28, 1359–1360. <https://doi.org/10.1897/09-119.1>.
- [24] Tarring, E., Robison-Smith, C., Cable, J., Durance, I., Harbottle, M., Ward, B.D., 2024. Detection of polyvinylpyrrolidone in *Daphnia magna*: development of a refractive index quantification method for water-soluble polymers in aquatic organisms. *Sci Total Environ* 935, 173428. <https://doi.org/10.1016/j.scitotenv.2024.173428>.
- [25] Nigro, L., Magni, S., Ortenzi, M.A., Gazzotti, S., Della Torre, C., Binelli, A., 2022. Are “liquid plastics” a new environmental threat? the case of polyvinyl alcohol. *Aquat Toxicol* 248, 106200. <https://doi.org/10.1016/j.aquatox.2022.106200>.
- [26] Guo, R., Ma, X., Hu, C., Jiang, Z., 2007. Novel PVA-silica nanocomposite membrane for pervaporative dehydration of ethylene glycol aqueous solution. *Polym (Guildf)* 48, 2939–2945. <https://doi.org/10.1016/j.polymer.2007.03.035>.
- [27] Yin, D., Xiang, A., Li, Y., Qi, H., Tian, H., Fan, G., 2019. Effect of plasticizer on the morphology and foaming properties of poly(vinyl alcohol) foams by supercritical CO₂ foaming agents. *J Polym Environ* 27, 2878–2885. <https://doi.org/10.1007/s10924-019-01570-4>.
- [28] Bui, T.D., Wong, Y., Thu, K., Oh, S.J., Kum, J.A., Ng, K.C., et al., 2017. Effect of hygroscopic materials on water vapor permeation and dehumidification performance of poly(vinyl alcohol) membranes. *J Appl Polym Sci* 134. <https://doi.org/10.1002/app.44765>.
- [29] LeBlanc, G.A., Surprenant, D.C., 1983. The acute and chronic toxicity of acetone, dimethyl formamide, and triethylene glycol to *Daphnia magna* (Straus). *Arch Environ Contam Toxicol* 12, 305–310. <https://doi.org/10.1007/bf01059407>.
- [30] Schladt, L., Ivens, I., Karbe, E., Rühl-Fehlert, C., Bomhard, E., 1998. Subacute oral toxicity of tetraethylene glycol and ethylene glycol administered to Wistar rats. *Exp Toxicol Pathol* 50, 257–265. [https://doi.org/10.1016/s0940-2993\(98\)80096-1](https://doi.org/10.1016/s0940-2993(98)80096-1).
- [31] Liu, G., Li, Y., Yang, L., Wei, Y., Wang, X., Wang, Z., et al., 2017. Cytotoxicity study of polyethylene glycol derivatives. *RSC Adv* 7, 18252–18259. <https://doi.org/10.1039/c7ra00861a>.
- [32] ECHA, 2026. Pentaethylene glycol. EC number: 225-341-4, CAS number: 4792-15-8. <https://echa.europa.eu/it/substance-information/-/substanceinfo/100.023.038>.
- [33] Cognitive Market Research, 2025. Dishwasher tablets market report. www.cognitivemarketresearch.com/dishwasher-tablets-market-report/author-details.
- [34] ZFIN, 2026. Zebrafish book. https://zfin.org/zf_info/zfbook/zfbk.html.
- [35] Magni, S., Binelli, A., Nigro, L., Roubeau Dumont, E., Eysseric, E., Sbarberi, R., et al., 2025. Assessment of the sub-lethal effects induced by three tire rubber-derived contaminants on zebrafish larvae. *Environ Toxicol Pharmacol* 120, 104863. <https://doi.org/10.1016/j.etap.2025.104863>.
- [36] Brunning, H., Brett Sallach, J., Boxall, A., 2025. Emissions of water-soluble polymers from household products to the environment: a prioritization study. *Environ Toxicol Chem* 44, 563–588. <https://doi.org/10.1093/etjnl/vgae030>.
- [37] OECD, 2013. Test No. 236: fish embryo acute toxicity (FET) test. OECD Guidelines for the Testing of Chemicals, Section 2. <https://doi.org/10.1787/9789264203709-en>.
- [38] Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. *Dev Dyn* 203, 253–310. <https://doi.org/10.1002/aja.1002030302>.
- [39] Schiwy, S., Bräunig, J., Alert, H., Hollert, H., Keiter, S.H., 2014. A novel contact assay for testing aryl hydrocarbon receptor (AhR)-mediated toxicity of chemicals and whole sediments in zebrafish (*Danio rerio*) embryos. *Environ Sci Pollut Res* 22, 16305–16318. <https://doi.org/10.1007/s11356-014-3185-0>.
- [40] Eliso, M.C., Billé, B., De Marco, G., Pulvirenti, E., Dal Bello, F., Rapisarda, P., et al., 2025. Embryotoxicity of polystyrene microplastics, alone and conjugated with bisphenol A, in the black sea urchin *Arbacia lixula*: a multi-biomarker approach. *J Hazard Mater* 499, 140139. <https://doi.org/10.1016/j.jhazmat.2025.140139>.
- [41] MetaAnalyst, 2025. <https://www.metaboolanalyst.ca>.
- [42] Laganà, A., Billé, B., Di Pietro, A., Facciola, A., Celesti, C., Iannazzo, D., et al., 2026. Toxicological and metabolomic assessment of the acute and sub-chronic effects of nanoceria (≤ 50 nm) on the human alveolar cells A549. *J Hazard Mater* 504, 141239. <https://doi.org/10.1016/j.jhazmat.2026.141239>.
- [43] Liu, Q., Yin, X., Languino, L.R., Altieri, D.C., 2018. Evaluation of drug combination effect using a Bliss independence dose-response surface model. *Stat Biopharm Res* 10, 112. <https://doi.org/10.1080/19466315.2018.1437071>.
- [44] Loewe, S., Muischnek, H., 1926. Effect of combinations: mathematical basis of problem. *Arch Exp Pathol Pharmacol* 114, 313–313.
- [45] Greco, W.R., Bravo, G., Parsons, J.C., 1995. The search for synergy: a critical review from a response surface perspective. *Pharmacol Rev* 47, 331–385.
- [46] Gagné, F., 2014. Biochemical ecotoxicology: principles and methods, 1st Edition. Elsevier, London. doi.org/978-0-12-0411604-7.
- [47] Magni, S., Gagné, F., André, C., Della Torre, C., Auclair, J., Hanana, H., et al., 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. <https://doi.org/10.1016/j.scitotenv.2018.03.075>.
- [48] Parenti, C.C., Ghilardi, A., Della Torre, C., Magni, S., Del Giacco, L., Binelli, A., 2019. Evaluation of the infiltration of polystyrene nanobeads in zebrafish embryo tissues after short-term exposure and the related biochemical and behavioural effects. *Environ Pollut* 254, 112947. <https://doi.org/10.1016/j.envpol.2019.07.115>.
- [49] 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* 72, 248–254. [https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3).
- [50] Zhang, J.L., Souders, C.L., Denslow, N.D., Martyniuk, C.J., 2017. Quercetin, a natural product supplement, impairs mitochondrial bioenergetics and locomotor behavior in larval zebrafish (*Danio rerio*). *Toxicol Appl Pharmacol* 327, 30–38. <https://doi.org/10.1016/j.taap.2017.04.024>.
- [51] Souders, C.L., Liang, X., Wang, X., Ector, N., Zhao, Y.H., Martyniuk, C.J., 2018. High-throughput assessment of oxidative respiration in fish embryos: advancing adverse outcome pathways for mitochondrial dysfunction. *Aquat Toxicol* 199, 162–173. <https://doi.org/10.1016/j.aquatox.2018.03.031>.
- [52] Cao, F., Liu, B., Hou, X., Shi, J., Li, P., Zhang, X., et al., 2025. Maternal polystyrene nanoplastics suppress zebrafish offspring development and locomotion through mitochondrial dysfunction. *Environ Pollut* 385, 127070. <https://doi.org/10.1016/j.envpol.2025.127070>.
- [53] Gibert, Y., McGee, S.L., Ward, A.C., 2013. Metabolic profile analysis of zebrafish embryos. *J Vis Exp* 4300. <https://doi.org/10.3791/43000>.
- [54] Agilent, Seahorse XFe24 Analyzer. <https://www.agilent.com/en/product/cell-analysis/real-time-cell-metabolic-analysis/xf-analyzers/seahorse-xfe24-analyzer-r740878>.
- [55] Bond, S.T., McEwen, K.A., Yoganantharajah, P., Gibert, Y., 2018. Live metabolic profile analysis of zebrafish embryos using a Seahorse XF24 extracellular flux analyzer. *Methods Mol Biol* 1797, 393–401. https://doi.org/10.1007/978-1-4939-7883-0_21.
- [56] Hagger, J.A., Jones, M.B., Lowe, D., Leonard, D.R.P., Owen, R., Galloway, T.S., 2008. Application of biomarkers for improving risk assessments of chemicals under the Water Framework Directive: a case study. *Mar Pollut Bull* 56, 1111–1118. <https://doi.org/10.1016/j.marpolbul.2008.03.040>.
- [57] Magni, S., Parolini, M., Della Torre, C., de Oliveira, L.F., Catani, M., Guzzinati, R., et al., 2017. Multi-biomarker investigation to assess toxicity induced by two antidepressants on *Dreissena polymorpha*. *Sci Total Environ* 578, 452–459. <https://doi.org/10.1016/j.scitotenv.2016.10.208>.
- [58] Vieira, M.G.A., Da Silva, M.A., Dos Santos, L.O., Beppu, M.M., 2011. Natural-based plasticizers and biopolymer films: a review. *Eur Polym J* 47, 254–263. <https://doi.org/10.1016/j.eurpolymj.2010.12.011>.
- [59] Sothornvit, R., Krochta, J.M., 2005. Plasticizers in edible films and coatings. *Innov Food Packag* 403–433. <https://doi.org/10.1016/b978-012311632-1/50055-3>.
- [60] Backhaus, T., Faust, M., 2012. Predictive environmental risk assessment of chemical mixtures: a conceptual framework. *Environ Sci Technol* 46, 2564–2573. <https://doi.org/10.1021/es2034125>.
- [61] Kortenkamp, A., 2014. Low dose mixture effects of endocrine disrupters and their implications for regulatory thresholds in chemical risk assessment. *Curr Opin Pharmacol* 19, 105–111. <https://doi.org/10.1016/j.coph.2014.08.006>.
- [62] Nicholson, J.K., Lindon, J.C., Holmes, E., 1999. Metabonomics: understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica* 29, 1181–1189. <https://doi.org/10.1080/004982599238047>.
- [63] Cappello, T., 2020. NMR-based metabolomics of aquatic organisms. *eMagRes* 9, 81–100. <https://doi.org/10.1002/9780470034590.emrstm1604>.
- [64] Bundy, J.G., Davey, M.P., Viant, M.R., 2008. Environmental metabolomics: a critical review and future perspectives. *Metabolomics* 5, 3–21. <https://doi.org/10.1007/s11306-008-0152-0>.
- [65] Zhang, J., Qian, L., Wang, C., Teng, M., Duan, M., Chen, X., et al., 2020. UPLC-TOF-MS/MS metabolomics analysis of zebrafish metabolism by spirotetramat. *Environ Pollut* 266, 115310. <https://doi.org/10.1016/j.envpol.2020.115310>.
- [66] Ribbenstedt, A., Posselt, M., Benskin, J.P., 2022. Toxicometabolomics and biotransformation product elucidation in single zebrafish embryos exposed to carbamazepine from environmentally relevant to morphologically altering doses. *Chem Res Toxicol* 35, 431–439. <https://doi.org/10.1021/acs.chemrestox.1c00335>.
- [67] Wilhelmi, P., Giri, V., Zickgraf, F.M., Haake, V., Henkes, S., Driemert, P., et al., 2023. A metabolomics approach to reveal the mechanism of developmental toxicity in zebrafish embryos exposed to 6-propyl-2-thiouracil. *Chem Biol Interact* 382, 110565. <https://doi.org/10.1016/j.cbi.2023.110565>.
- [68] Zhang, Y., Chen, M., Baral, K., Chen, C., Meng, Y., Zhang, M., et al., 2025. The combined toxicity assessment of polystyrene microplastics and di(2-ethylhexyl) phthalate on cardiac development in zebrafish embryos. *Ecotoxicol Environ Saf* 303, 118867. <https://doi.org/10.1016/j.ecoenv.2025.118867>.
- [69] Vignat, C., Cappello, T., Fu, Q., Lajoie, K., De Marco, G., Cléradeau, C., et al., 2019. Imidacloprid induces adverse effects on fish early life stages that are more severe in Japanese medaka (*Oryzias latipes*) than in zebrafish (*Danio rerio*). *Chemosphere* 225, 470–478. <https://doi.org/10.1016/j.chemosphere.2019.03.002>.
- [70] Roy, U., Conklin, L., Schiller, J., Matysik, J., Berry, J.P., Alia, 2017. Metabolic profiling of zebrafish (*Danio rerio*) embryos by NMR spectroscopy reveals multifaceted toxicity of β-methylamino-L-alanine (BMAA). *Sci Rep* 7. <https://doi.org/10.1038/s41598-017-17409-8>.
- [71] Xu, M., Legradi, J., Leonards, P., 2023. A comprehensive untargeted metabolomics study in zebrafish embryos exposed to perfluorohexane sulfonate (PFHxS). *Sci Total Environ* 887, 163770. <https://doi.org/10.1016/j.scitotenv.2023.163770>.

- [72] Ganesan, R., Vasantha-Srinivasan, P., Sadhasivam, D.R., Subramanian, R., Vimalraj, S., Suk, K.T., 2021. Carbon nanotubes induce metabolomic profile disturbances in zebrafish: NMR-based metabolomics platform. *Front Mol Biosci* 8, 688827. <https://doi.org/10.3389/fmolb.2021.688827>.
- [73] Liu, Y., Liu, S., Huang, J., Liu, Y., Wang, Q., Chen, J., et al., 2023. Mitochondrial dysfunction in metabolic disorders induced by per- and polyfluoroalkyl substance mixtures in zebrafish larvae. *Environ Int* 176, 107977. <https://doi.org/10.1016/j.envint.2023.107977>.
- [74] Bashirova, N., Poppitz, D., Klüber, N., Scholz, S., Matysik, J., Alia, 2023. A mechanistic understanding of the effects of polyethylene terephthalate nanoplastics in the zebrafish (*Danio rerio*) embryo. *Sci Rep* 13, 1891. <https://doi.org/10.1038/s41598-023-28712-y>.
- [75] Hua, J., Zhang, T., Chen, X., Zhu, B., Zhao, M., Fu, K., et al., 2024. Behavioral impairments and disrupted mitochondrial energy metabolism induced by polypropylene microplastics in zebrafish larvae. *Sci Total Environ* 947, 174541. <https://doi.org/10.1016/j.scitotenv.2024.174541>.
- [76] Fu, J., Gong, Z., Kelly, B.C., 2019. Metabolomic profiling of zebrafish (*Danio rerio*) embryos exposed to the antibacterial agent triclosan. *Environ Toxicol Chem* 38, 240–249. <https://doi.org/10.1002/etc.4292>.
- [77] Feng, M., Luo, J., Wan, Y., Zhang, J., Lu, C., Wang, M., et al., 2022. Polystyrene nanoplastic exposure induces developmental toxicity by activating the oxidative stress response and base excision repair pathway in zebrafish (*Danio rerio*). *ACS Omega* 7, 32153–32163. <https://doi.org/10.1021/acsomega.2c03378>.
- [78] Li, J., Chen, Y., Chen, Y., Xie, H., Wu, G., Zhang, Y., et al., 2026. Polystyrene microplastics and nanoplastics induce neurotoxicity in zebrafish via oxidative stress and neurotransmitter disruption. *Comp Biochem Physiol C Toxicol Pharmacol* 300, 110397. <https://doi.org/10.1016/j.cbpc.2025.110397>.
- [79] Ding, P., Xiang, C., Yao, Q., Li, X., Zhang, J., Yin, R., et al., 2024. Aged polystyrene microplastics exposure affects apoptosis via inducing mitochondrial dysfunction and oxidative stress in early life of zebrafish. *J Environ Manag* 367, 121995. <https://doi.org/10.1016/j.jenvman.2024.121995>.
- [80] Schieber, M., Chandel, N.S., 2014. ROS function in redox signaling and oxidative stress. *Curr Biol* 24, R453–R462. <https://doi.org/10.1016/j.cub.2014.03.034>.
- [81] Holmström, K.M., Finkel, T., 2014. Cellular mechanisms and physiological consequences of redox-dependent signalling. *Nat Rev Mol Cell Biol* 15, 411–421. <https://doi.org/10.1038/nrm3801>.
- [82] Hu, M., Palić, D., 2020. Micro- and nano-plastics activation of oxidative and inflammatory adverse outcome pathways. *Redox Biol* 37, 101620. <https://doi.org/10.1016/j.redox.2020.101620>.
- [83] Audira, G., Siregar, P., Chen, J.R., Lai, Y.H., Huang, J.C., Hsiao, C.-D., 2020. Systematical exploration of the common solvent toxicity at whole organism level by behavioral phenomics in adult zebrafish. *Environ Pollut* 266, 115239. <https://doi.org/10.1016/j.envpol.2020.115239>.
- [84] Lionetto, M.G., Caricato, R., Calisi, A., Giordano, M.E., Schettino, T., 2013. Acetylcholinesterase as a biomarker in environmental and occupational medicine: new insights and future perspectives. *Biomed Res Int* 2013, 321213. <https://doi.org/10.1155/2013/321213>.
- [85] Sarangi, P., Sahoo, P.K., Pradhan, L.K., Bhoi, S., Sahoo, B.S., Chauhan, N.R., et al., 2024. Concerted monoamine oxidase activity following exposure to di-2-ethylhexyl phthalate is associated with aggressive neurobehavioral response and neurodegeneration in zebrafish brain. *Comp Biochem Physiol C Toxicol Pharmacol* 283, 109970. <https://doi.org/10.1016/j.cbpc.2024.109970>.
- [86] Sarmah, S., Marrs, J.A., 2016. Zebrafish as a vertebrate model system to evaluate effects of environmental toxicants on cardiac development and function. *Int J Mol Sci* 17, 2123. <https://doi.org/10.3390/ijms17122123>.
- [87] Zicarelli, G., Faggio, C., Blahova, J., Riesova, B., Hesova, R., Doubkova, V., et al., 2024. Toxicity of water-soluble polymers polyethylene glycol and polyvinyl alcohol for fish and frog embryos. *Sci Total Environ* 933, 173154. <https://doi.org/10.1016/j.scitotenv.2024.173154>.
- [88] Chang, Y.S., Park, S.M., Rah, Y.C., Han, E.J., Koun, S.I., Chang, J., et al., 2021. vivo assessment of the toxicity of electronic cigarettes to zebrafish (*Danio rerio*) embryos following gestational exposure, in terms of mortality, developmental toxicity, and hair cell damage. *Hum Exp Toxicol* 40, 148–157. <https://doi.org/10.1177/0960327120947785>.
- [89] Di Sante, M., Antonucci, S., Pontarollo, L., Cappellaro, I., Segat, F., Deshwal, S., et al., 2023. Monoamine oxidase A-dependent ROS formation modulates human cardiomyocyte differentiation through AKT and WNT activation. *Basic Res Cardiol* 118, 4. <https://doi.org/10.1007/s00395-023-00977-4>.
- [90] Parenti, C.C., Magni, S., Ghilardi, A., Caorsi, G., Della Torre, C., Del Giacco, L., et al., 2021. Does triclosan adsorption on polystyrene nanoplastics modify the toxicity of single contaminants? *Environ Sci Nano* 8, 282–296. <https://doi.org/10.1039/d0en00961j>.
- [91] Zindler, F., Beedgen, F., Brandt, D., Steiner, M., Stengel, D., Baumann, L., et al., 2019. Analysis of tail coiling activity of zebrafish (*Danio rerio*) embryos allows for the differentiation of neurotoxicants with different modes of action. *Ecotoxicol Environ Saf* 186, 109754. <https://doi.org/10.1016/j.ecoenv.2019.109754>.
- [92] Tierney, K.B., 2011. Behavioural assessments of neurotoxic effects and neurodegeneration in zebrafish. *Biochim Biophys Acta* 1812, 381–389. <https://doi.org/10.1016/j.bbadis.2010.10.011>.
- [93] Basnet, R.M., Zizioli, D., Taweedet, S., Finazzi, D., Memo, M., 2019. Zebrafish larvae as a behavioral model in neuropharmacology. *Biomedicines* 7, 23. <https://doi.org/10.3390/biomedicines7010023>.
- [94] Könnemann, S., Von Wyl, M., Vom Berg, C., 2022. Zebrafish larvae rapidly recover from locomotor effects and neuromuscular alterations induced by cholinergic insecticides. *Environ Sci Technol* 56, 8449–8462. <https://doi.org/10.1021/acs.est.2c00161>.
- [95] Roussel, Y., Paradis, M., Gaudreau, S.F., Lindsey, B.W., Bui, T.V., 2020. Spatiotemporal transition in the role of synaptic inhibition to the tail beat rhythm of developing larval zebrafish. *eNeuro* 7, ENEURO.0508-18.2020. <https://doi.org/10.1523/eneuro.0508-18.2020>.
- [96] Rowson, Z., Setzer, R.W., Padilla, S., Friedman, K.P., Carstens, K.E., 2025. A data analysis approach for locomotor behavioral responses in developmentally exposed zebrafish. *NAM J* 1, 100033. <https://doi.org/10.1016/j.namjnl.2025.100033>.
- [97] Yang, Y.T., Zheng, Z.Y., Wei, X.P., Meng, Y., Zhou, J.X., Li, S.Y., et al., 2025. Toxicological interactions of cosmetic and personal care additives mixtures: an update based on measurement and simulation. *Environ Res* 266, 120566. <https://doi.org/10.1016/j.envres.2024.120566>.
- [98] Ritz, C., 2010. Toward a unified approach to dose–response modeling in ecotoxicology. *Environ Toxicol Chem* 29, 220–229. <https://doi.org/10.1002/etc.7>.
- [99] Sánchez-Bayo, F., Goka, K., 2007. Simplified models to analyse time- and dose-dependent responses of populations to toxicants. *Ecotoxicology* 16, 511–523. <https://doi.org/10.1007/s10646-007-0158-9>.