University of Study of Milan

Environmental Science

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Liquid plastics as a new environmental problem? An integrated risk assessment of polyvinyl alcohol, polyethylene glycol, polyvinyl pyrrolidone and polyacrylic acid

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XXXVII cycle

Academic year 2024-2025

ABSTRACT

Water-soluble polymers (WSPs), also known as "liquid plastics," are synthetic polymers that dissolve, disperse, or swell in water due to their hydrophilic groups. Despite their extensive use in various industrial and domestic products, these polymers are not currently regulated under European plastic strategies or chemical regulations, resulting in their pervasive presence in aquatic ecosystems. This study aimed to evaluate the ecotoxicity of four commonly used WSPs—polyvinyl alcohol (PVA), polyvinylpyrrolidone (PVP), polyethylene glycol (PEG), and polyacrylic acid (PAA)—using the freshwater fish *Danio rerio* and the crustacean *Daphnia magna* as model organisms. The impacts were assessed at multiple levels of biological organization, including molecular, cellular, physiological, organismal, and population levels.

Firstly, the study focused mainly on PVA standard, examining its acute and chronic effects on *D. rerio* and *D. magna*. Acute toxicity tests showed no significant effects at environmentally relevant concentrations, while chronic toxicity assessments revealed few behavioral changes in both *D. magna* and *D. rerio*, with slight enhancements in swimming performance under certain conditions. Subsequent studies expanded to include PVP, PEG, and PAA, uncovering significant dose-response relationships and behavioral alterations in *D. rerio* under varying light conditions. Proteomic analyses indicated that PVP had the most substantial impact on *D. rerio* embryos, followed by PEG and PAA, affecting genetic processes, signalling pathways, and eye development. In *D. magna*, PEG exhibited acute toxicity at 0.5 mg/L, while PVA notably impacted the proteome, altering proteins involved in stress responses, reproduction, and energy allocation. Biomarker analyses highlighted biochemical and physiological effects, with changes in heart rate and acetylcholinesterase activity. Behavioral assessments indicated stress responses in swimming patterns, suggesting potential ecological implications. To investigate the possible long-term and hereditary effects, the transgenerational impacts of PEG and PVA were evaluated on *D. magna*. This study revealed two generational effects, such as changes in DNA methylation and juvenile counts, which were significative between the F_0 and F_1 generations. However, these changes did not persist through the F_4 generation, indicating that the induced epigenetic modifications were not heritable over multiple generations.

Our findings underscore the necessity for comprehensive environmental assessments to evaluate the ecological risks associated with WSPs. This multi-level and integrative approach provided a detailed ecotoxicological profile of these WSPs, laying a foundation for future studies on their long-term and transgenerational effects. Additionally, studies focusing on their

effects across different ecosystems—including terrestrial and marine environments—are necessary to obtain a complete understanding of their ecological impact.

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1 INTRODUCTION

1.1 From Innovation to Environmental Challenge: The Journey of Plastics

The world has undergone significant transformations over the past centuries due to remarkable inventions, among which plastics stand out. Defined by the International Union of Pure and Applied Chemistry as "polymeric materials that may contain other substances to enhance performance and/or reduce costs", plastics have become indispensable in modern society. Their low production cost, lightweight nature, and durability make them the most economical and durable materials in commerce (Frias and Nash, 2019). We are living in the so-called “Plastic Age,” where plastic infrastructure, industrial, and consumer products play a crucial role in every aspect of our daily lives (Osborn and Stojkovic, 2014).

The development of plastic materials began in the 19th century with natural substances like chewing gum and shellac, which exhibited plastic properties (Harrison and Hester, 2018). This initial phase was followed by the explosion of synthetic chemistry, which drove the expansion of the plastic industry and led to the creation of a wide range of polymers, including both chemically modified natural materials (rubber, nitrocellulose, collagen) and modern synthetic ones, derived from petrochemicals (Andrady and Neal, 2009). Global production of plastic increased from around 2 million tons (Mt) in 1950 to 400.3 Mt reached in 2022 (Rafey and Siddiqui, 2021; Plastic Europe, 2023).

Nowadays, plastics are categorized as 'bio-based/organic polymers' or 'engineered' plastics. Engineered plastics are developed using unrefined petroleum, flammable gas, and coal, with approximately 4% of the world's petroleum and gas production used as raw material and an additional 3-4% expended for energy in their production (Verma et al., 2016; Alhazmi et al., 2021; Hopewell et al., 2009). In contrast, bio-based plastics are produced from sustainable raw materials such as carbohydrates, vegetable fats/oils, and other natural substances. Of all these plastics, approximately 50% are used for single-use applications like packaging and disposable consumer items.

Despite their valued features, the widespread use of plastics has resulted in significant environmental problems (Fig. 1). Ineffective waste management practices and the continuous plastic release contribute to substantial volumes of plastic waste (Hu et al., 2019). By the end of 2015, an estimated 5800 Mt of mismanaged plastic waste had been released into the environment worldwide (Geyer et al., 2017). Factors such as urbanization, economic development, and population growth significantly contribute to the accumulation of plastic in natural habitats, posing critical threats to both the environment and human health (Amasuomo and Baird, 2016; Hu et al., 2019) (Fig. 1).

Much of this pollution originates from diverse anthropogenic sources and reaches the oceans through various pathways (Rochman, 2020). Predominantly, plastic waste accumulates in terrestrial environments due to its manufacturing, usage, and disposal on land (Hurley et al., 2020) (Fig. 1). Agricultural plastics, including polytunnel films, wrappings, and packing materials, significantly contribute to this waste stream. The problem is further exacerbated by illegal dumping and direct waste inputs, as terrestrial environments act as plastic sinks where human activities, wind, and water transport plastic litter (Hurley et al., 2020; Magni et al., 2024). In fact, these contaminants can be transported by wind from landfills, overflowing garbage bins, natural disasters, accidental spills, or sewage overflows, with rivers acting as dominant pathways (Lebreton et al., 2017). Freshwater ecosystems, such as rivers, dams, lakes, and urban drainage networks, act as major repositories for plastic debris (Azevedo-Santos et al., 2021). Rivers are primary conduits for transporting plastics into marine environments, with nearly 1,000 rivers worldwide accounting for approximately 80% of annual fluvial plastic emissions, according to the United Nations Environment Programme (UNEP) (Hurley et al., 2020; Chen et al., 2021). Additionally, wind plays a crucial role in dispersing small-sized plastic particles across wide geographic areas within urban environments (Azevedo-Santos et al., 2021).

One of the biggest issues with plastics is that most are not biodegradable and are extremely persistent in the environment. Their degradation dependent on local environmental factors such as ultraviolet light exposure, temperature, and oxygen levels (Swift and Wiles, 2004). This persistence is intensified as plastics can degrade into smaller particles—mesoplastics (MePs), microplastics (MPs), and nanoplastics (NPs)—which can enter organisms, exert adverse effects, and accumulate along the food chain (Toussaint et al., 2019; Suman et al., 2020; Ding et al., 2021; Yin et al., 2022).

The first documented instance of plastic debris in wildlife was observed in the 1960s when plastic fragments were found in the guts of seabirds (Suaria et al., 2020). Today, an estimated 8 to 10 Mt of plastics, including macroplastics (MaPs) and MPs, enter the ocean each year, constituting 80% of all marine pollution. This pollution leads to the death of over 100,000 marine species annually, affecting both coastal and deep-sea ecosystems (Cózar et al., 2014; van Sebille et al., 2015). The floatability and durability of plastics, along with wind and ocean currents, facilitate their dispersal across vast expanses of the ocean (Eriksen et al., 2014; Jambeck et al., 2015) (Fig. 1). As a result, plastics in marine environments manifest as floating debris, seafloor deposits, and shoreline pollution, posing significant ecological and environmental challenges (Welden, 2020).

In summary, while plastics have revolutionized various aspects of our modern life due to their versatility and cost-effectiveness, their environmental and health impacts have significant challenges. Addressing these issues requires a balanced approach that maximizes the benefits of plastics while minimizing their adverse effects on the environment and human health.

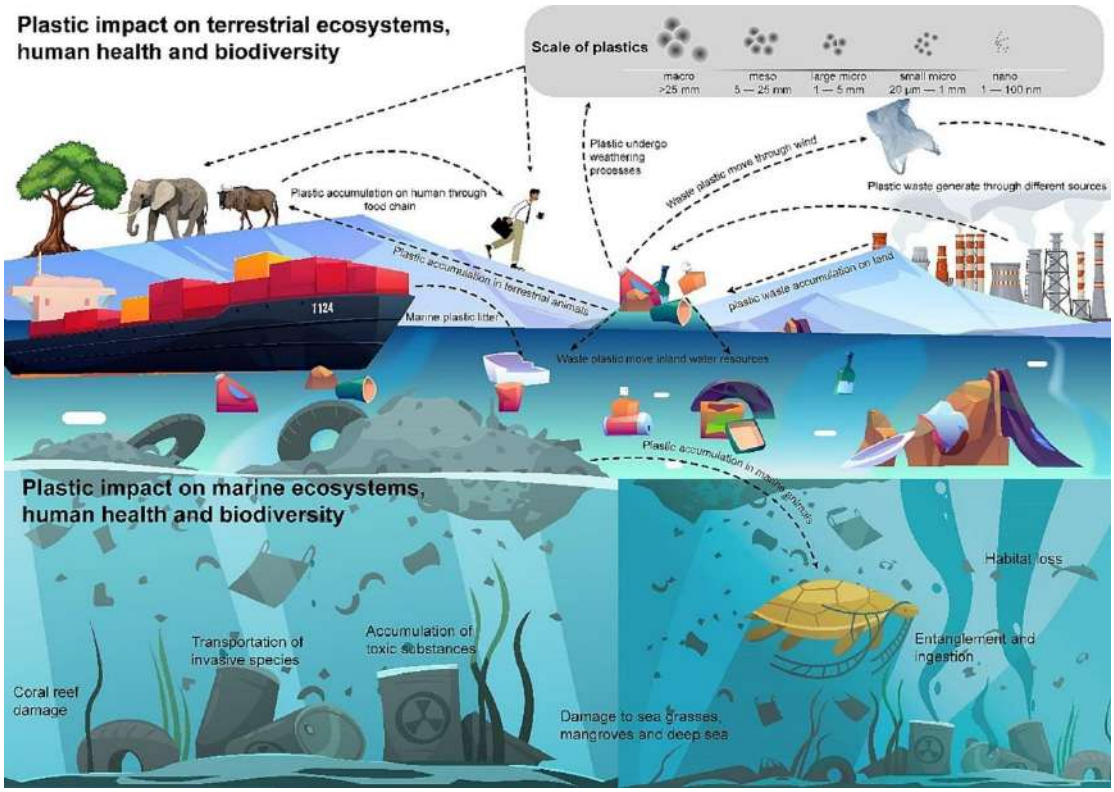


Fig. 1. Plastics in terrestrial and marine environments (Pilapitiya et al., 2024)

1.2 **Conventional and Unconventional Plastics**

The National Oceanic and Atmospheric Administration (NOAA) defines MPs as particles ranging from 0.1 to 5,000 μm (Thompson et al., 2004), and NPs as particles from 0.001 to 0.1 μm (EU Commission, 2011). According to the International Organization for Standardization (ISO), MPs are any solid plastic particles insoluble in water with dimensions between 1 μm and 1,000 μm, while NPs are plastic particles smaller than 1 μm (according to OECD, nanoparticles are up to 100 nm). Based on a recent classification by Hartmann et al. (2019), MPs range between 1,000 μm and 1 μm, NPs between 1 μm and 0.001 μm, mesoplastics (MePs) between 1 mm and 10 mm, and macroplastics (MaPs) from 1 cm upwards.

As we can see from the wide range of classifications of plastic and the derived fragments, the topic of plastic pollution has attracted much attention and the scientific community is trying to standardize the plastic research, in order to compare and evaluate the environmental impact of these debris. However, all these classifications are based on specific characteristics of the plastic including their size and insolubility in water. In fact, the micro-nanoplastics (MNPs) can appear as pellets, films, fibers, and fragments, and they can be classified as primary or secondary based on their origin. Primary MPs include plastic powders used in molding and microspheres in cosmetics, while secondary MPs result from the fragmentation of larger waste through prolonged UV light exposure and physical abrasion (Laskar et al., 2019).

Currently, MNPs are present in large quantities worldwide (Amelineau et al., 2016; Pradel et al., 2021; Loughlin et al., 2021; Materic et al., 2021). Researchers estimate that there are between 15 and 51 trillion MP particles floating on the ocean's surface (Van Sebille et al., 2015). They have been found in the ocean's deepest parts (Jamieson et al., 2019) and Arctic Sea ice (Obbard et al., 2014). The widespread distribution of plastic particles has numerous ecological impacts, particularly in aquatic environments. They can cause biological effects such as changes in gene and protein expression (Paul-Pont et al., 2016; Magni et al., 2019), inflammation (Von Moos et al., 2012), disruption of feeding behavior (Cole et al., 2015), decreased growth and reproductive success (Au et al., 2015; Sussarellu et al., 2016), larval development changes (Nobre et al., 2015), reduced filtration and respiration rates (Paul-Pont et al., 2016), decreased survival (Au et al., 2015; Cui et al., 2017), and oxidative stress (Magni et al., 2018, 2019). Given that plastic is now an indispensable material in our society, its impact on human and animal health, natural populations, and ecosystems is a significant and ongoing concern. The enormous production of plastic waste highlights the challenges in managing it, making plastic disposal one of the major problems of modern global society. Reuse and recycling are specific

actions available to reduce the environmental impacts of plastics, as they lower petroleum consumption, carbon dioxide emissions, and the amount of wastes.

Despite the significant focus and numerous studies on conventional plastics, some polymers have been largely ignored and not included in plastic regulations due to their specific properties. Unconventional plastics, such as end-of-life tires (ELTs), bioplastics and water-soluble polymers (WSPs) fall into this category. ELTs, although not classified as plastics by ISO, contain significant amounts of synthetic polymers, leading some researchers to include them in this category (Lassen et al., 2015; Hartmann et al., 2019). Each year, about 1.5 billion tires are produced globally (Mashiri et al., 2015), and this number is expected to rise with global population growth (Mohajerani et al., 2020). Once their life cycle ends, ELTs were initially disposed in landfills. However, this system soon became unsustainable due to their non-biodegradable nature, potential to serve as habitats for disease-carrying pests and high flammability (Jang et al., 1998). Current legislation now promotes their recycling, encouraging their use as alternative fuels or in civil applications. However, this may inadvertently lead to increased release of MPs into the environment (Magni et al., 2022). Similarly, among unconventional plastics, bioplastics stand out as they are expected to gradually replace fossil-based materials in the coming decades. These polymers, sourced from renewable raw materials like cellulose, proteins, and lipids, offer a promising alternative. While they offer a lower carbon footprint and enhanced waste management possibilities, several challenges persist. Although bioplastics share characteristics with conventional plastics, their recyclability differs, and they can emit methane in landfills (Atiwesh et al., 2021; De Felice et al., 2024). In 2022, global bioplastics production amounted to 2.23 Mt, representing just 1% of total plastic output (European Bioplastics, 2023). Nevertheless, predictions indicate a significant 36% increase in bioplastics usage by 2027, driven by advances in polymers like polylactic acid and polyhydroxyalkanoates. However, concerns persist regarding waste management. While bioplastics ideally biodegrade within six months in industrial composting facilities, real-world degradation in natural environments may take 3–5 years (Ncube et al., 2020). Given current waste disposal practices, there's a risk of bioplastic accumulation and subsequent degradation, potentially leading to the formation of MPs similar to conventional plastics.

Water-soluble polymers (WSPs) also present potential environmental impacts due to their distinctive properties and wide applications. Their solubility in water allows for various industrial and consumer uses, including adhesives, coatings, and textiles. However, their environmental effects are not yet understood, making it crucial to investigate their behaviour and impacts in various ecosystems. The lack of comprehensive studies and regulations

highlights the need for scientific attention to better manage and mitigate their environmental impact.

1.3 **Water soluble polymers (WSPs)**

WSPs, often referred to as "liquid plastics," are a unique category of synthetic polymers capable of dissolving, dispersing, or swelling in water due to the presence of hydrophilic groups in their polymeric backbone. Since they do not conform to the conventional definitions of plastic, which typically mandate solidity and insolubility, WSPs are not monitored and considered in current European plastic strategies or circular economy action plans (Rozman and Kalcikova, 2021). Besides, WSPs are not even regulated as chemicals since they are not under the European REACH (Registration, Evaluation, Authorization, and Restriction of Chemicals) legislation. This regulatory gap persists due to the perceived low environmental impact of WSPs, attributed to their high molecular weight. Consequently, these polymers have been employed across a wide range of applications without regulatory oversight, leading to their widespread release to aquatic ecosystems. Furthermore, the only possible estimations of the production volume of WSPs in Europe is based on the annual production of their respective monomers, assuming WSP synthesis as the primary application (Huppertsberg et al., 2020). These estimates suggest production volumes ranging from thousands to Mt for each WSP type. In detail, for the polymers on which this study focused on, the estimations are: polyvinylpyrrolidone (PVP) > 103 t/y (ECHA (I)); polyacrylic acid (PAA) = 105−106 t/y (ECHA (II)); polyvinyl alcohol (PVA) = 105−106 t/y (ECHA (III)); and polyethylene glycol (PEG) > 106 t/y (ECHA (IV)). This underscores the considerable potential for their release into natural environments, further reinforcing the concern that the presence of WSPs in ecosystems can be considered ubiquitous. They are discharged into sewage systems and surface water bodies due to their utilization across various industrial and everyday products, including textiles, personal care products, cosmetics, pharmaceuticals, pesticides and fertilizers, paints, flocculants, food additives (Duis et al., 2021). Despite recent efforts to detect WSPs, limited information is available regarding their environmental concentrations due to analytical challenges (Huppertsberg et al., 2020), as will be described in detail later.

The characteristics of the WSPs that were assayed in this three-year study will now be described.

1.3.1 Polyvinyl Alcohol (PVA)

PVA (Fig. 2) is a versatile compound that finds application across many industrial and domestic applications (Nagarkar et al., 2019; Mohammadi et al., 2022). In the textile industry, PVA is used as a sizing agent (Reddy et al., 2014) to provide fabrics with stiffness and resistance to abrasion, while the adhesive industry also benefits from PVA properties in woodworking glues, paper adhesives, and as a binder in paper coatings (Gadhave and Dhawale, 2022). Additionally, its film-forming properties are crucial in packaging, as it acts as a barrier against moisture and oxygen, thereby extending the shelf life of food products (Nagarkar and Patel, 2019). Moreover, the supposed PVA biodegradability and non-toxic characteristics have facilitated its use in environmentally friendly products like biodegradable plastics and packaging materials (Lal et al., 2020). In the medical field, PVA has received approval from the Food and Drug Administration (FDA) for clinical uses in humans due to its biocompatibility and moisture-retaining properties (Chong et al., 2013; Nagarkar and Patel, 2019). It is a key component in contact lenses, ensuring user comfort (Solaro et al., 2000), and in hydrogels used for wound dressings (Jang and Lee, 2003; Jayasekara et al., 2004; Fernandes et al., 2013). Additionally, researchers are exploring PVA-based hydrogels for drug delivery applications due to their ability to encapsulate and release drugs in a controlled manner for targeted therapies (Salunkhe et al., 2013). PVA is also used in surgical procedures as a haemostatic agent to control bleeding and as a lubricant in certain medical devices (Dorkhani et al., 2023). In the electronics industry, PVA is employed as a dielectric material in capacitors and as a component in the manufacture of optical films used in liquid crystal displays (LCDs) (Zhou et al., 2023). It also plays a role in the production of polyvinyl butyral (PVB), a specific material used in laminated safety glass for automotive and architectural applications (Kumar et al., 2016).

However, Julinova et al. (2018) revealed the potential ubiquitous presence of PVA in water systems suggesting how these substances pose a certain risk to aquatic organisms. In fact, PVA biodegradability varies with environmental conditions, and in certain cases, for instance in soil, it can degrade much slower than in aquatic ecosystem (Chiellini et al., 2003). In this context, a recent petition was launched in the United States (U.S.) to the Environmental Protection Agency (EPA) to regulate the use of PVA in PVA-based products, such as detergent pods. The petition cites concerns about the biodegradation of PVA and requests the removal of PVA from the EPA Safer Choice Program and Safer Chemical List (Magni et al., 2024). The petition argues that the current classification of PVA as a safer chemical may not adequately reflect its environmental impact, particularly in aquatic ecosystems where its persistence could pose significant risks to aquatic life.

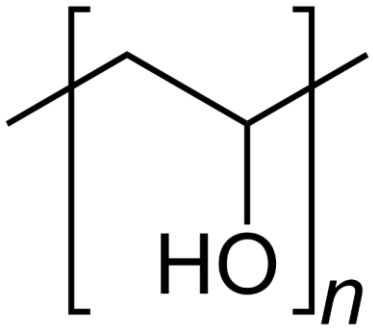


Fig. 2 Chemical structure of PVA

1.3.2 Polyethylene Glycol (PEG)

PEG (Fig. 3) is a multifunctional compound with extensive applications across some industrial and everyday applications (Ozdemir et al., 2023) spacing from the pharmaceutical to the cosmetic one, due to its unique properties, such as solubility in water and biocompatibility. In the 1990s, Enzon Pharmaceuticals-USA introduced “Adage”, the first FDA -approved PEGylated product (Levy et al., 1988). This first approval opened the way for extensive research and clinical trials on PEG-drug conjugates (Ozdemir et al., 2023). Today, numerous FDA-approved PEGylated products are available on the market for various applications. In the pharmaceutical field, PEG plays a crucial role as excipient, enhancing drug solubility and delivery (Kolate et al., 2014; Ozdemir et al., 2023). Its ability to form stable protein complexes makes it invaluable for protein crystallization and purification in biotechnology (Hekmat et al., 2015). Additionally, its laxative properties also make it a crucial component for treating constipation (DiPiro et al., 1986). It is also used in intravenous injections to solubilize poorly water-soluble drugs, thereby enhancing their bioavailability (D’Souza and Shegokar, 2016). The process is defined as “PEGylation”, meaning the way in which PEG chains attach to therapeutic molecules, prolonging drug circulation in the body, reducing immunogenicity, and enhancing therapeutic efficacy (Kolate et al., 2014; D’Souza and Shegokar, 2016; Ozdemir et al., 2023). About industrial applications, PEG acts as a lubricant and plasticizer, contributing to the flexibility and durability of materials (Ozdemir et al., 2023). It is also used in the manufacturing of resins and in the production of ceramics, where it acts as a binder and a dispersant (Jang et al., 2015, Wu et al., 2020). In the cosmetics industry, PEG acts as an emulsifier, surfactant, and humectant, improving the stability and texture of creams, lotions,

and shampoos. PEGs with molecular weights ranging from 20,000 to 8,000,000 are specifically intended for cosmetic use and have been deemed safe by the Cosmetic Ingredient Review Expert Panel. However, these specific types are not mentioned in the US FDA Inactive Ingredient Database (IID) (D'Souza and Shegokar, 2016), which primarily lists excipients used in approved drug products. Although PEGs are widely used in pharmaceuticals and personal care products, their specific types and molecular weights are not always included (D'Souza and Shegokar, 2016). This gap in regulatory documentation underscores the need for comprehensive information on PEGs, given their diverse applications and potential human health and environmental impacts. PEG 6000 is also employed as a food additive, serving as an anti-foaming agent, thickener, and dispersing agent, thereby improving the texture and consistency of various food products (Cox et al., 2021). The Joint FAO/WHO Expert Committee on Food Additives recommends not more than 10 mg/kg/daily of 1,4-dioxane present in PEGs (D'Souza and Shegokar, 2016).

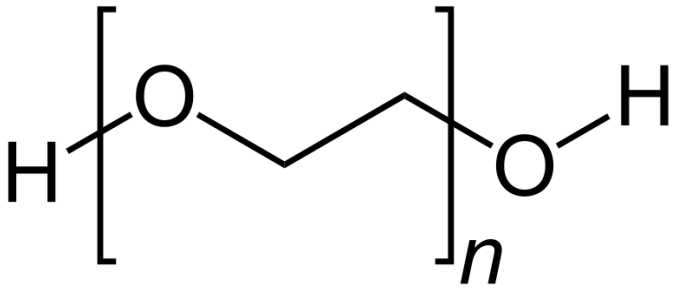


Fig. 3 Chemical structure of PEG

1.3.3 Polyvinylpyrrolidone (PVP)

PVP (Fig. 4) is a multifunctional substance widely used in pharmaceuticals, cosmetics, and medical field (Kurakula and Rao, 2020). Initially used as a plasma volume expander (Bühler et al., 2005), PVP found its way into the hair spray market by the 1950s, replacing shellac resin as a hair fixative agent. Today, PVP is valuable across many applications, including pharmaceuticals, biomedicine, cosmetics, and the food industry (Kurakula and Rao, 2020).

In the pharmaceutical sector, PVP is used as tablet binder, disintegrant, and solubilizing agent, aiding in the formulation of solid dosage forms with enhanced drug release profiles (Kurakula and Rao, 2020). Its film-forming properties make it ideal for wound dressings and transdermal drug delivery systems (Suksaeree et al., 2017). Additionally, PVP also forms complexes with various substances, making it valuable for drug stabilization and taste masking in oral formulations (Teodorescu et al., 2019; Kurakula and Rao, 2020). PVP acts as a film former, hair fixative, and viscosity enhancer in the cosmetics industry, contributing to the development of hair styling products, gels, and mousses (Burnette and Heldreth, 2017; Kurakula and Rao, 2020). It is incorporated into a variety of cosmetic products including eyeliners, mascara, eye shadows, lipsticks, hair sprays, hair setting lotions, shampoos, nail shaving creams, polishes, and moisturizers. Its use in cosmetics is well-established (Burnette and Heldreth, 2017), with the US FDA reporting its use in 799 cosmetic products as of 2014 (Burnette and Heldreth, 2017).

In the medical field, PVP is utilized in ophthalmic and contact lens solutions to enhance solubility and clarity (Kurakula and Rao, 2020). It is also effective in wound dressings, creating a protective barrier that aids in healing (Trott et al., 2012). Furthermore, PVP-based solutions are used as surgical scrubs and pre-operative skin antiseptics due to their film-forming and antiseptic properties (Kurakula and Rao, 2020). Beyond these applications, PVP is used as a stabilizer in food packaging. Its easy processability, biocompatibility, and non-antigenicity have led to its approval by the US FDA as a safe polymer for biological experiments, especially in the pharmaceutical and biomedical fields (D'Souza et al., 2004; Saxena et al., 2006; Smith et al., 2006; Ng et al., 2011).

In the food industry, PVP is recognized as a food additive with the code E 1201. The European Food Safety Authority (EFSA) recently re-evaluated its safety positively and considered extending its use in food for special medical purposes in tablet and coated tablet forms (EU Commission Regulation 2022/1038) (EFSA, 2020).

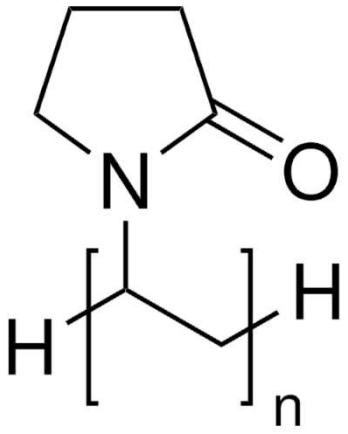


Fig. 4 Chemical structure of PVP

1.3.4 Polyacrylic acid (PAA)

PAA (Fig. 5) is a multifunctional substance utilized in several industries such as personal care products, detergents, water treatment, adhesives, and pharmaceuticals (Saunders et al., 2010; Huppertsberg et al., 2020; Patil and Ferritto, 2013; Rivas et al., 2018; Rozman et al., 2021). It serves multiple functions such as a super-absorbent, emulsifier, stabilizer, thickener, suspending agent, and sequestrant (Buron et al., 2022). PAA-containing products are predominantly designed for single-use or wash-off applications, such as in detergents and cleaning products (Pecquet et al., 2019). Its water-absorbing properties make it valuable in disposable diapers (Chazovachii et al., 2021), as chelating agent in laundry and dish soap (Cahn et al., 2005), and as thickener in shampoos (Wiyanto et al., 2016).

In the medical field, PAA plays a significant role in wound care, pharmaceutical formulation, and tissue engineering (Arkaban et al., 2022; Dalei et al., 2022). PAA-based hydrogels are used in wound dressings, and PAA serves as a tablet binder and disintegrant in pharmaceuticals, aiding in drug dissolution and absorption (Park and Park, 1993; Yang et al., 2004). As bioadhesives, polyacrylic-based water-soluble polymers (WSPs) form hydrogen bonds with polysaccharides and proteins under acidic conditions (Sahoo et al., 2011; Wang et al., 2009). These hydrogels are explored for drug delivery applications, enabling targeted drug release in response to specific stimuli for effective treatment (Dalei et al., 2022). Beyond its medical and technical applications, PAA is used in the agriculture industry as a soil conditioner, improving soil structure and water retention (Laftah, 2013; Teodorescu et al., 2009; Dalei et al., 2022).

Despite its widespread use, there is limited comprehensive environmental monitoring and effect data available for PAA, underscoring the necessity for more extensive research on its ecological impacts. For instance, both polyacrylamides (PAMs) and PAAs show significant resistance to microbial degradation under environmental conditions, resulting in low soil degradability rates that do not exceed 10% per year. This degradation rate is dependent on their structural composition (Dell'Ambriogio et al., 2019), since linear PAMs tend to break down more quickly than cross-linked PAMs, whereas cross-linked PAAs show even greater resistance. According to the REACH Regulation, these properties are sufficient to classify all PAM/PAA polymers as persistent substances (ECHA 2017). Moreover, these degradation rates exceed the threshold values set by the European Union for persistent chemicals, indicating the necessity for ecotoxicity testing with terrestrial species (EU 2002).

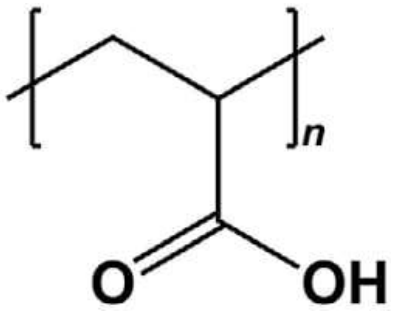


Fig. 5 Chemical structure of PAA

1.4 **Sources and detection**

Nowadays, the current knowledge of WSPs might be comparable to the initial phase of microplastics research because we have a general awareness of their environmental presence, but we lack detailed information regarding their distribution, concentrations, and impacts (Huppersberg et al., 2020). Given the extensive use of these polymers, which are unregulated as either chemicals or physical agents, their environmental presence is likely ubiquitous (Fig. 6).

Depending on their applications, these polymers may be released into the environment through both direct and indirect pathways. For instance, WSPs can enter into sewage systems and surface waters through their use in personal care items, cosmetics, washing agents, textiles, and pharmaceuticals. Additionally, they can enter these systems via rain runoff from construction sites, and from coatings and paint formulations (Duis et al., 2021). Their incorporation into pesticides and fertilizers results in their direct release onto agricultural land (Krogh et al., 2003; Koltzenburg et al., 2014; Xiong et al., 2018), while their application as flocculants in wastewater and drinking water treatment processes leads to their accumulation in sewage sludge (Petrovic and Barcelo, 2000; Pocurull et al., 2009; Koltzenburg et al., 2014; Duis et al., 2021).

Despite their assumed ubiquitous presence into the environment, quantitative and qualitative data on the occurrence of WSPs remain scarce, with existing studies covering only a limited range of polymer types and chain lengths (Petrovic and Barcelo, 2000). This lack of comprehensive data can be attributed to a general unawareness within the scientific community (Arp and Knutsen, 2020) and the significant analytical challenges involved. Current analytical methods for WSPs are generally suited for relatively pure products or formulations with few high molecular weight constituents (Sainju et al., 2023). Moreover, methods such as size exclusion chromatography (SEC) combined with UV, refractive index, or evaporative light scattering detection lack specificity and may struggle with the complex matrices in environmental samples, which contain natural macromolecules like humic substances. Furthermore, they may lack the sensitivity to detect WSPs at environmental concentrations, typically in the mg/L range, compared to the g/L range in product formulations (Lechner et al., 2010). As a result, current polymer analysis methods are not yet suitable for detecting WSPs in the environment matrices. This fact is also underscored by the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), which recently reported that monitoring these polymers in the environment is extremely challenging due to the lack of specific analytical methods (ECETOC, 2020). Consequently, new studies have started to propose innovative techniques for detecting WSPs in environmental samples. For instance,

Huppertsberg et al. (2020) suggested the modifying mass spectrometry-based trace-analytical methods as a promising approach for WSP analysis in environmental samples. On the same line Pauelsen et al. (2023) with the analytical combination of size-exclusion chromatography hyphenated with electrospray ionization-high-resolution mass spectrometry (SEC-ESI-HRMS) measured PEG concentrations in water samples at concentrations ranged from below the limit of detection (LOD) to 11 μg/L in surface waters (with 11 out of 18 samples exceeding 1 μg/L), while 400 μg/L in influent and 20 μg/L in effluent of wastewater treatment plants (WWTPs) were measured in Denmark (Table 1). Furthermore, more than 100 of newly identified polyethylene and polypropylene oxides (EO/PO copolymers) compounds were detected in the wastewater effluents in Denmark using a liquid chromatograph coupled to a 6600 quadrupole-time-of-flight (QTOF) by Tisler et al. (2021). Antic et al. (2011) quantified the presence of PVP in effluents from WWTPs in Düren, Germany, by continuous-flow off-line pyrolysis-GC/MS. The analysed concentrations ranged from 0.9 mg/L to 7.1 mg/L, as well as in river water impacted by urban wastewater emissions, with levels around 0.18 mg/L (Table 1).. With the same technique, Vidovic et al. (2022) uncovered the presence of poly(N-vinylcaprolactam) in WWTP effluents of Aachen, Germany, reaching concentrations up to 0.07 mg/L (Table 1). Additionally, Vidovic et al. (2023) proposed an analytical method combining on-line and off-line pyrolysis GC/MS to detect the emission of polyethyleneimine in influent and effluent waters from various treatment plants in Germany (from 0.08 mg/L to 0.55 mg/L) (Table 1). Wang et al. (2021) measured the presence of PEG in fresh snowfall in Montreal, Canada, at a concentration of 21.9 ng/mL with a ultra-trace quantification method for MNPs based on nanostructured laser desorption/ionization time-of-flight mass spectrometry (NALDI-TOF-MS) (Table 1). Similarly, Freeling et al. (2019) detected PEG at the average concentrations of 7.4 μg/L in over 30 WWTPs in Germany, using high performance liquid chromatography electrospray tandem mass spectrometry (HPLC-MS/MS) and UHPLC coupled to quadrupole time-of-flight (UHPLC-QTOF) (Table 1). Lastly, Sainju et al. (2023) found PEG concentrations in the range of 2.1–33.5 μg/L in England rivers throught the use of 1D 1H and 2D 1H Diffusion Ordered spectroscopy (DOSY) Nuclear Magnetic Resonance (NMR) for WSP analysis in a complex environmental matrix (Table 1).

Notably, a recent study by Tarring et al. (2024) has, for the first time, quantified the presence of these polymers in an aquatic organism. They developed a new method using GPC with a refractive index detector to detect and quantify PVP at concentrations above 0.05 mg/mL and 0.2 mg/mL, respectively. This method was applied to *D. magna*, which were exposed to 50 mg/L of PVP for 48 hours. This study determined the limit of detection (LOD) and limit of

quantification for PVP in an organism, underlining the capability of these WSPs to pass through the biological membranes. Interdisciplinary collaboration is required to address both polymer quantification and their ecotoxicological effects. Given the limited information about the impact of these polymers, these new findings will help to better define their effects on freshwater organisms.

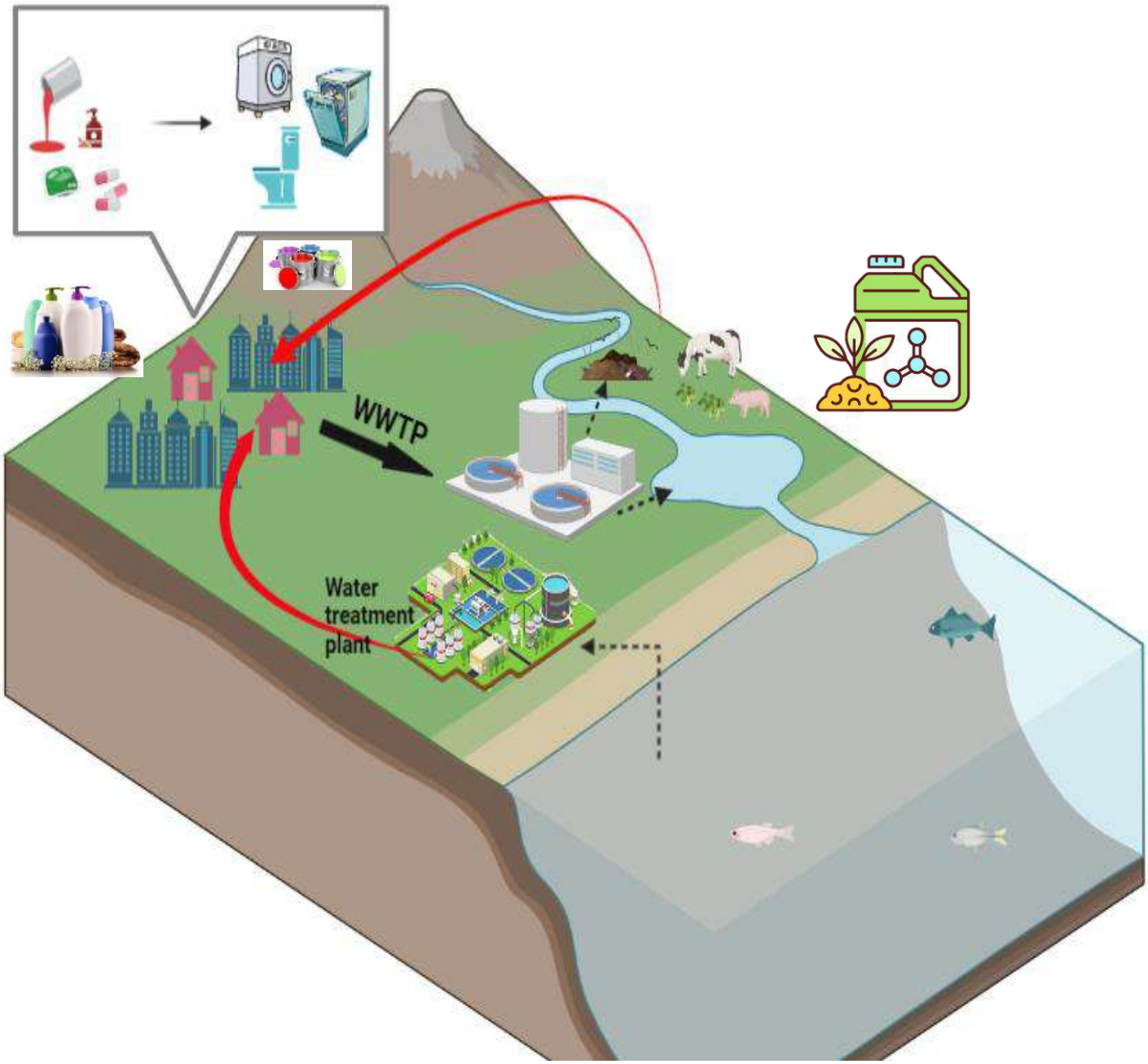


Fig. 6 Release of WSPs in the environment

Polymer	Country	Sample Site	Concentration	Analytical Method	Reference
PEG	Denmark	Surface waters	Below LOD-11 µg/L	SEC-ESI-HRMS	Pauelsen et al. (2023)
PEG	Denmark	WWTPs Influent	400 µg/L	SEC-ESI-HRMS	Pauelsen et al. (2023)
PEG	Denmark	WWTPs effluent	20 µg/L	SEC-ESI-HRMS	Pauelsen et al. (2023)
PVP	Germany	WWTPs effluent	0.9 mg/L -7.1 mg/L	Continuous-flow off-line pyrolysis-GC/MS	Antic et al. (2011)
PVP	Germany	River water impacted by urban wastewater emissions	0.18 mg/L	Continuous-flow off-line pyrolysis-GC/MS	Antic et al. (2011)
PNVCL	Germany	WWTP effluents	0.07 mg/L	Continuous-flow off-line pyrolysis-GC/MS	Vidovic et al. (2022)
PEI	Germany	Treatment plants influent and effluent	0.08 mg/L - 0.55 mg/L	Oon-line and off-line pyrolysis GC/MS	Vidovic et al. (2023)
PEG	Canada	Fresh snowfall	21.9 ng/mL	NALDI-TOF-MS	Wang et al. (2021)
PEG	Germany	WWTPs	7.4 µg/L	HPLC-MS/MS coupled to UHPLC-QTOF	Freeling et al. (2019)
PEG	England	Rivers	2.1–33.5 µg/L	1D 1H and 2D 1H DOSY NMR	Sainju et al. (2023)

Tabel 1. Detection and Analysis of Water-Soluble Polymers (WSPs) in Different Environments. **SEC-ESI-HRMS**-analytical combination of size-exclusion chromatography hyphenated with electrospray ionization-high-resolution mass spectrometry; **LOD**- limit of detection; **WWTPs**-wastewater treatment plants; **PVP**- polyvinylchloride; **PNVCL**-Poly (N-vinylcaprolactam); **GC/MS**-gas chromatography-mass spectrometry; **PEI**-polyethyleneimine; **NALDI-TOF-MS** -ultra-trace quantification method for MNPs based on nanostructured laser desorption/ionization time-of-flight mass spectrometry; **UHPLC-QTOF**- high performance liquid chromatography electrospray tandem mass spectrometry and UHPLC coupled to quadrupole time-of-flight; **1D 1H and 2D 1H DOSY NMR-** 1D 1H and 2D 1H Diffusion Ordered spectroscopy Nuclear Magnetic Resonance

1.5 Effects on organisms

Studies on the ecological risk assessment of WSPs are relatively scarce, and significant effects are primarily observed at high exposure concentrations that often exceed measured environmental levels. For instance, research on PVA toxicity typically focuses on extremely high concentrations that are not representative of the potential natural ones. The calculated LC_{50} for *Ceriodaphnia dubia* after 48 hours of exposure was 238.32 mg/L, with the Lowest Observed Effect Concentration (LOEC) ranging from 9.87 to 172.64 mg/L (Trudel et al., 2001; Magni et al., 2024). A study by Alonso-López et al. (2021) examined the effects of PVA on the sea urchin *Paracentrotus lividus*, revealing LOEC values ranging from 3.33 to 10 g/L, while 28-day exposure studies observed no toxicity in amphipods *Hyalella azteca* and *Leptocheirus plumulosus* at concentrations of 5.55 and 0.70 mg/L, respectively (Trudel et al., 2001; Magni et al., 2024). Similarly, a 96-hour exposure of PVA to fathead minnow *Pimephales promelas* and sheepshead minnow *Cyprinodon variegatus* showed no measurable toxicity (Trudel et al., 2001; Magni et al., 2024). Regarding polyacrylic acid (PAA), both *D. magna* and fish demonstrated low toxicity (LC/EC50 (48 h) > 200 mg/L for *D. magna*; LC/EC50 (96 h) > 200 mg/L for fish) (Wang et al., 2023). Nevertheless, Mondellini et al. (2022) reported reproductive effects in *D. magna* at concentrations above 5 mg/L for PAA, PVA, PEG, and PVP. Rozman and Kalčíková (2021) found that acrylate copolymers at 100 mg/L impacted bioluminescence and oxygen consumption in nitrifying bacteria *Allivibrio fischeri*, while showing no effect on the motility of *D. magna* over a 48-hour exposure period. Weston et al. (2009) discovered no significant acute effects of polyacrylamide (PAM) on aquatic organisms such as *Hyalella azteca*, *Chironomus dilutes, Ceriodaphnia dubia*, *Pimephales promelas*, and *Selenastrum capricornutum*, with LC_{50} values exceeding 100 mg/L, although *D. magna* exhibited higher sensitivity with an LC_{50} of 14 mg/L. Additional data on PEG ecotoxicity include findings by Hatami et al. (2019) which indicated alterations in *Cyprinus carpio* following a 21-day exposure to 10 mg/L of PEG, while Nascimento et al. (2021) observed neurotoxicity in *Physalaemus cuvieri* tadpoles after short exposures to 5 and 10 mg/L of PEG. In contrast, Duis et al. (2021) reported low acute and chronic toxicity in some crustaceans, whereas Siniakova et al. (2023) noted changes in luminescence intensity and colour of *Obelia longissimi* in the presence of the same polymer. A recent study by Robison-Smith et al. (2024) proposed that PVP might interact with the mucosal layer or gastrointestinal tract of organisms, potentially disrupting natural synergistic relationships or nutrient uptake. In this study, fish exposed to 0.01 mg/L PVP for 45 days and 1 mg/L PVP for 28 days did not show evidence of increased stress or energy demand despite experiencing significant growth reduction. Moreover, Julinova et al. (2018) highlighted

the potential hazardous effects of WSPs on entire ecosystems due to their interactions with persistent organic pollutants (POPs), thereby enhancing their bioavailability within the trophic chain.

These findings underscore the complex and multifaceted nature of WSP impacts on aquatic organisms, suggesting further research to elucidate their ecological consequences and inform effective mitigation strategies. In fact, the assumption of non-toxicity of WSPs is based solely on the performance of acute and chronic (eco)toxicity tests, as stated by the ECHA. These apical effects assays are those required by national and international legislations and are at this time the only endpoints considered for environmental risk assessment (ERA) of pollutants. In recent years, however, the need to perform sub-organism assays, especially for emerging contaminants, has been increasingly recognized (Sanderson and Solomon, 2009), as significant effects detected at the lower levels of the biological organization may still lead to effects that can still indirectly result in the death of the organism, impacting also the population.

2 AIMS OF THE PROJECT AND EXPERIMENTAL DESIGN

The primary objective of this study was to evaluate the ecotoxicity of four WSPs (PVP, PVA, PEG, PAA) to address the existing knowledge gap and provide data that could inform better regulation and risk assessment of these polymers. Besides, it is crucial to emphasize the importance of studying chronic effects on freshwater organisms, since the current literature is predominantly focused on acute toxicity tests, which rely on parameters such as the No Observed Effect Concentration/Lowest Observed Effect Concentration (NOEC/LOEC) and the Effective Concentration at 50% (EC50) (Magni et al., 2024). While these studies are relevant, they provide limited data, as they mainly focus on immediate effects and do not explore long-term consequences on organisms. Our work aims to fill this gap by analysing chronic effects, not limited to traditional mortality indicators, but considering a broader range of sublethal impacts that may emerge over extended periods.

To achieve this, a multi-step approach was assessed using the freshwater fish *D. rerio* and the crustacean *D. magna*, both widely used in ecotoxicology studies (Fig. 7). The effects of the four WSPs were investigated at molecular, cellular, physiological, organismal, and population levels.

By selecting different endpoints corresponding to these levels, the aim was to obtain comprehensive information on the modes of action of all the WSPs and understand how molecular-level impacts could reflect organismal and population-level effects.

Our initial study focused on the potential ecotoxicological impact of PVA (Par. 5.1.1). Given its slow degradation and widespread presence, both acute and chronic effects were investigated of both PVA standard powder and a commercial PVA-based bag. Firstly, to determine the useful concentrations, a range-finding acute test on *D. rerio* was conducted, exposing the specimens to 0.001 mg/L, 0.5 mg/L, 1 mg/L, 0.5 g/L, and 1 g/L. Since the highest concentrations caused acute effects and did not align with environmental levels, the lower concentrations were selected (0.001 mg/L, 0.5 mg/L, and 1 mg/L) for all subsequent studies. Thus, in the following study, *D. magna* adults and *D. rerio* embryos were exposed to the selected concentrations of solubilized PVA and PVA-based commercial bag. Acute toxicity was assessed through immobilization/mortality rates, while sub-lethal effects were measured through several endpoints at cellular (activity of the neuro-enzyme monoamine oxidase-MAO) and organism level (behavioral alterations in swimming performance) (Par. 5.1.1).

Building on the findings of our first study, we expanded our research to include and compare the effects of other commonly used WSPs: PVP, PEG, PAA (Par. 5.2.1). Since behavioral endpoints can reflect the alterations at molecular, cellular and physiological levels, this second study was focused on the swimming behaviour of *D. rerio* embryos exposed until 120 hours post-fertilization (hpf) to these WSPs, under varying light/dark stimuli intensities (300 lx, 2200 lx, and 4400 lx). This study aimed to introduce a novel methodological approach to evaluate the toxicity of WSPs by emphasizing swimming performance and locomotor behaviour. From this study, it was clear that all the WSPs affected the locomotor performance of *D. rerio*, following this scale of toxicity: PVP > PEG >> PAA (Par. 5.2.1).

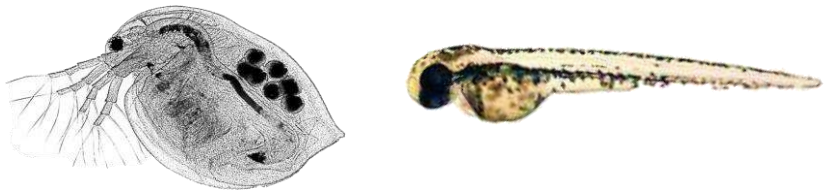
To better understand whether this toxicity scale is also reflected at lower levels of biological organization, the third study aimed to investigate the molecular and cellular mechanisms underlying the observed behavioural changes (Par. 5.2.2). It was employed a comprehensive suite of biochemical endpoints, including oxidative stress, detoxifying performance, and neurotoxicity, along with high-throughput proteomics to examine the chronic toxicity of PVP, PEG, and PAA. Moreover, by analysing the heart rate, it was possible to correlate phenotypic alterations with molecular changes, providing the mode of action for these WSPs. The integration of proteomics allowed to define protein variations across the whole proteome, enhancing our understanding of the toxicity mode of action of these polymers. At the end of these three studies, it was possible to confirm the scale of toxicity of these WSPs for *D. rerio* model, defining that PVP>PEG>>PAA>PVA.

To extend our findings from vertebrate models to invertebrates, our fourth study evaluated the effects of the same four polymers on *D. magna* (Par. 5.3.1). Using the same concentrations and

exposure durations as in previous studies, the same both physiological and biochemical endpoints were assessed in *D. magna* over 14 days. Behavioral parameters were measured such as swimming performance and phototaxis, along with biochemical biomarkers like MAO and acetylcholinesterase (AChE) activities and glycogen content. This study aimed to compare the modes of action of WSPs across different biological models and levels of organization, providing a WSPs comprehensive ecotoxicological profile.

Besides, to further understand the long-term ecological impacts of WSPs, the last research investigated the transgenerational effects of PEG and PVA on *D. magna*. By exposing successive generations (F_0, F_1, F_2, F_3) to 1 μg/L of these two polymers, the aim was to observe potential population-level effects and the transmission of the impact across generations (Par. 5.3.2). To achieve this, four successive generations were exposed to the contaminants. Two experimental groups were exposed for all the generations to the WSP, while for other two experimental groups the exposure was limited to the parental generation (F_0), with subsequent generations (F_1 onwards) returned to clean water. This approach was designed to observe whether the effects could be transmitted across generations even after the contaminant was removed. The endpoints analyzed in this study included global DNA methylation and reproduction.

Through this series of interconnected studies, both acute and chronic effects of these four WSPs were analysed on both vertebrate and invertebrate aquatic models. This multi-level and integrative approach not only elucidates the potential hazards posed by these polymers, but also lays the basement for future studies on their long-term and transgenerational effects. Our findings emphasize the need for comprehensive environmental assessments and regulatory measures to mitigate the ecological risks associated with WSPs.



Daphnia magna *Danio rerio*

Fig. 7 Organisms models selected for this study: the Cladocera *Daphnia magna* and the embryo of *Danio rerio*

3 SUMMARY OF RESULTS

This project integrates findings from five investigations focused on WSPs, evaluating their impacts on the two biological models *D. magna* and *D. rerio* to provide a comprehensive understanding of biological responses to their exposures. Initially, acute evaluations of PVA powder and PVA-based bags on both the model organisms revealed no significant differences in viability, indicating the absence of acute effects. Besides, no chronic effects in both *D. magna* and *D. rerio* were observed concerning the parameter of distance moved and the activity of AChE (Par. 5.1.1). On the contrary, PAA, PEG, and PVP impacted various locomotor parameters in *D. rerio*: both PAA and PEG influenced thigmotaxis behaviour, while PVP showed pronounced effects on locomotion parameters, as well as PEG, which exhibited heterogeneous effects. These results allowed us to observe how these polymers can alter the response of these organisms to stress (such as light), which can be encountered in natural conditions. Therefore, these findings are the first to support the hypothesis of a potential hazard posed by these polymers to freshwater organisms (Par. 5.2.1). In support to these first results, proteomic analyses highlighted significant influence of PVP, followed by PEG and PAA. Up-regulation of DNA-binding proteins (DBPs), RNA-binding proteins (RBPs), and translation initiation factors (eIFs) suggested impacts on genetic processes and development in *D. rerio*. Additionally, WSP exposure up-regulated signalling proteins, including GTP-binding proteins, potentially affecting receptor signalling and protein synthesis pathways. Protein binding, transport, and photoreception activity were also modulated, implying effects on eye development and vision. Biomarker assessments revealed that PVP and PAA induced changes in *D. rerio* heart rate, oxidative stress markers, and AChE activity, indicative of cardiotoxic and neurotoxic effects. In contrast, PEG did not significantly alter biomarkers compared to the control group. These results allowed us to identify the mode of action and confirm the effects of these polymers on a vertebrate embryo (Par. 5.2.2).

Also, the effects of PVA, PAA, PEG, and PVP on D. *magna* were comprehensively assessed across proteomic, physiological, and behavioral endpoints. While PEG exhibited acute toxicity at 0.5 mg/L, PVA had the most significant impact on the proteome, altering proteins involved in stress responses, reproduction, and energy allocation. Biomarker analyses highlighted variations in heart rate and AChE activity, indicative of neurotoxic effects in response to WSP exposures. Behavioral assessments revealed altered responses in swimming patterns, reflecting potential ecological implications for energy utilization and survival strategies in the presence of WSPs (Par. 5.3.1).

In conclusion, these integrated findings demonstrate the importance of measure endpoints on multiple biological levels, from molecular responses to behavioral adaptations, in assessing the ecotoxicological risks posed by emerging contaminants such as WSPs. Future research should continue to explore the mechanism of action of WSP and their long-term impacts on aquatic ecosystems to effectively inform regulatory measures and environmental management strategies. Therefore, the last study conducted about the transgenerational effects of PEG and PVA was essential to evaluate for the first time the potential hereditary effects and their impact across multiple generations. Thanks to epigenetic studies, it was determined that there are no long-term effects. In detail, multigenerational effects (changes in DNA methylation and in the number of juveniles) were observed between the F_0 and F_1 in individuals exposed to 1µg/L of PEG but the effects did not persist until the F_4, suggesting no heritability of these changes (Par. 5.3.2). This indicates that, while initial generations may experience certain epigenetic modifications due to exposure, these modifications are not passed down through subsequent generations, ultimately diminishing over time.

4 FINAL REMARKS

This comprehensive study has elucidated the ecotoxicological impacts of four widely used—PVA, PVP, PEG, and PAA—on both vertebrate (*D. rerio*) and invertebrate (*D. magna*) freshwater models. Our findings reveal several molecular, metabolic, and behavioral changes in organisms exposed to these WSPs, with PVP, PEG, and PAA demonstrating notable toxicity. Despite these acute and chronic effects, our epigenetic analyses suggest that these changes are not heritable across multiple generations, implying a limited long-term ecological impact on *D. magna*.

Future research should aim to deepen the understanding of the molecular mechanisms driving these toxic effects and explore the accumulation potential of these polymers. Conducting more extensive transgenerational studies on PVP and PAA is essential to fully assess their long-term ecological consequences. It is equally important to investigate the behavior of these polymers in the environment and to understand the potential synergistic effects of WSP mixtures once they enter aquatic ecosystems. Additionally, studies focusing on their impacts across different ecosystems—including terrestrial and marine environments—are necessary to obtain a holistic understanding of their ecological footprint.

Given the widespread use of these polymers in numerous commercial products, future studies should also focus on their interactions with various additives to develop more sustainable and less harmful alternatives. This study lays the groundwork for future studies on these relatively

unexplored polymers, highlighting the need for comprehensive risk assessment and regulatory frameworks. Such measures are critical to mitigate the environmental risks posed by WSPs, ensuring sustainable management practices.

5. ORIGINAL PAPER

5.1 Comparison of the effects of a standard powder and the commercial product

5.1.1 *Are "liquid plastics" a new environmental threat? The case of polyvinyl alcohol.*

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. Aquatic Toxicology, 248,106200.

5.2 Analysing the effects of WSPs on *Danio rerio*

5.2.1 *Assessment of behavioural effects of three water-soluble polymers in zebrafish embryos.*

Nigro, L., Magni, S., Ortenzi, M.A., Gazzotti, S., Della Torre, C., Sbarberi, R., Signorini, S., Binelli, A., 2023. Assessment of behavioural effects of three water-soluble polymers in zebrafish embryos. Science of The Total Environment, 893,164843.

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

Binelli, A., Nigro, L., Sbarberi, R., Della Torre, C., Magni, S., 2023. To be or not to be plastics? protein modulation and biochemical effects in zebrafish embryos exposed to three water-soluble polymers. Science of The Total Environment.

5.3 Analysing the effects of WSPs on *Daphnia magna*

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

Nigro, L., Magni, S., Ortenzi, M.A., Gazzotti, S., Della Torre, C., Sbarberi, R., Signorini, S., Binelli, A., 2024. Unveiling the multilevel impact of four water-soluble polymers on *Daphnia magna:* From proteome to behaviour (a case study) Journal of Hazardous Materials, 469, 134000

5.3.2 *Transgenerational Effects of Water-Soluble Polymers on Daphnia magna at Environmentally Relevant Concentrations*

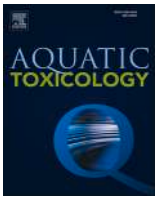
In preparation

Aquatic Toxicology 248 (2022) 106200

Contents lists available at ScienceDirect

Aquatic Toxicology

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



Are "liquid plastics" a new environmental threat? The case of polyvinyl alcohol

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ARTICLE INFO

Keywords:
Water-soluble polymers
Freshwaters
Behaviour
Neurotoxicity

ABSTRACT

Despite the pollution induced by plastics become a well-known and documented problem, bringing many countries to adopt restrictions about their production, commercialization and use, the impact of another emerging category of synthetic polymers, represented by the Water-Soluble Polymers (WSPs), also known as "liquid plastics", is overlooked by scientific community. WSPs are produced in large quantities and used in a wide plethora of applications such as food packaging, pharmaceuticals and personal care products, cosmetics and detergents, with a consequent continuous release in the environment. The aim of this study was the investigation of the possible toxicity induced by polyvinyl alcohol (PVA), one of the main produced and used WSPs, on two freshwater model organisms, the crustacean *Daphnia magna* and the teleost *Danio rerio* (zebrafish). We evaluated the effects of solubilized standard PVA powder and PVA-based commercial bags for carp-fishing, at 3 different concentrations (1 μg/L, 0.5 mg/L and 1 mg/L), through the exposures for 14 days of *D. magna* (daphnids; age < 24 h) and for 5 days of zebrafish embryos (up to 120 h post fertilization - hpf). As acute effects we evaluated the immobilization/mortality of specimens, while for chronic toxicity we selected several endpoints with a high ecological relevance, as the behavioural alteration on swimming performance, in real-time readout, and the activity of monoamine oxidase (MAO), a neuro-enzyme with a potential implication in the organism movement. The results showed the lack of significant effects induced by the selected substances, at all tested concentrations and in both model organisms. However, considering the wide plethora of available WSPs, other investigations are needed to provide the initial knowledge of risk assessment of these compounds contained in some consumer products.

1. Introduction

Plastics represent one of the main inventions of the 20th Century due to their low cost, mechanical properties, light weight, stability, and durability (Raddadi and Fava, 2019). In addition, these materials are suitable for a wide plethora of uses, and their production reached the 367 million tons in 2020 (PlasticsEurope, 2021). Consequently, the pollution induced by plastics represents an emerging global concern, well documented in scientific literature (Magni et al., 2019, 2021; Binelli et al., 2020, 2022; Talbot and Chang, 2022 and citations therein). However, another emerging form of plastic pollution, represented by the Water-Soluble Polymers (WSPs), also called "liquid plastics", is overlooked by scientific community. WSPs are substances that can be water-soluble under specific conditions of pH or temperature but may become insoluble if such conditions change. Consequently, they can affect the viscosity of the aqueous solution and can be modified, through water dispersion or dissolution, in gelled, stabilized, concentrated, and emulsified formulations (Ammar et al., 2019).

Being the conventional plastics characterized by solid state and insolubility in water (Hartmann et al., 2019), WSPs escape from the current legislations to limit the plastic pollution (Lam et al., 2018). In addition, WSPs are not registered under the Regulation, Evaluation, Authorization and Restriction of Chemicals (REACH) of the European Union (EU) and, consequently, there are not concrete evidence on their production volume, but also there are many gaps about their presence and effects in the environment (Arp and Knutsen, 2020; Huppertsberg et al., 2020). Furthermore, this scenario is complicated by the heterogeneous variety of WSPs available for many different industrial

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https://doi.org/10.1016/j.aquatox.2022.106200
Received 7 April 2022; Received in revised form 14 May 2022; Accepted 16 May 2022
Available online 17 May 2022

applications and in some consumer products. Indeed, the presence of some of which is already detected in wastewaters (Antić et al., 2011; Mairinger et al., 2021) because polyacrylamide (PAM) and its co-polymers are used as flocculants in Wastewater Treatment Plants (WWTPs), while polypropylene oxide (PPO) and polyethylene glycol (PEG; also known as polyethylene oxide - PEO) are added in paints and fertilizers as dispersing agents. In addition, PEG and polyvinylpyrrolidone (PVP) are used in pharmaceuticals and personal care products (PPCPs), while polyacrylic acid (PAA) as excipient in cosmetics (Patil and Ferritto, 2013; Penlidis et al., 2018; Rivas et al., 2018; Huppertsberg et al., 2020; Rozman and Kalčíková, 2021). Another well-known WSP, the polyvinyl alcohol (PVA or PVOH), is widely used in the production of textile and industrial fibers, adhesives, binders, water-soluble films for packaging materials, and in detergent pods (DeMerlis et al., 2003; Gaaz et al., 2015). This WSP is produced by polyvinyl acetate (PVAc; FAO, 2004) and its wide use is associated to its theoretical biodegradability, chemical and thermal stability, resistance to organic solvents and high-water solubility (Julinová et al., 2018). Other specific properties, such as biocompatibility, made the PVA useful in the biomedical and pharmaceutical fields, as the production of contact lenses, synthetic tear eye-drops, surgical sponges, and drug delivery (DeMerlis et al., 2003; Muppalaneni and Omidian 2013; Gaaz et al., 2015).

PVA-based products represent the largest volume of WSP produced in this century, reaching 650,000 tons/year worldwide (Xu et al., 2018). However, despite the massive application of PVA, previous studies reported its degradation as a slow process that can occur only under specific environmental conditions (Chiellini et al., 2003; Rolsky and Kelkar, 2021). Indeed, the physical properties of PVA as density, crystallinity and solubility are related to hydrolysis degree, crystal precipitation, molecular mass, and moisture (Saunders et al., 2012; Gaaz et al., 2015). To support this evidence, Suaria and co-authors (2016) showed that the percentage of PVA in the Mediterranean Sea was about 1.2 % of the total floating particles $>$ 700 μm, pointing out the environmental persistence of this polymer.

Based on this very complicated scenario, new evidence regarding the possible ecotoxicological impact of this polymer is then required. The aim of the present study was the evaluation of the effects of 3 different concentrations (1 μg/L, 0.5 mg/L and 1 mg/L) of solubilized standard PVA powder and PVA-based commercial bags for carp-fishing, on two model organisms well representative of the aquatic ecosystem: the crustacean *Daphnia magna* (daphnids; age $<$ 24 h, exposure of 14 days) and the teleost *Danio rerio* (zebrafish embryos; exposure from 0 to 120 h post fertilization - hpf). We assessed the acute toxicity as immobilization/mortality, and the chronic toxicity evaluating the behavioural alteration on swimming performance, in real-time readout, as well as the activity of the neuro-enzyme monoamine oxidase (MAO), as neurotoxicity endpoint just linked to movement. The monitoring of behavioural alterations is a sensitive biomarker to evaluate the xenobiotic impact, considering that some chemicals, as pesticides or microorganism products, and nanoparticles are able to alter the locomotor-based behaviour (Bownik, 2017; Simão et al., 2019 and citations therein). Therefore, based on the ability of PVA to affect viscosity of the aqueous media, the measurement of behavioural parameters as horizontal and vertical movement and positive/negative phototaxis ratio could highlight eventual modulation of key ecological functions as predator/prey relationship and the capacity to get food (Bownik et al., 2017; Horzmann et al., 2018). The evaluation of MAO activity fits coherently with this aspect, considering the role of this enzyme family in the degradation of monoamines (e.g., catecholamines and indolamines), in turn involved in important physiological mechanisms, as movement and reproduction in both vertebrate and invertebrates (Pearson, 1993; Campos et al., 2012, 2013; McCoole et al., 2012; Bellot et al., 2021).

2. Materials and methods

2.1. PVA powder and PVA-based bag characterization

Standard PVA powder was purchased from Sigma Aldrich. The producer declared a molecular weight (Mw) of 89,000 - 98,000 Da, a viscosity in water (4 % at 20°C) of 11.6 - 15.4 cps and a molar degree of hydrolysis of 99.0-99.8 %.

Due to the absence of technical information about the PVA-based bags (TKING; size of 100 × 140 mm), a commercial product commonly used as bait container in fishing activity, we deeply investigated its chemical/physical characteristics through an integrated analytical approach. In particular, we used the Proton Nuclear Magnetic Resonance (^{1}H-NMR), the Fourier-Transform Infrared Spectroscopy (FT-IR) and the Gas Chromatography-Mass Spectrometry (GC-MS) to identify the eventual presence of additives as well as the hydrolysis degree of PVA-based bags. For their characterization we used as reference standard the PVA powder Mowiol® 4-98 by Kuraray Europe GmbH, purchased from Sigma Aldrich. The producer declared a Mw of about 27,000 Da, a viscosity in water (DIN 53015) of 4.0-5.0 mPa x s, a molar degree of hydrolysis on 98.0 - 98.8% and 97.5 ± 2.5 % *non*-volatile components (water and organic solvents). About the ^{1}H-NMR, the 400 MHz spectra were recorded on a Bruker Ultrashield 400 spectrometer at 298 K. Samples were prepared dissolving about 10 mg of PVA in heavy water (D_2O). The FT-IR spectra were obtained through a Spectrum 100 spectrophotometer (Perkin Elmer) in attenuated total reflection (ATR) mode using a resolution of 4.0 and 256 scans, in a range of wavenumber between 4,000 and 400 cm^{-1}, using air at standard temperature and environmental moisture (23°C and 50 % RH) as background. Lastly, the GC-MS analysis was performed using an ISQ™ QD single quadrupole GC-MS (Thermo Fisher) and an Agilent technology VF-5ms (30 m x 0.25 mm i.d. x 0.25 μm) GC column. 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 %) with a flow rate of 1.2 mL/min, injection temperature 250°C, injection volume of 1 μL, and a split flow of 6.0 mL/min. MS apparatus transfer line and ion source temperatures were set at 270°C with a delay time of 5 min. The m/z range was set between 45 and 1,000. In total, 0.5 mg of the samples were dispersed in 1 mL of dichloromethane (CH_2Cl_2; HPLC purity) and stirred at room temperature for 24 h. The solution was filtered and then analysed.

2.2. Preparation of PVA solutions and exposure tests

For the PVA powder and PVA-based bag exposures we used both zebrafish (embryos; up to 120 hpf) and *D. magna* specimens (daphnids; age $<$ 24 h). The PVA powder and PVA-based bag solutions, used in the zebrafish exposures, were prepared in deionized water with 0.1 g/L Instant Ocean®, 0.1 g/L sodium bicarbonate ($NaHCO_3$), 0.2 g/L calcium sulphate ($CaSO_4$), 0.1 % methylene blue and aerated for 15 min before the use. To obtain a complete solubilization of compounds, the solutions were heated up to 100°C. On the other hand, the solutions for the *D. magna* exposures were prepared using commercial mineral water with conductivity 415 μS/cm at 25°C, pH 7.7, 57.1 mg/L hydrogen carbonate (HCO_3^-), 21 mg/L Calcium (Ca^{2+}), 1.7 mg/L Magnesium (Mg^{2+}), 1.9 mg/L Sodium (Na^+), 1.8 mg/L Potassium (K^+), 16.9 mg/L sulphate (SO_4^-), 1.6 mg/L nitrate (NO_3^-), 0.2 mg/L Fluoride (F^-), 5.9 mg/L silicon dioxide (SiO_2) and aerated for 15 min.

2.2.1. Zebrafish exposures

Regarding zebrafish, the fertilized eggs were provided by the facility of the Department of Earth and Environmental Sciences of the University of Milan Bicocca, according to the Italian laws, rules and regulations (Legislative Decree no. 116/92; authorization n. 0020984 - 12/02/2018). Considering the lack of information about the presence of WSPs in the aquatic environment, we performed a preliminary range-finding

test on zebrafish embryos (and not on *D. magna*) to 1 μg/L, 0.5 mg/L, 1 mg/L, 0.5 g/L and 1 g/L of PVA-based bag to select the exposure concentrations. We detected an acute effect (100 % mortality within the 120 hpf) starting to 0.5 g/L and for this reason we performed the subsequent exposures for chronic toxicity evaluation to 1 μg/L, 0.5 mg/L and 1 mg/L of PVA powder and PVA-based bag, carried out in static conditions and at 28°C. Zebrafish embryos were exposed, in triplicate, from 0 to 120 hpf within 50 mL Petri dish with 20 organisms for each treatment group. Viability and mortality were daily reported. At the end of the exposure, we performed the analysis of behavioural alteration and subsequently the specimens were frozen at -80°C for the measurement of MAO activity.

2.2.2. *D. magna* exposures

Regarding the *D. magna* exposures, we used specimens (daphnids, age < 24 h) derived from Daphtoxkit F ephippia (MicroBio Tests). For the ephippia hatching, 2 L of standard freshwater was prepared (UNI EN ISO 6341, 2013) with sodium bicarbonate ($NaHCO_3$; 129.5 mg), calcium chloride dihydrate ($CaCl_2$ x $2H_2O$; 588 mg), magnesium sulphate heptahydrate ($MgSO_4$ x $7H_2O$; 264.5 mg), potassium chloride (KCl; 11.5 mg) and aerated for 15 min before the use. Subsequently, the ephippia were placed in a micro-sieve, washed using tap water to eliminate the storage medium, and transferred in a Petri dish with 15 mL of pre-aerated standard water. The ephippia incubation was carried out for 72 h at 20°C under continuous illumination at 6000 lx. Before the test the daphnids were fed for 2 h with a suspension of the blue-green alga *Spirulina* spp. The exposure was conducted for 14 days in semi-static conditions at 20.0°C with a photoperiod of 16 h light (1500 lx) and 8 h dark, according to the *D. magna* Reproduction Test of Organisation for Economic Co-operation and Development (OECD) guideline 211 (2012). During the exposure, organisms were fed daily with the *Spirulina* spp. (3 μg/μL) and the yeast *Saccharomyces cerevisiae* (1 μg/μL), renewing the media solutions 3 times *per* week. The exposure was conducted in triplicate and for each treatment group we used 4 different beakers (50 mL) containing 5 specimens each one. Viability and immobilisation were daily reported. At the end of the exposure, we performed the analysis of behavioural alteration and subsequently the specimens were frozen at -80°C for the measurement of MAO activity.

2.3. Behavioural alteration on zebrafish and D. magna

In the last decades, many ecotoxicological tests were developed to evaluate the behavioural alterations, as coiling, touch-induced escape response, optomotor and optokinetic response and light-dark challenge test (Ahmad et al., 2012 and citations therein). In this study, we conducted several experiments to evaluate both the horizontal and vertical movements and positive/negative phototaxis ratio as behavioural parameters, using the light intensity as stimulus. Potential horizontal movement alterations, induced by PVA and PVA-based bag, on the swimming activity of both zebrafish embryos and *D. magna* specimens, were evaluated using the DanioVision™ video tracking system (Noldus IT, Wageningen, Netherlands). For each treatment group we used 18 specimens (54 total embryos or daphnids for each treatment). Each specimen was put in a single well of a 24-multiplate, in 3 mL of water (without tested contaminants to avoid the eventual direct effects induced by PVA on the viscosity of aqueous media) and submitted to 2 cycles of alternating dark period/low intensity light period, 2 cycles of dark period/high intensity light period and 2 cycles of dark period/-highest intensity light periods (Table 1). In detail, 10 min of adaptation were followed by 2 cycles of 5 min of dark and 5 min of low intensity light at 300 lx, 2 cycles of 5 min of dark and 5 min of light at 2200 lx and 2 cycles of 5 min of dark and 5 min of highest intensity light at 4400 lx (100% DanioVision™ illumination), recording the swimming activity at 30 frames/s (Table 1). These light intensities were chosen on the basis of lx detected in an oligotrophic lake in which the genus *Daphnia* lives (300–2000 lx; Tilzer et al., 1995), as well as on previous studies aimed to evaluate the behavioural alteration on *D. magna* induced by other emerging contaminants at different light stimuli (Spulber et al., 2014; González et al., 2018; Nikitin et al., 2019; Simão et al., 2019; Bedroissant et al., 2020; Fuertes and Barata, 2020; Zheng et al., 2021). The total duration of each analysis for each multiwell was 1.10 h (10 min for each cycle). During the entire test the temperature was maintained at 28°C for zebrafish, by the DanioVision™ temperature control unit, and at 20°C for *D. magna* though a room temperature. The data were acquired every 30 s for 60 min and analysed using the software EthoVision XT (Noldus IT, Wageningen, Netherlands) by measuring the total distance moved (mm).

Table 1
Sequence of dark/light conditions used in the behavioural parameter evaluation (horizontal movement, vertical migration and phototaxis).

End-point	Species		Sequence of dark/ light conditions	Time (min)
Horizontal movement	*D. magna* ✓	zebrafish ✓	dark	5
			300 lx	5
			dark	5
			300 lx	5
			dark	5
			2200 lx	5
			dark	5
			2200 lx	5
			dark	5
			4400 lx	5
			dark	5
			4400 lx	5
Vertical migration	*D. magna* ✓		dark	5
			300 lx	5
			2200 lx	5
			4400 lx	5
Phototaxis	*D. magna* ✓		dark	5
			300 lx	5
			2000 lx	5
			4400 lx	5

Considering that *D. magna* specimens in the environment migrate also vertically along the water column based on photoperiod, we evaluated their vertical migration and positive/negative phototaxis ratio. An experimental chamber was designed aligning 9 cylindrical glass cuvettes (5 × 1; h x diameter), containing an individual each one. For each group, 9 *D. magna* specimens of the same experimental treatment were distributed among the 9 vials filled with 3 mL of water (without tested contaminants to avoid the eventual direct effects induced by PVA on the viscosity of aqueous media). In total, 18 specimens of the same treatment group of each experimental replicate were analysed. A visible light LED lamp (4000 K) of 25 cm, mounted on the top of the cuvettes, was used to provide the light stimuli at 300 lx, 2200 lx and 4400 lx (Table 1). The dark condition (80 lx) was obtained positioning the lamp at 2 m from the chamber. Animals were acclimated in dark conditions for 10 min before the video recording. In detail, 5 min of dark period (80 lx) were followed by 15 min of increasing light intensities: 5 min at 300 lx, 5 min at 2200 lx and 5 min at 4400 lx (measured by HoldPeak 881d Digit lx meter on the top of the water column), with a total duration of 20 min for each experiment (Table 1). Video-tracking was recorded by a Basler acA1300-60gm GigE camera with an optical 8 mm HR 2•2″ F1.4 lend and a resolution of 1,280 × 1,024 pixels, positioned squarely 32 cm from the rack containing the experimental chambers. The GigE camera was connected through a Power PoE single injector (Ace series) to the EthoVision XT 11.5 software (Noldus IT, Wageningen, Netherlands) and the chamber was surrounded by a black paper sheet to avoid the entrance of interfering light in the system. After videorecording at 25 frames/s (fps), EthoVision XT 12 video tracking software was used for analysing the movement of each animal. The individual tracks, acquired every 30 s for 20 min, were analysed using the software EthoVision XT

(Noldus IT, Wageningen, Netherlands) determining the total distance moved (mm) for each animal. Lastly, considering that some chemicals can alter phototaxis (Rivetti et al., 2016), we also evaluated the *D. magna* positive/negative phototaxis ratio in response to different light intensities. The analysis was performed by splitting the experimental chamber (the same used for the vertical migration assessment) in two different zones (upper, zone 1; lower, zone 2) through the EthoVision XT 11.5 software. The movement of each animal in both zones was measured as the mean distance moved (mm) every 5 min (5 min of dark period, 5 min at 300 lx, 5 min at 2200 lx and 5 min at 4400 lx; Table 1). Subsequently, the ratio between the distance moved in the two zones was calculated. Through this ratio, it was possible to define in which zone the *D. magna* specimens conducted the major movement under the different light conditions, defining the positive (toward light, evaluated in zone 1) or negative phototaxis (far to light, evaluated in zone 2).

2.4. Neurotoxicity biomarker

Contextually to the evaluation of behavioural alteration, we also assessed the potential neurotoxicity of selected substances, on 3 pools of 20 specimens for each group, following the procedure described by Gagné (2014) and Magni et al. (2018, 2021). The homogenates were obtained pottering the specimens in 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) - sodium hydroxide (HEPES--NaOH) buffer (pH = 7.4), in a ratio 1:10 W/V, with 100 mM sodium chloride (NaCl), 0.1 mM dithiothreitol (DTT) and protease inhibitor. Subsequently, the homogenates were centrifuged at 1,000 g for 20 min at 4°C. We quantified the proteins in the S1 fraction using the Bradford method (Bradford, 1976), to normalize the MAO activity. The kinetics of MAO was measured in the S1 fraction using 1 mM tyramine as substrate, 10 μM dichlorofluorescein diacetate in a 140 mM NaCl, 10 mM HEPES-NaOH buffer, pH = 7.4, 1 mg/mL peroxidase and 10 mM of 3-amino-1,2,4-triazole (catalase inhibitor). We measured the fluorescence for 3 min at 485 nm (excitation) and 530 nm (emission) at the EnSight™ multimode plate reader (PerkinElmer).

2.5. Statistical analysis

STATISTICA 7.0 software was used to perform Statistical analyses on biomarker and acute effect data. The significant differences between treated and control were assessed by two-way analysis of variance (two-way ANOVA) for the horizontal and vertical movement (treatment and time as variables) and by one-way ANOVA for phototaxis, MAO activity (treatment as variable) and acute effects, followed by Bonferroni Correction test ($p < 0.05$ as significant cut-off).

3. Results and discussion

3.1. Material characterization

The ^{1}H NMR spectra were recorded to determine the actual hydrolysis degree of Mowiol® 4-98 in the form of powder and the PVA-based bag. The calculation was made according to previous approaches (Budhlall et al., 2000): spectra of Mowiol® and PVA-based bag (Fig. 1A and B, respectively) are characterized by a peak centred at 1.96 ppm, which can be attributed to the methyl group (Fig. 1A and B, in red) of the

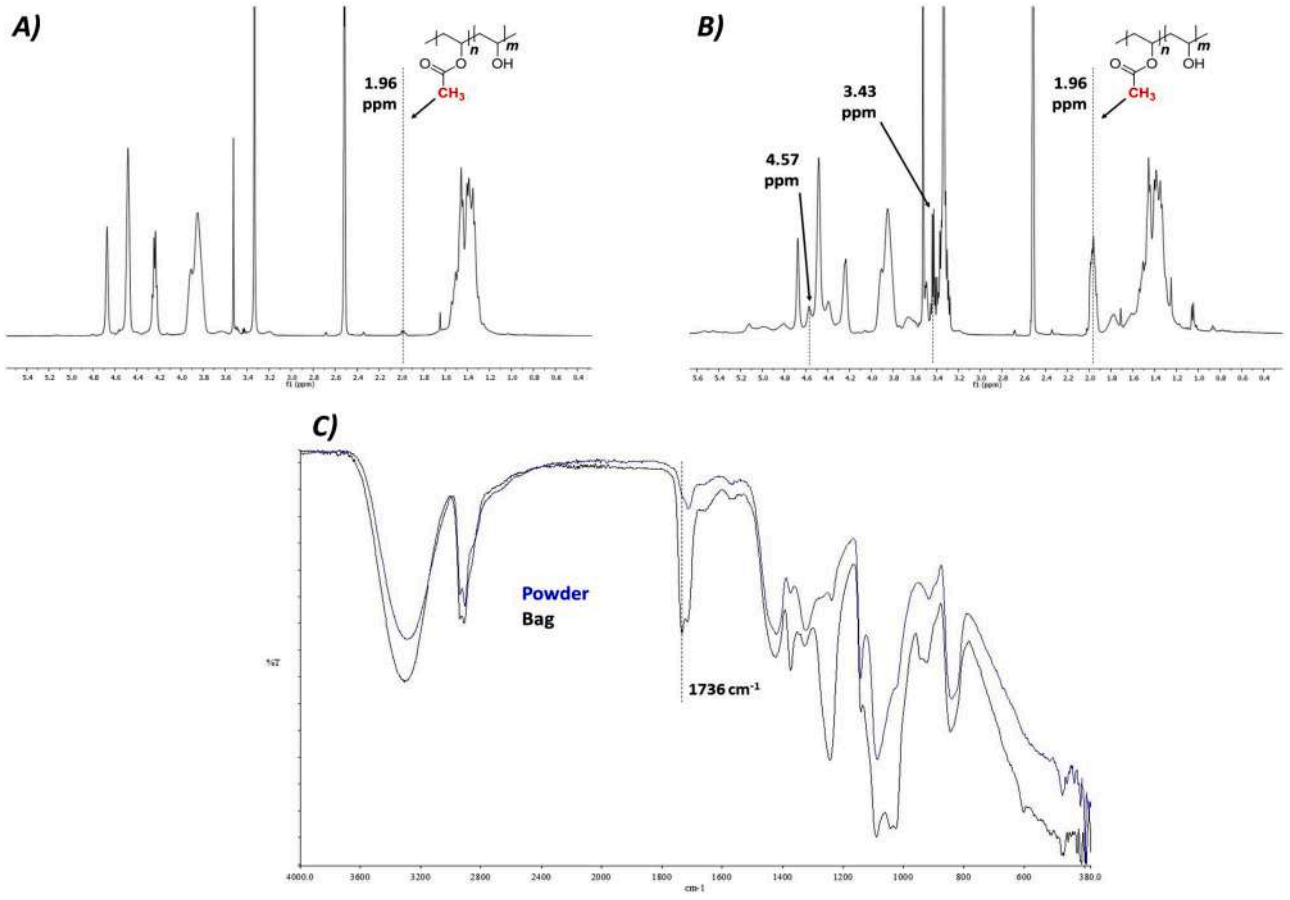

Fig. 1. (A) ^{1}H-NMR spectrum of Mowiol® and (B) of PVA-based bag. The peaks centred at 1.96 (methyl group of the acetyl group of the *non*-hydrolysed repeating units), 4.57 and 3.43 ppm (additives in the PVA-based bag) were highlighted by arrows in the spectra. (C) Superimposed FT-IR spectra for Mowiol® (blue infrared spectrum) and PVA-based bag (black infrared spectrum). The strong band at 1,736 cm^{-1} was attributed to the carbonyl stretching of the acetyl moieties on the partially hydrolysed PVA-based bag.

acetyl group of the *non*-hydrolysed repeating units. Given the higher hydrolysis degree, the peak is only slightly detectable in the spectrum of the Mowiol® (Fig. 1A), while it is well visible in the PVA-based bag (Fig. 1B). The integration of the peak, compared to the integration of the signals of the -CH and -CH_2 groups of the chain, allowed us the calculation of the hydrolysis degree of the samples assessed to be 98 % for the Mowiol® and 85 % for the PVA-based bag. In this context, superimposed FT-IR spectra for PVA-based bag and Mowiol® are reported in Fig. 1C. The main difference between the two spectra is located in the strong band at 1,736 cm^{-1} which was attributed to the carbonyl stretching of the acetyl moieties on the partially hydrolysed PVA-based bag. The analysis did not allow the detection of additives which are likely embedded in the material itself and therefore not detectable as such through FT-IR. However, as shown in Fig. 1A and B, the spectrum of the PVA-based bag highlighted the presence of signal that cannot be attributed to Mowiol® and is therefore likely due to additives (peaks at 4.57 and 3.43 ppm) which are not present in the Mowiol®. To characterize the additives, both the Mowiol® and the PVA-based bag were subjected to solvent extraction with dichloromethane and GC-MS chromatograms were recorded on the extracts. As shown in Fig. 2A, no peaks are detectable in the Mowiol® extract, while three main peaks are detectable in the chromatogram of the PVA-based bag (Fig. 2B) extract at 10.22, 13.78, and 17.00 min, which were assigned by NIST2017 database to triethylene glycol (TEG), tetraethylene glycol (TetraEG) and pentaethylene glycol, respectively. The presence of these three species also finds confirmation in the peaks at 4.57 and 3.43 ppm detected in the ^{1}HNMR spectrum reported in Fig. 1B. These additives, derived from ethylene glycol (EG), were probably used as plasticizer in the PVA-based bag production, considering the EG use in the polyester industry, *non*-volatile antifreeze, and plasticizer (Guo et al., 2007; Yin et al., 2019). In addition, TEG/PVA blend, due to its high hygroscopic property, is used in dehumidification applications (Bui et al., 2017). For this reason, the addition of TEG in the PVA product could reduce the moisture absorption by PVA, increasing both product conservation and quality. Based on these evidence, the identification of TEG, TetraEG and pentaethylene glycol in the PVA-based bag could be interesting in the context of ecotoxicological effects of considered materials.

3.2. Toxicity evaluation

Regarding the acute effects induced by PVA powder and PVA-based bag on both *D. magna* and zebrafish specimens, coherently with the results obtained in the range-finding test, we did not observe significant differences in the viability parameter. In detail, in *D. magna* specimens all treatment groups showed a viability $\geq$ to 77 % (78 % for 1 μg/L, 77 % for 0.5 mg/L and 82 % for 1 mg/L PVA powder and 85 % for 1 μg/L and 83 % for both 0.5 and 1 mg/L of PVA-based bag), perfectly comparable with a value of 88 % in the control. In the same manner, in zebrafish we obtained a viability $\geq$ to 86 % in all treatments (92 % for 1 μg/L, 93 % for 0.5 mg/L and 95 % for 1 mg/L PVA powder and 92 % for 1 μg/L, 86 % for 0.5 mg/L and 92 % for 1 mg/L of PVA-based bag), with 98 % of viability in the control.

After the confirmation of the absence of acute effects, we evaluated the potential chronic toxicity on the selected biological models. Concerning the behavioural alteration, after 14 days of exposure to PVA powder and PVA-based bag, the *D. magna* horizontal swimming showed a similar behavioural response among the treatments without significant differences compared to control (Fig. 3A and B). Only the *D. magna* specimens exposed to 0.5 mg/L of PVA powder showed a higher, but not significant, swimming performance during the entire light/dark 60 min cycle (Fig. 3A). The increase of distance moved under the different lights could be related to the phototaxis, to find darker zones to make themselves less visible to visual-oriented predators, whose activity increases at high light intensities (Tałanda et al., 2018; Simão et al., 2019). However, no differences, compared to control, are induced by the two tested materials on positive/negative phototaxis ratio (Fig. 4). Since phototaxis is also related to *D. magna* vertical migration to limit the predation during the day, we also checked this parameter, but no differences were observed between treated and controls (Fig. 5A and B).

Moving to the results of the tests on zebrafish, which represents the potential zooplankton predator, the behavioural analysis did not show again any differences in the horizontal swimming, compared to control (Fig. 3C and D). In general, differently to *D. magna*, the total distance moved was higher under dark compared to light conditions. In line with previous studies (Llanos et al., 2018; Basnet et al., 2019; Hussain et al., 2020), the reduction of locomotion in response to light stimuli test was

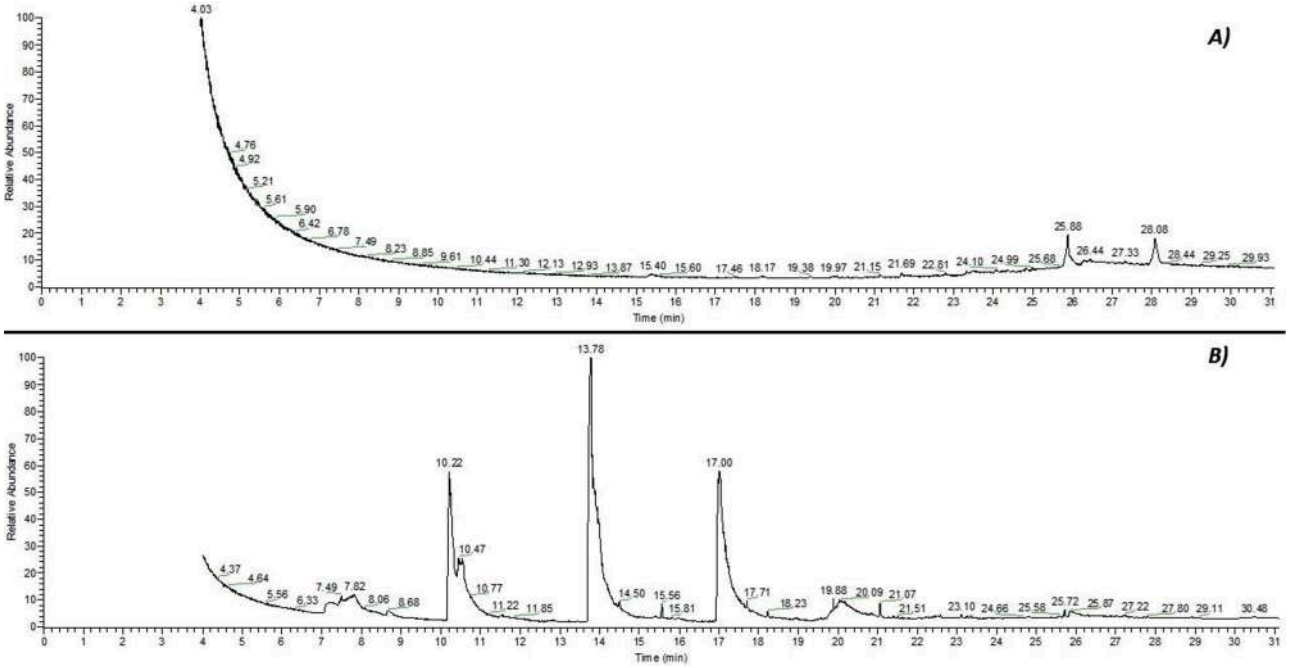

Fig. 2. GC-MS chromatograms relative to the extracts of Mowiol® (A) and PVA-based bag (B). The peaks in the chromatogram of PVA-based bag extract were referred to additives triethylen glycol (TEG; 10.22 min), tetraethylen glycol (TetraEG; 13.78 min) and pentaethylen glycol (17.00 min).

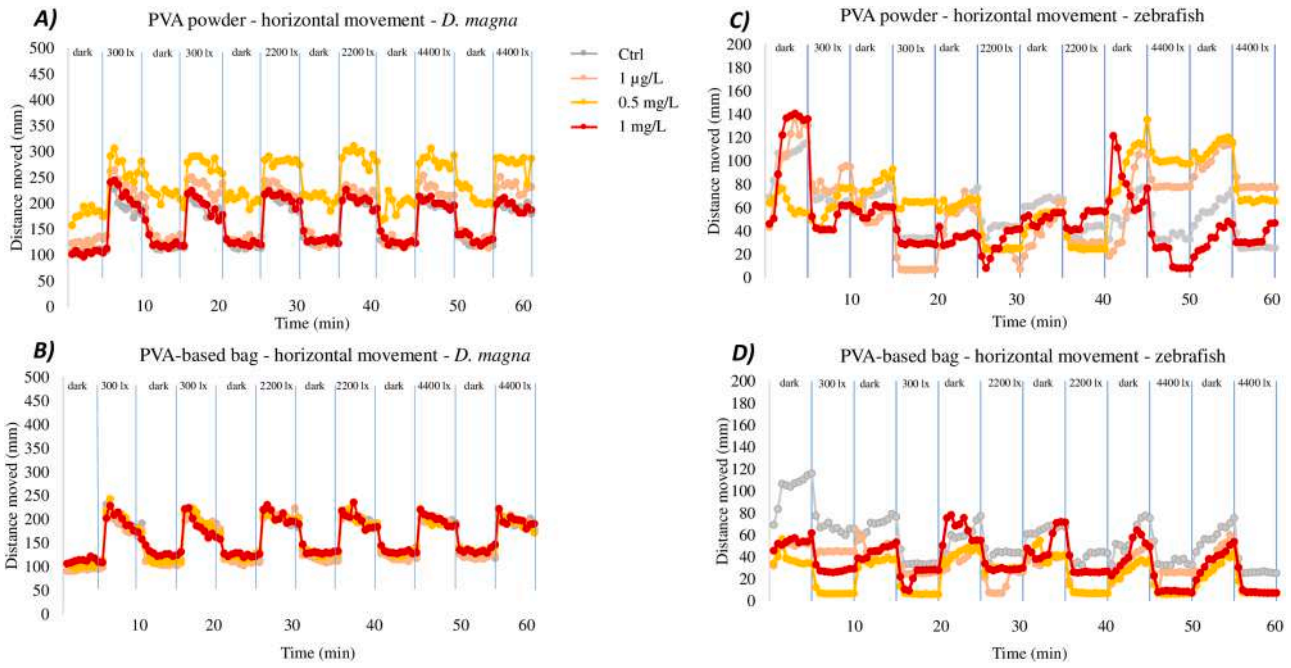

Fig. 3. Horizontal movement (mean value; the standard deviations (SDs) were removed from the graphs to increase the readability of presented results, see the Supplementary materials for SD) of *D. magna* (A, B) and zebrafish embryos (C, D) at the end of exposure (14 days and 120 hpf, respectively) to PVA and PVA-based bag (exposure in triplicate; n = 18 specimens *per* treatment; two-way ANOVA). The measurements (every 30 s) were conducted as the distance moved across consecutive 2 cycles of 5 min of dark and 5 min of low intensity light at 300 lx, 2 cycles of 5 min of dark and 5 min of light at 2200 lx and 2 cycles of 5 min of dark and 5 min of highest intensity light at 4400 lx. We used the same control groups for both PVA powder and PVA-based bag treatments.

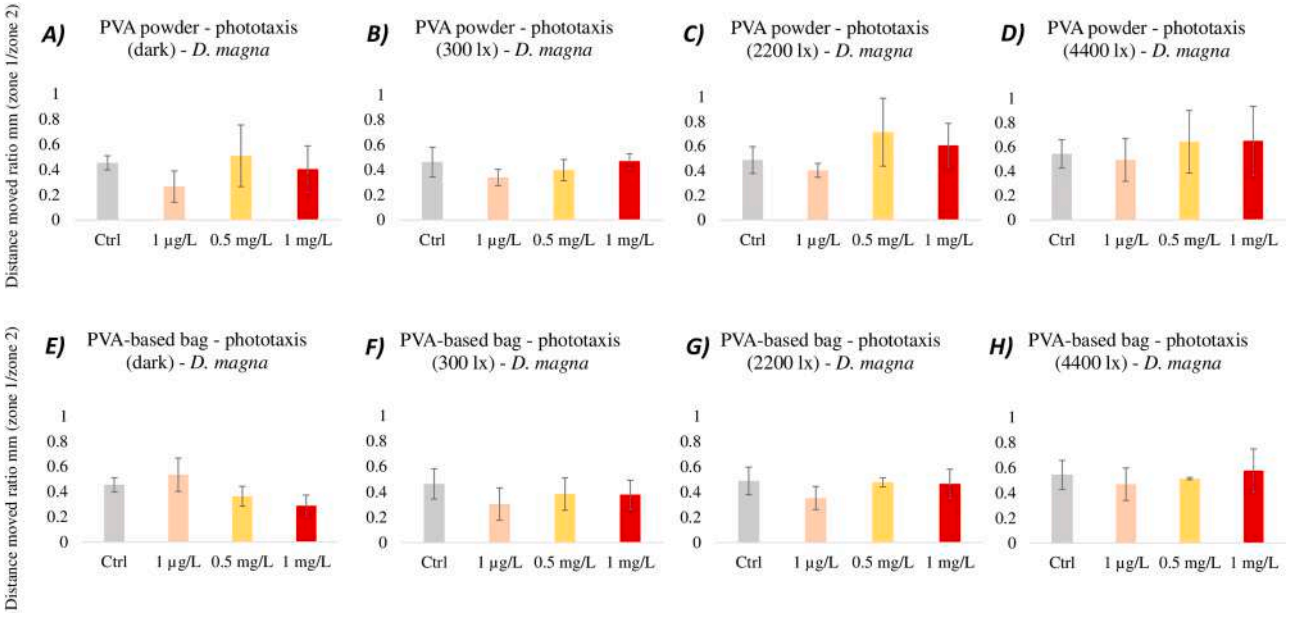

Fig. 4. Positive/negative phototaxis ratio (mean ± standard deviation; SD) of *D. magna* at the end of exposure (14 days) to PVA powder (A,B,C and D, the letters are referred to different light intensities) and PVA-based bag (E,F,G and H; exposure in triplicate; n = 18 specimens *per* treatment; one-way ANOVA). The measurements were conducted as the distance moved across consecutive 5 min of dark (80 lx), low intensity light at 300 lx, light at 2200 lx and highest intensity light at 4400 lx.

observed under all light intensities. This phenomenon is known as "freezing", a common anxiety index (Champagne et al., 2010) together with erratic movements, thigmotaxis and scototaxis, characterized by the absence of movement, apart from gills and eyes (Ahmad et al., 2012). In general, adult specimens present freezing behaviour if exposed to anxiogenics, as illumination conditions and environmental characteristics (inner/outer and opaque/transparent zones of the tank; Egan et al., 2009; Champagne et al., 2010), while young larvae remain in freezing condition for more time (Thirumalai and Cline, 2008; Colwill and Creton, 2011). In this context, the light conditions are a pivotal parameter to zebrafish embryos, which can decrease or increase their swimming activity depending on light intensity (Padilla et al., 2011).

Moving to the results obtained after our exposure assays, the freezing behaviour was more visible in the control groups than in other exposure groups, although no significant differences were observed (Fig. 3 C and D). Interestingly, zebrafish specimens did not show the natural freezing

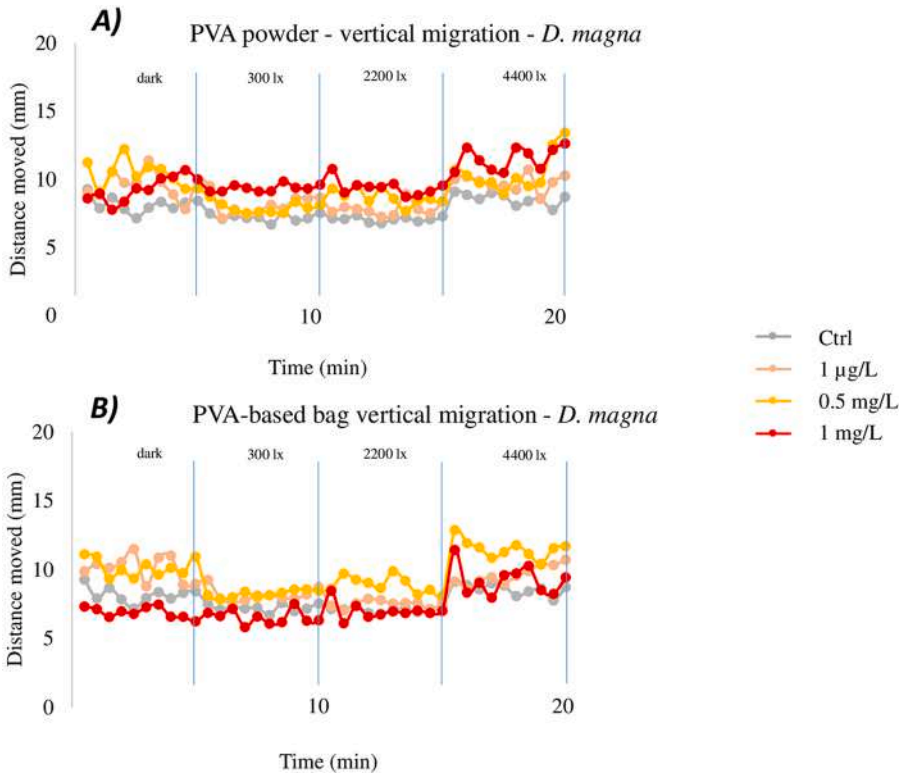

Fig. 5. Vertical migration (mean value; the standard deviations (SDs) were removed from the graphs to increase the readability of presented results, see the Supplementary materials for SD) of *D. magna* at the end of exposure (14 days) to PVA powder (A) and PVA-based bag (B; exposure in triplicate; n = 18 specimens *per* treatment; two-way ANOVA). The measurements (every 30 s) were conducted as the distance moved across consecutive 5 min of dark (80 lx), low intensity light at 300 lx, light at 2200 lx and highest intensity light at 4400 lx. We used the same control groups for both PVA powder and PVA-based bag treatments.

behaviour in the treated to 1 µg/L and 0.5 mg/L PVA powder when exposed for the first time to 4400 lx, while the groups exposed to 1 mg/L PVA powder and the control showed a rapid fall of movement performance when exposed for the first time at the same light condition (Fig. 3C). This strange behavior, the cause of which should be investigated further in the future, is also confirmed in the second exposure at 4400 lx, albeit to a lesser extent than the first.

The lack of significant alteration was obtained, once again, in zebrafish exposed to PVA-based bag in all the treatment groups, although a *non*-significant decrease in the distance moved can be observed for all three treatments for the two initial dark phases and for the exposure to 300 lx (Fig. 3D). Furthermore, a general decrease of the movement under 2200 lx and 4400 lx conditions was detected for all treatment groups, while the specimens exposed to 0.5 mg/L PVA-based bag showed a lower movement during all the analysis, compared to control (Fig. 3C and D). To deeply investigate the effects of selected

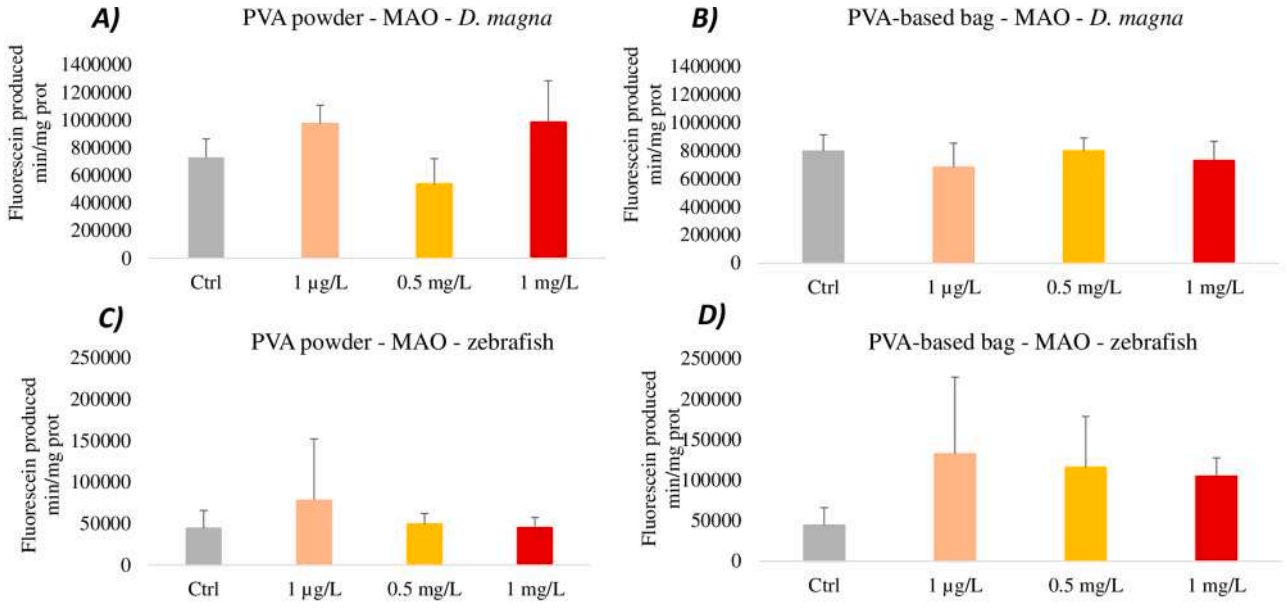

Fig. 6. MAO activity (mean ± standard deviation; SD) in *D. magna* (A, B) and zebrafish embryos (C, D) at the end of exposure (14 days and 120 hpf, respectively) to PVA and PVA-based bag (for *D. magna*: exposure in triplicate with 4 beakers for each treatment - 5 specimens *per* beakers, n = 3 pools of 20 specimens *per* treatment; for zebrafish: exposure in triplicate with 1 Petri dish for each treatment with - 20 specimens *per* Petri dish; n = 3 pools of 20 specimens *per* treatment; one-way ANOVA). We used the same control groups for both PVA powder and PVA-based bag treatments.

materials on the movement of exposed organisms, we also evaluated the MAO activity. After the exposure, *non*-significant increase in the biological trend of MAO activity was observed in both *D. magna* and zebrafish embryos (Fig. 6). In detail, *D. magna* showed an increase of MAO activity at 1 μg/L and 1 mg/L of PVA powder (Fig. 6A), while zebrafish at 1 μg/L of PVA powder (Fig. 6C) and at all tested concentrations of PVA-based bag (Fig. 6D).

As final consideration, the lack of significant differences for the MAO activities of the exposed organisms with the relative controls confirms that the two tested materials, and therefore the PVA which represents their major component, does not seem to have any negative effect on the two selected biological models, at least for the tested concentrations and for the measured end points. Indeed, we must remember that the preliminary range-finding had highlighted an extensive mortality in zebrafish embryos exposed to higher PVA concentrations. The lack of effects observed in this study suggests the absence of ecotoxicological differences between the standard PVA powder and PVA-based bag, highlighting how the additives detected in the commercial product did not have a key role in the toxicity. Lastly, we would highlight the need to improve the knowledge regarding the impact of these emerging contaminants on the aquatic environment, making possible the eventual toxicity comparison between experiments on WSP toxicity, actually not feasible due to the absence of data.

4. Conclusions

This study is surely the first to investigate the potential ecotoxicological effect of one of the most widely used WSPs using two different biological models. The presented results showed a lack of toxicity induced by standard PVA powder and a commercial PVA-based bag on selected freshwater species investigating both apical parameters, such as any effects on behaviour, and biochemicals such as the measurement of MAO activity. However, other investigations are necessary in this field for the following reasons: 1) could be interesting to evaluate also the impact of these contaminants at molecular and cellular levels, using biomarkers of cellular stress, oxidative damage and cyto-genotoxicity and “omics” techniques, to propose a possible WSP mechanism of action; 2) the presented results are referred to PVA, but considering the wide plethora of produced and used WSPs, as PEG, PVP and PAA, the future studies could also consider the impact of these substances; 3) a pivotal aspect in the ecotoxicology of WSP could be their monitoring in the aquatic environment, to certify their presence, as well as to provide environmental relevant concentrations useful for the laboratory exposures.

CRediT authorship contribution statement

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

Declaration of Competing Interest

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

Acknolodgements

This study was funded by Line 2 grant of the Research Support Plan (PSR) 2020 assigned to Dr. Stefano Magni by Department of Biosciences of the University of Milan.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2022.106200.

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Science of the Total Environment 893 (2023) 164843

Contents lists available at ScienceDirect

Science of the Total Environment

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

Assessment of behavioural effects of three water-soluble polymers in zebrafish embryos

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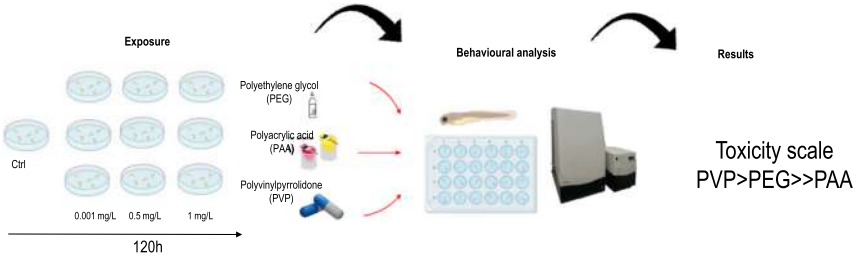
HIGHLIGHTS

- The ecotoxicological effects of three water-soluble polymers (WSPs) were evaluated.
- Tested WSPs were PolyVinyl Pyrrolidone, PolyEthylene Glycol, PolyAcrilyc Acid.
- Many endpoints of the swimming behaviour of zebrafish embryos were monitored.
- Significant changes in swimming parameters were measured for all WSPs.
- The whole dataset suggests a toxicity scale: PVP > PEG ≫ PAA.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Henner Hollert

Keywords:
Ecotoxicology
Risk assessment
Aquatic ecosystems
Synthetic polymers
Swimming behaviour
Liquid plastics

ABSTRACT

The water-soluble polymers (WSPs) are widely used in many industrial applications and are present in several commonly used products due to their physical-chemical characteristics: as their name indicates, despite being synthetic polymers, they are able to solubilize in water. Because of this peculiar property, both the qualitative-quantitative evaluation in aquatic ecosystems and their potential (eco)toxicological effects have been neglected until now. The aim of this study was to evaluate the possible effects of three of the most widely used WSPs as polyacrylic acid (PAA), polyethylene glycol (PEG) and polyvinyl pyrrolidone (PVP) on the swimming behaviour of zebrafish (*Danio rerio*) embryos after the exposure to different concentrations (0.001, 0.5, 1 mg/L). The exposure lasted from the eggs' collection up to 120 h post fertilization (hpf) also using three different light intensity (300 lx, 2200 lx, 4400 lx) to better evaluate any effects related to different gradients of light/dark transitions. In order to analyze individual behavioural changes in embryos, their swimming movements were tracked and a number of parameters for locomotion and directionality were quantified. The main results showed that all three WSPs resulted in significant ($p \leq 0.05$) variations in different movement parameters, suggesting a possible toxicity scale: PVP > PEG ≫ PAA.

1. Introduction

The emerging environmental problem related to the presence of plastics in the environment has neglected until now a particular category of polymers that could represent a further danger especially to aquatic ecosystems, the so-called water-soluble polymers (WSPs) also known as "liquid plastics". This kind of polymers are suitable for a wide range of applications since they could act as suspending agents, thickeners, stabilizers, coagulants, flocculants, filming agents, binders, humectants, and lubricants in aqueous media (Petrovic and Barcelo, 2000; Umoren et al., 2006; Pocurull et al., 2009; Kadajji and Betageri, 2011; Koltzenburg et al., 2014). Inevitably, this widespread use often facilitates both direct and indirect release especially into the aquatic environments where they dissolve,

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http://dx.doi.org/10.1016/j.scitotenv.2023.164843
Received 20 March 2023; Received in revised form 18 May 2023; Accepted 10 June 2023
Available online 14 June 2023

disperse, or swell and consequently modify the physical properties of the resulting aqueous matrix (Kadajji and Betageri, 2011), as well as representing other potentially dangerous compounds for the biocoenosis. The presence of WSPs in the ecosystems can now be considered ubiquitous: their release in sewage system and surface water could be due to their use in washing agents, textiles, personal care products, cosmetic and pharmaceutical industries or to rain runoff from constructions, coatings, and paint formulations (Duis et al., 2021). Furthermore, their application as constituents of pesticides and fertilizers causes their release directly onto fields (Krogh et al., 2003; Koltzenburg et al., 2014; Xiong et al., 2018) and their usage as flocculants in wastewaters and drinking waters determines their presence in the sewage sludge (Petrovic and Barcelo, 2000; Pocurull et al., 2009; Koltzenburg et al., 2014; Duis et al., 2021). In this context, Julinova et al. (2018) highlighted that WSPs can interact with the persistent organic pollutants (POPs) making them more bioavailable for the trophic chain and potentially hazardous for the entire ecosystem.

Despite their wide production and use, WSPs have not been registered under REACH (regulation, evaluation, authorization, and restriction of chemicals; UE 1907/2006) yet, even if the possible REACH registration is current being reviewed as mentioned in the Wood report (EU, 2020). Moreover, the WSPs do not enter the polymer size-based classification, escaping the current legislations towards physical contaminants as plastics. For all these reasons, they are not monitored or included in current European plastic strategies or circular economy action plans (Rozman and Kalcikova, 2021).

The production volume of these WSPs in Europe can be estimated considering the annual production of the respective monomers, assuming WSP synthesis as their main application (Huppertsberg et al., 2020). This aspect results in an estimated production volume in the range of thousands to millions of tons for each WSPs: polyvinylpyrrolidone (PVP) > 10^3 t/y (ECHA (I)); polyacrylic acid (PAA) = $10^5 - 10^6$ t/y (ECHA (II)), polyvinyl alcohol (PVA) = $10^5 - 10^6$ t/y (ECHA (III)), and polyethylene glycol (PEG) > 10^6 t/y (ECHA (IV)), clearly indicating that a huge release in the natural environment can occur.

Among the most commonly used WSPs, PVA, PAA, PEG and PVP stand out for the wide range of applications in which they are used (Halake et al., 2014). Notwithstanding their widespread use, the environmental occurrence and concentration of these WSPs are unknown and only two studies are available about their environmental levels: Antić et al. (2011) measured a PVP concentration between 0.9 mg/L and 7 mg/L in effluents from wastewater treatment plants, and of about 0.1 mg/L in river water affected by municipal wastewater emissions in Düren (Germany). Moreover, Wang et al. (2021) showed that PEG is present in fresh falling snow with a concentration of 219 μg/L in the city of Montréal (Canada).

Even from the ecotoxicological point of view, few and *non*-conclusive information is available related to tests on acute and chronic toxicity evaluation. The recent study of Rozman and Kalcikova (2021) showed how a very high concentration (100 mg/L) of an acid-based WSP used in personal care products and cosmetics was able to inhibit the bioluminescence by 73 % and the oxygen consumption by 88 % in the bacterium *Aliivibrio fischeri*, while the same concentration had no effect on the motility of the cladoceran *Daphnia magna* exposed for 48 h. Some polyacrylamide (PAM) flocculants with MWs between 5 and 17 × 10^6 g/mol demonstrated no acute effects on larvae and juveniles of four mussel species (Buczek et al., 2017), as well as 5 mg/L concentrations of PEG did not affect any biochemical parameter in carp (*Cyprinus carpio*) during a 21-day exposure, while higher concentration (10 mg/L) induced alterations in some blood biochemical parameters of fish (Hatami et al., 2019). Mondellini et al. (2022) in a recent study showed how PEG, PVP, PAA and PVA did not exert any acute effect up to 50 mg/L on *D. magna* although effects on reproduction were observed for animals exposed to PVA (5 mg/L and 10 mg/L), PEG (5 mg/L and 10 mg/L), PAA (10 mg/L) and PVP (5 mg/L).

In this context, to evaluate the mode of action of emerging contaminants it is possible to evaluate endpoints at different levels of biological organization and we chose the behavioural parameters, not considered in the abovementioned studies, since they summarize the potential alterations occurred at molecular, cellular and physiological levels.

Therefore, this study evaluated for the first time the effects of some WSPs on swimming behaviour of zebrafish embryos, an effect of high ecological relevance and considered as a new methodological approach for toxicity evaluation since swimming ability is representative of motility performance and locomotor behaviour that play a key role in the feeding, social and defensive activities of zebrafish throughout its entire life cycle (Colwill and Creton, 2011a). In addition, this work is a part of a whole research aimed on the toxicity evaluation of WSPs at different levels of biological organization, from behavioural alterations, considered in the present research, to other molecular and cellular endpoints as biomarkers and proteomics.

In detail, in the present study, we evaluated the behavioural effects of exposures at different concentrations of PVP, PEG and PAA on the teleost *D. rerio* (zebrafish) embryos by the measure of several endpoints related to swimming performances. We conducted the exposures in static conditions, exposing the embryos for 5 days up to 120 h post fertilization (hpf) to increasing concentrations (0.001, 0.5 and 1 mg/L) of the three WSPs, selected based on our previous study on PVA (Nigro et al., 2022) and on the few environmental data above mentioned. Another variable to be considered in these studies is the molecular weight (MW) of the chosen WSPs, since each one of them can be produced in different grades, i.e. different MWs. The chemical-physical properties of each WSP are influenced by their MW and, in turn, their environmental fate is influenced as well as a direct consequence: e.g., the higher is the MW, the higher is the degradation time. Moreover, on a general level, the higher is the MW of the polymer, the less soluble it is.

Furthermore, our experiments were conducted not only by alternating consecutive cycles of light/dark stimuli using only one light intensity, as normally carried out in video-tracking studies, but using also three different intensities (300 lx, 2200 lx and 4400 lx) to evaluate if different dark/light transition gradients can influence the effects on swimming behaviour caused by the WSPs administered.

2. Materials and methods

2.1. Preparation of WSP solutions

Standards of PAA (CAS number: 9003-01-4), PEG (CAS number: 25322-68-3) and PVP (CAS number: 9003-39-8) powders have been purchased by Sigma-Aldrich (Merk Life Science, Milan, Italy), selecting those available with a low MW: ~450,000 Da for PAA, 1900–2200 Da for PEG and 10,000 Da for PVP.

The three WSP stock solutions (250 mg/L) were prepared the day before the exposures in "zebrafish water" constituted by deionized water with 0.1 % methylene blue ($C_{16}H_{18}ClN_3S$), 0.1 g/L sodium bicarbonate ($NaHCO_3$), 0.1 g/L Instant Ocean® and 0.2 g/L calcium sulphate ($CaSO_4$). To obtain a complete solubilisation of the WSPs, the solutions were heated following the procedure of Nigro et al. (2022) and then maintained overnight at 28 °C. These stock solutions were subsequently diluted in "zebrafish water" to obtain the three concentrations selected for the following ecotoxicological tests and aerated for 15 min. Based on our first PVA study (Nigro et al., 2022), we performed the exposures to controls and three different concentrations (0.001, 0.5 and 1 mg/L) of all the three WSPs, which are close to the few environmental level measured by Antić et al. (2011). Moreover, these low concentrations do not represent any problem in terms of solubility in water (100 mg/mL for PVP, 0.256 mg/mL for PEG, while PAA is swellable in water but at high concentration it originates thixotropic solutions), a situation that could interfere with the results obtained.

2.2. Determination of WSP concentrations in the exposure medium

The nominal concentration of the three WSPs at all the concentrations into zebrafish medium were investigated through an integrated analytical

approach. We used the Gas Chromatography-Mass Spectrometry (GC–MS) and the High-Performance Liquid Chromatography (HPLC). Due to the interference of the salts into zebrafish medium with the instruments, was not possible to check the concentrations during the entire exposure test. For this reason, the analysis has been carried out related to the same WSP dilutions in milli Q® water with ^{1}H NMR (Nuclear Magnetic Resonance) spectroscopy to control the working solution concentrations before the tests.

The nominal concentration of WSPs in milli Q® water solutions were determined through a two-step procedure on 1 L of the 1 mg/L solution for each tested WSP. The first step involved the complete evaporation of the water by means of a rotary evaporator apparatus: the solution was put in a 2 L one-neck round bottom flask and heated up to about 65 °C in the rotary evaporator. After reducing the volume of the solution to about 50 mL, some mL of milli Q® water were poured with a pipette on the walls of the flask to recover any possible solid residue present after the partial evaporation. The solution was then transferred to a 100 mL one neck round bottom flask and the evaporation was continued using the same conditions. The evaporation of the solvent allowed to recover the solute and determine its weight. The second step was focused on the assessment and verification of the structure of the recovered WSP through the ^{1}H NMR spectroscopy. Samples were prepared by dissolving the recovered WSP into 0.7 mL of deuterated dimethyl-sulfoxide (DMSO-d_6) and spectra were recorded by a Bruker Ultrashield 400.

2.3. Zebrafish embryo exposures

The fertilized eggs were provided by the facility of the Department of Earth and Environmental Sciences of the University of Milan Bicocca, according to the Italian laws, rules and regulations (Legislative Decree no. 116/92; authorization n. 0020984-12/02/2018). In detail, adult AB wild-type pairs, deriving from the European Zebrafish Resource Center (Karlsruhe Institute of Technology, Germany), were bred in a ZebTec Active Blue circulating system (Tecniplast, Buguggiate, Italy). Breeders were maintained at 28 °C, pH 7.5, 500 μS with 14 and 10 h light-dark cycle. Organisms were fed three times *per* day with Zebrafeed (Sparos Lda, Olhão, Portugal).

We used thirty zebrafish embryos both for controls and for each treatment group that were exposed in triplicate in static conditions from the eggs' collection to 120 hpf, and maintained under constant darkness at 28 °C in an incubator within 50 mL Petri dishes. Embryos were exposed to increasing concentrations (0.001, 0.5 and 1 mg/L) of the three WSPs following the OECD 236 (2013) with some modifications. Embryos were observed every 24 h under a stereomicroscope to assess the possible occurrence of mortality (egg coagulation, lack of somite formation, *non*-detachment of the tail and lack of heartbeat; OECD 236, 2013) or the presence of sub-lethal morphological anomalies (scoliosis, development delay, edemas, malformations, eye development, etc.), according to Schiwy et al. (2015). The dead embryos were removed from the Petri dishes. At the end of exposures, we analysed the swimming behaviour.

2.4. Behavioural alteration on zebrafish embryos

Potential alterations induced by the exposure of the three WSPs on the swimming activity of zebrafish embryos were evaluated using the DanioVision™ video tracking system (Noldus IT, Wageningen, Netherlands). Video data were recorded from the three independent experiments at a sample rate of 30 frames/s *via* a high-speed infrared camera.

For each experimental group we used 18 specimens (54 total embryos for each treatment) of the 30 previously exposed. At the end of exposures, each specimen was put in a single well of a 24-multiplate with 3 mL of "zebrafish water" (see Section 2.1) and undergone to six consecutive cycles of light/dark phases whose duration was 5 min each one. The three light phases were characterized by different intensities (300 lx, 2200 lx and 4400 lx) to also evaluate the potential effect of this environmental condition on the possible alterations of swimming behaviour due to the WSP exposures, bearing in mind that there is no unanimity in using a light with a fixed intensity (Legradi et al., 2015). The choice to use for the behavioural assessment zebrafish embryos at 120 hpf, as well as being motivated by the limitations imposed by the law on animal welfare, is justified by the fact that already after 96–120 hpf zebrafish embryos develop the ability to swim and exhibit a wide spectrum of behaviours, such as hunting, avoidance, thigmotaxis (Colwill and Creton, 2011a, 2011b; Schnörr et al., 2012), representative of motility performance and locomotor behaviour that play a key role in zebrafish nutrition, social and defensive activities (Colwill and Creton, 2011a). We have not only followed the most recent indications suggesting that it is more appropriate to characterize the behaviour of zebrafish embryos using both light and dark conditions, as they increase the chances of detecting behavioural phenotypes (Tuz-Sasik et al., 2022), but we also studied transitions from light to dark (and *vice versa*) less strong than those normally performed in zebrafish embryo tests (4400 lx-darkness; Yu et al., 2021; Tuz-Sasik et al., 2022; Zheng et al., 2022;), employing two other light intensities (300 lx and 2200 lx) mimicking natural light conditions (Simao et al., 2019). Moreover, to reduce the variability of the embryos' responses, we used only the second light/dark and dark/light transition periods for data analysis, while the first ones were considered as acclimation phases, following the suggestion made by Leuthold et al. (2019) and Haigis et al. (2022). Specifically, the two lower light intensities were similar to those measured from spring to summer in the surface of an oligotrophic lake (Tilzer et al., 1995), while 4400 lx is considered a high-intensity light stimulation for zebrafish (Zheng et al., 2021). The first three dark/light cycles for each light intensity were used exclusively as specific acclimation periods for the different conditions used during the tests, while the only subsequent dark/light cycles were considered for the evaluation of swimming behaviour. In detail, the entire experimental protocol for each exposure tests was summarized in Fig. 1.

Data were acquired every 30 s (for a total analysis of 60 min, Fig. 1) and different behavioural parameters have been analysed by the software EthoVision XT 11.5 (Noldus IT, Wageningen, Netherlands). In detail, we measured the "total distance moved", that is the distance travelled by the organism from the previous track to the final one (mm), the "change in distance moved" in the illumination transition from dark to light condition (and *vice versa*), represented by the distance moved (mm) in the first 30 s of light phase minus the distance moved in the last 30 s of dark phase (or *vice versa*), the "cumulative duration", that is the total time (s) in which the animal is in a specific area (centre or boundaries) of the well to highlight the eventual embryo thigmotaxis. Furthermore, we measured also the "mean velocity", which is the distance moved by the embryo *per* unit of time (mm/s), the "mean mobility", defined by the percentage changes of position (%) of the detected animal between the current and previous tracks and the "relative turn angle", that is the change in direction of the embryo between two consecutive tracks (degrees).

2.5. Statistical analysis

STATISTICA 7.0 software was used to perform all the statistical analyses on behavioural effect data. Eventual outliers have been removed thought the use of Box and Whiskers plots. The significant differences ($p \leq 0.05$) between treated and control for the average of all the behavioural parameters and for the analyze of viability were assessed by one-way ANOVA (treatment as variable) followed by the Bonferroni *post-hoc* test ($p \leq 0.05$). To compare the dose-response of every WSP, analysis of simple regression among the treatments has been carried out to identify the significant correlation ($p \leq 0.05$).

3. Results

3.1. Characterization of the WSPs in water

Direct measurement of the concentration of the three WSP solutions in "zebrafish water" was impossible to obtain either by HPLC or GC–MS due to

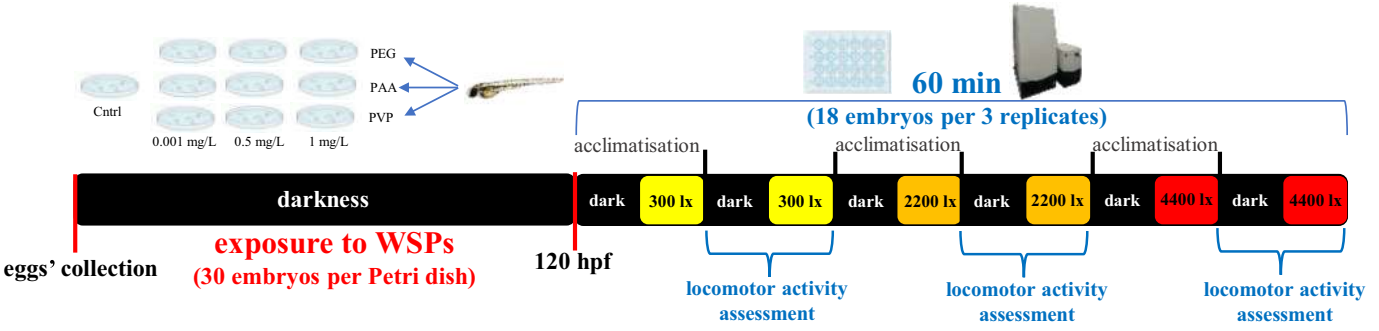

Fig. 1. Experimental protocol to evaluate the potential alteration induced by the exposure of the three WSPs on the swimming activity of zebrafish embryos. Six consecutive cycles of light/dark phases whose duration was 5 min each one; the three light phases were characterized by different intensities (300 lx, 2200 lx and 4400 lx)

the analytical interference represented by the salts used. This did not allow the check of the daily concentrations in the Petri dishes. Being the WSPs soluble in water, and being them stable in standard environmental conditions, we did not expect a change in water concentration. Besides, polymers cannot be analysed using chromatographic techniques specific for low molecular weight organic compounds such as GC, HPLC or similar. Therefore, to certify that the chosen nominal concentrations were achieved and to evaluate their maintenance during exposures, we had to carry out a check in distilled water. The check was carried out to determine both the concentration and the chemical integrity of the WSPs after the dissolution. As indicated in Section 2.2, the milli Q® water solutions of each WSP with 1 mg/L concentration were used to determine the nominal concentrations. One L of each solution was evaporated to dryness and the amount of WSP initially dissolved determined by weight. Table 1 reports the weighted amount of each WSP compared to the recovered amount after the evaporation of the solvent. As data show, the recovered amounts of WSPs are close to the ones weighted for the preparation of the initial solutions, showing the robustness of the applied methodology. Once the WSPs were recovered, they were analysed through ^{1}H NMR, in order to verify the chemical integrity of the solutes, as well as to determine that what was recovered were actually the WSPs and to confirm the absence of organic contaminants. Spectra of the recovered WSPs were recorded using DMSO-d_6 as solvent and compared to the spectra of the standard WSPs, confirming that all the samples were preserved in their structural integrity and free of organic contaminants (Supplementary Materials, SM, Figs. S1, S2 and S3).

3.2. Mortality and anomaly detection

Regarding the acute toxicity induced by the three WSPs on zebrafish specimens, we did not observe significant lethal effects, registering a mean viability of 84 % between the experimental groups. After the confirmation of the absence of any lethal effect and morphological anomalies, whose presence affects the swimming performance in zebrafish (Schiwy et al., 2015), we evaluated the behavioural effects in treated and control specimens using the DanioVision™ video tracking system.

3.3. Behavioural alterations

Due to the high number of tests performed, only the results of experiments in which we observed a significant difference ($p \leq 0.05$) between treated and control are reported in the text, while the other results are available in the Supplementary Materials (SM).

3.3.1. Effects of PAA

After the exposure to PAA the only behavioural parameter significantly affected ($p = 0.004$) by the intermediate concentration (0.5 mg/L) of this polymer was the cumulative duration (Fig. 2). We detected three opposite effects for this parameter when zebrafish embryos were subjected to the three different light intensities. Under the cycle with low intensity (300 lx) we detected a slight *non*-significant drop in the time spent in the center of the well for all tested concentrations with respect to controls, while under the cycle with intermediate intensity (2200 lx) embryos generally spent more time in the center of the well than in the border, but reaching significant differences ($p = 0.004$) compared to controls only for the middle exposure under dark condition (Fig. 2). For results obtained under the cycle with the highest light intensity (4400 lx), it was not possible to define a clear response, since under dark condition for the first two concentrations there was a slight reduction in the time spent at the center of the well, while for the highest one there was exactly the opposite (Fig. 2). A different trend was observed under the light condition at 4400 lx, with a general tendency of the exposed embryos to stay more in the center of the well.

Albeit not significant, a slight decrease, compared to control, in the distance moved, mean velocity (Fig. S4) and mean mobility (Fig. S5) was observed after the exposure to all PAA concentrations under the cycles with lowest light intensities (300 lx and 2200 lx). On the contrary, only under the dark phase in the cycles at 4400 lx a general decrease in the trend of these parameters was observed on exposed organisms, while under the light phase exposed embryos exhibited a growing trend for these parameters, albeit not in a significant way. Furthermore, the measurement of the relative turn angle (Fig. S6) and of change in distance travelled under the dark/light and light/dark transition were not significantly affected (Fig. S7).

3.3.2. Effects of PVP

The effects of PVP appeared to be higher than those observed after exposures to PAA. Indeed, the locomotor response of zebrafish under all the dark/light cycles was affected after the exposure to all the PVP concentrations ($p \leq 0.05$). In detail, the mean of total distance moved decreased under the light phase at 300 lx with significant differences ($p = 0.005$, $p = 0.01$) compared to controls for the two extreme tested concentrations (0.001 and 1 mg/L; Fig. 3). Under the exposure to 2200 lx light intensity, the highest concentrations of PVP (0.5 mg/L and 1 mg/L) determined a significant decrease in distance moved ($p = 0.006$, $p = 0.002$). On the contrary, for the exposure to 4400 lx light phase we observed a decrease (not significant) in the distance travelled by zebrafish embryos (Fig. 3). The same drop was also observed in the dark phases, with significant lower values than controls for the lowest ($p = 0.02$) and highest concentrations ($p = 0.02$) of PVP in the dark phase, that followed the 300 lx and 2200 lx phase, respectively (Fig. 3).

Table 1
Comparison of the amounts of WSPs weighted for the preparation of the solutions with the recovered amounts after evaporation of the solvent.

WSP	Weighted amount (mg)	Recovered amount (mg)
PAA	1.00	0.89
PVP	1.00	0.88
PEG	1.00	0.91

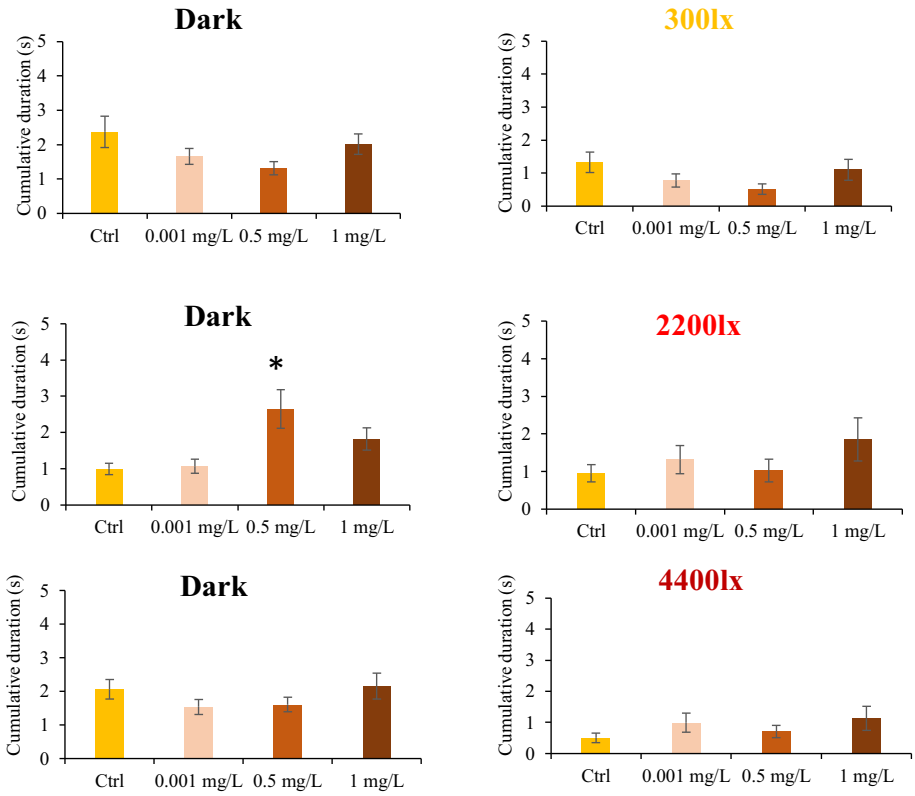

Fig. 2. Cumulative duration assessed on zebrafish exposed to three different concentrations of PAA. Exposure in triplicate, N = 54 specimens *per* treatment. Data are expressed as mean ± SE. Asterisks indicate the significant differences (*p < 0.05) compared to control (one-way ANOVA, Bonferroni post-hoc test).

Unlike what was observed for the PAA, the two parameters that measured the mobility and velocity of the embryos showed a significant ($p \leq 0.05$) decrease compared to controls, but exclusively in the dark/light cycles referred to the two lowest light intensities (Figs. 3, 4). The trend for both endpoints was very similar to the distance moved trend parameter. A significant decrease in mean velocity was observed for exposure to 0.001 mg/L ($p = 0.008$) and 1 mg/L ($p = 0.02$) under the 300 lx phase and even under the light phase at 2200 lx after the exposure to 0.001 mg/L ($p = 0.02$), 0.5 mg/L ($p = 0.01$), 1 mg/L ($p = 0.0007$).

Regarding the darkness condition before both light intensities of 300 lx and 2200 lx, in line with distance moved results, embryos were significantly affected after exposure to the lowest ($p = 0.02$) and highest ($p = 0.005$) concentrations of PVP (Fig. 3).

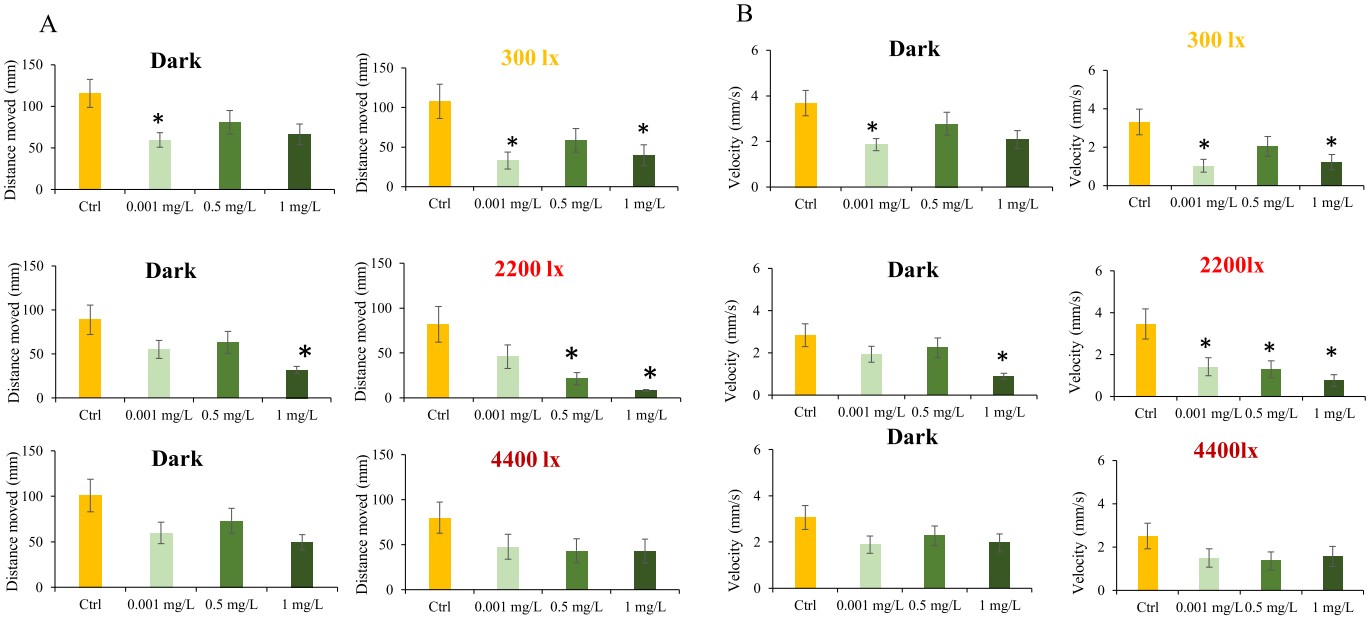

Fig. 3. Distance moved (A) and Velocity (B) assessed on zebrafish exposed to three concentrations of PVP. Exposure in triplicate, N = 54 specimens *per* treatment. Data are expressed as mean ± SE. Asterisks indicate the significant differences (*p < 0.05) compared to control (one-way ANOVA, Bonferroni post-hoc test).

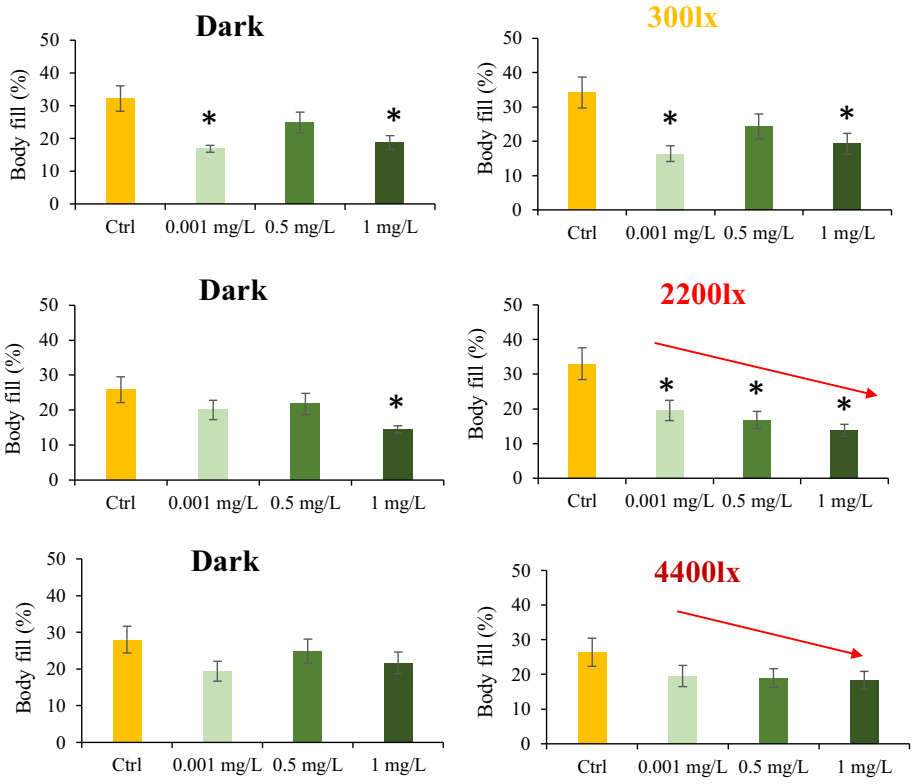

Fig. 4. Mobility assessed on zebrafish exposed to three concentrations of PVP. Exposure in triplicate, N = 54 specimens *per* treatment. Data are expressed as mean ± SE. Asterisks indicate the significant differences (*p < 0.05) compared to control (one-way ANOVA, Bonferroni post-hoc test). Arrows indicate the significant correlation between concentrations.

The mobility parameter reflected the trends just described with some small differences: in the dark before 300 lx a significant decrease was observed for both concentrations of 0.001 mg/L (p = 0.0001) and 1 mg/L (p = 0.005), and under highest light intensities a dose-dependent decrement (p = 0.04) was registered (Fig. 4). Regarding the light phase at 300 lx the mobility parameter was significantly affected (p = 0.002, p =

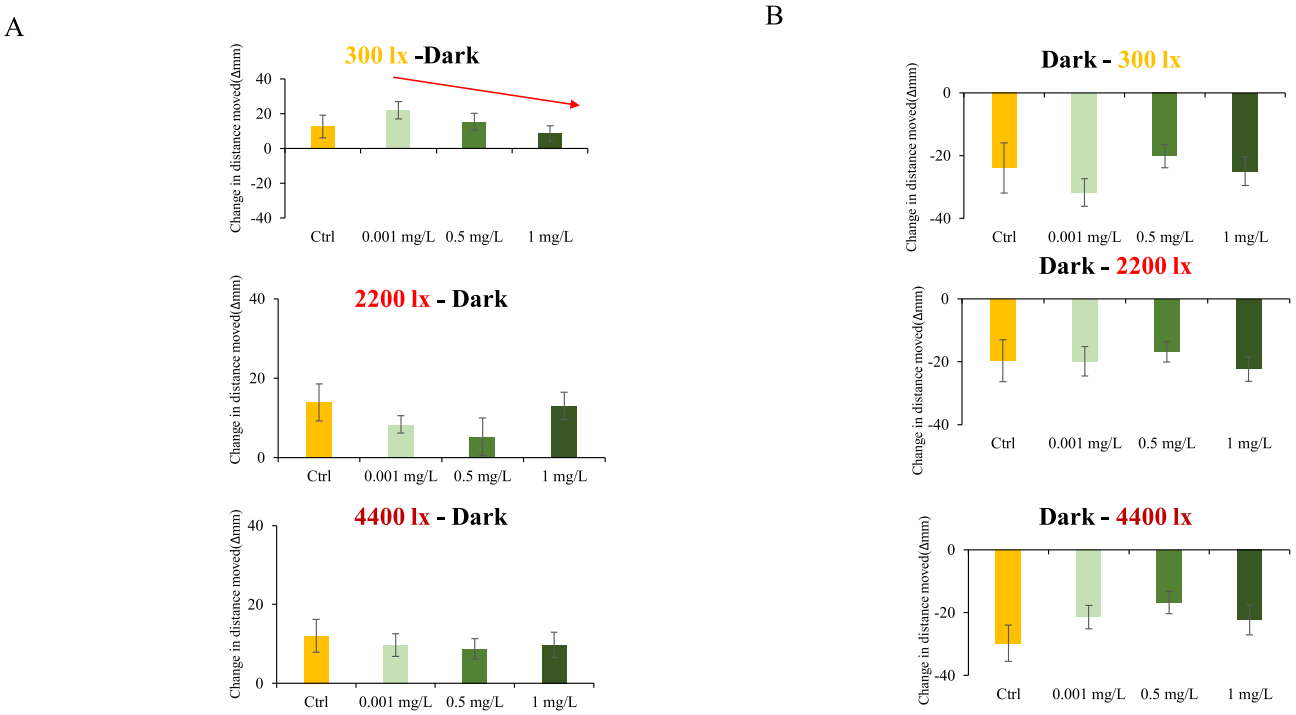

Fig. 5. Change in distance moved from light to dark condition (A) and *vice versa* (B) on zebrafish exposed to three different concentrations of PVP. Exposure in triplicate, N = 54 specimens *per* treatment. Data are expressed as mean ± SE. Arrows indicate significant correlation between concentrations.

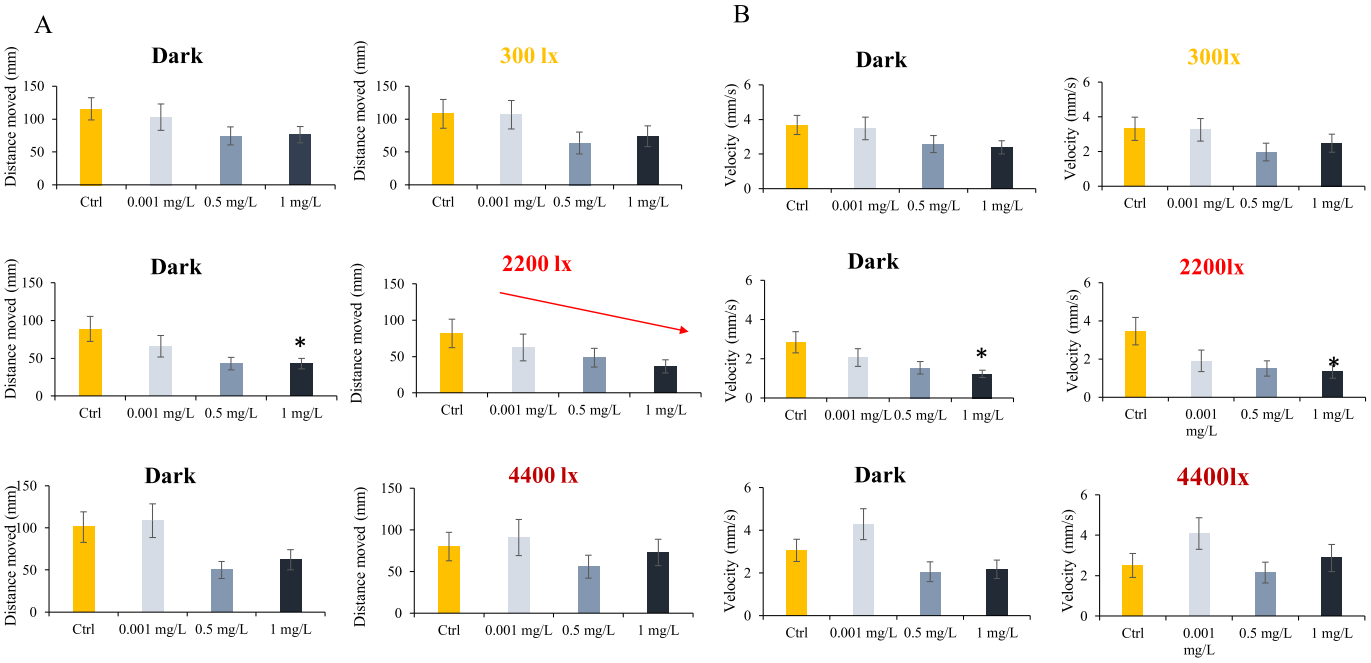

Fig. 6. Distance moved (A) and Velocity assessed (B) on zebrafish exposed to three concentrations of PEG. Exposure in triplicate, N = 54 specimens *per* treatment. Data are expressed as mean ± SE. Asterisks indicate the significant differences (*p < 0.05) compared to control (one-way ANOVA, Bonferroni post-hoc test). Arrows indicate significant correlation between concentrations.

0.02) in embryos exposed to the extreme tested concentrations (0.001 mg/L, 1 mg/L), while under light phase at 2200 lx significantly low values than control were observed for 0.001 mg/L (p = 0.015), 0.05 mg/L (p = 0.002) and 1 mg/L (p = 0.0001) showing a lower dose-dependent responsivity (p = 0.01) of the exposed organisms. Besides, a significant decrement (p = 0.03) in mobility was observed in embryos exposed to the highest concentration (1 mg/L) under dark condition before 2200 lx phase.

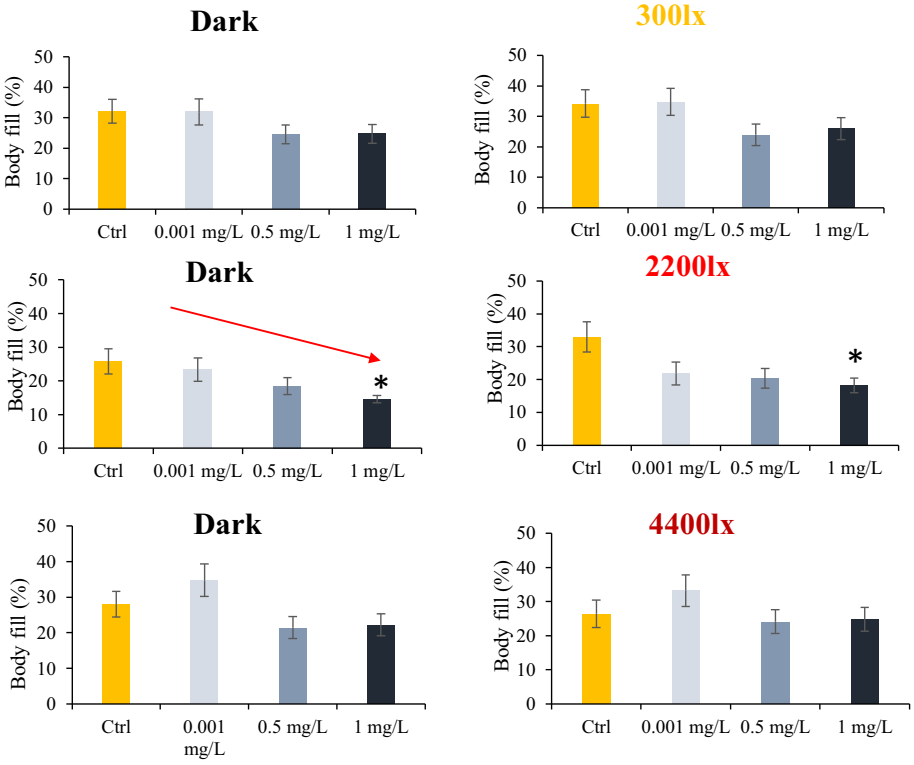

Fig. 7. Mobility assessed on zebrafish exposed to three concentrations of PEG. Exposure in triplicate, N = 54 specimens *per* treatment. Data are expressed as mean ± SE. Asterisks indicate the significant differences (*p < 0.05) compared to control (one-way ANOVA, Bonferroni post-hoc test).Arrows indicate significant correlation between concentrations.

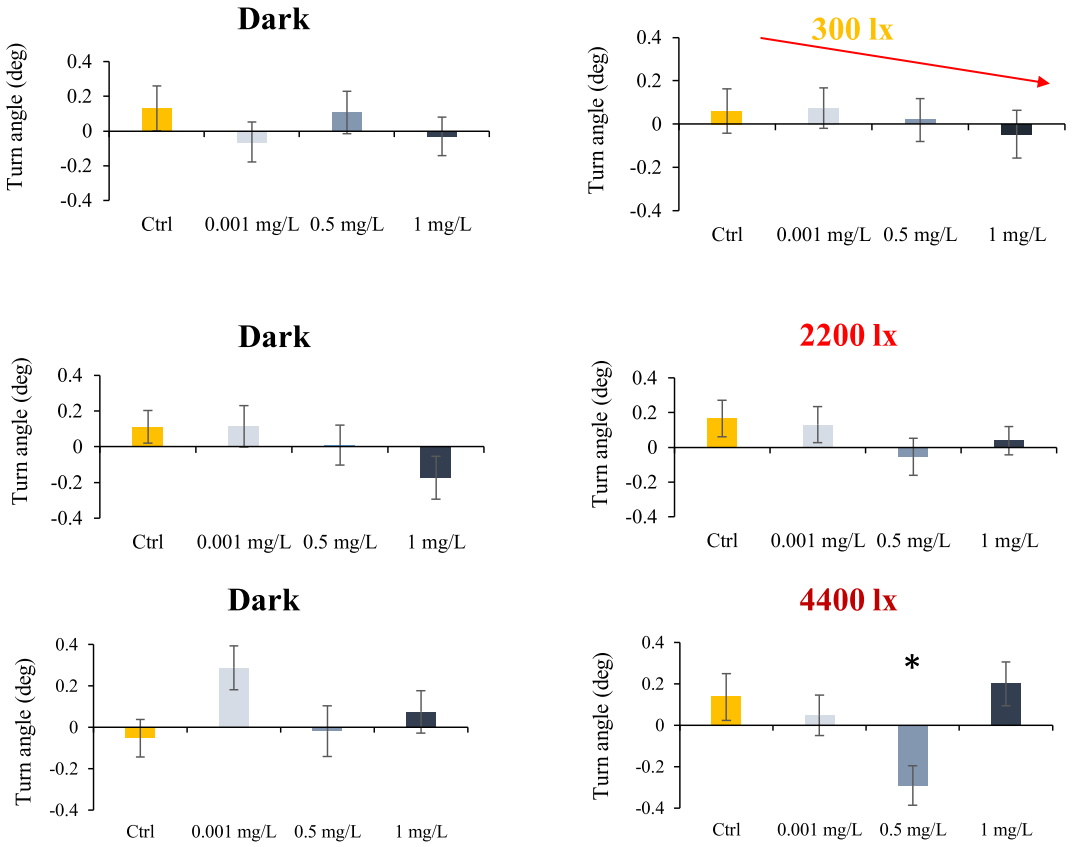

Fig. 8. Turn angle assessed on zebrafish exposed to three different concentrations of PEG. Exposure in triplicate, N = 54 specimens *per* treatment. Data are expressed as mean ± SE. Asterisks indicate the significant differences (*p < 0.05) compared to control (one-way ANOVA, Bonferroni post-hoc test). Arrows indicate significant correlation between concentrations.

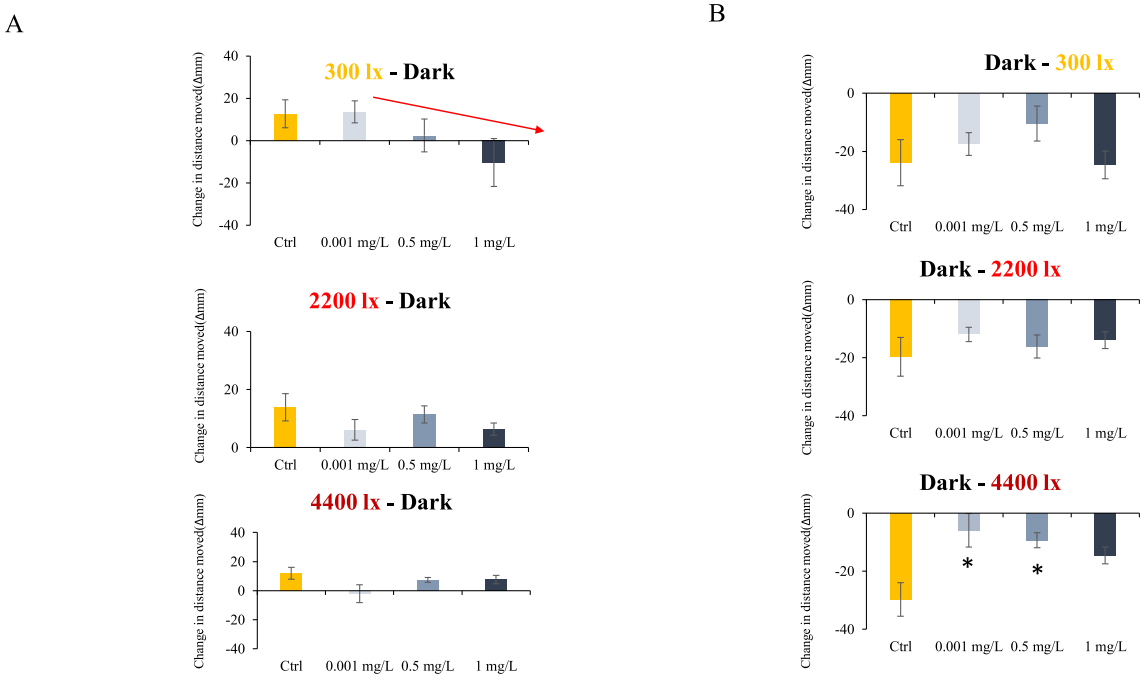

Fig. 9. Change in distance moved from light to dark condition (A) and *vice versa* (B) on zebrafish exposed to three different concentrations of PEG. Exposure in triplicate, N = 54 specimens *per* treatment. Data are expressed as mean ± SE. Asterisks indicate the significant differences (*p < 0.05) compared to control (one-way ANOVA, Bonferroni post-hoc test). Arrows indicate significant correlation between concentrations.

In contrast, the relative turn angle (Fig. S8) and the variation of the distance travelled under the light/dark transitions (Fig. 5) showed no significant differences compared to control group, although a dose-dependent decline ($p \leq 0.05$), in response to the change from 300 lx to dark condition, was observed. The measurement of cumulative duration in the center or border of the well showed a general not significant trend under the light compared to the dark conditions (Fig. S9).

3.3.3. Effects of PEG

The effects on swimming behaviour caused by PEG were more similar to those produced by PVP than by PAA. In general, in all dark/light phases we observed a decrease in the distance moved of the embryos as the concentration of administered PEG increased, with significantly lower values ($p = 0.05$) than controls for the highest concentration (1 mg/L) at the dark before light intensity of 2200 lx (Fig. 6). Besides, a dose-dependent decrease ($p = 0.04$) was observed at 2200 lx light phase. The exposure to 2200 lx was the condition that amplified the effects of WSPs, considering that the highest concentration of PEG (1 mg/L) determined a significant decrease in mobility ($p = 0.02$) and velocity ($p = 0.03$) parameters only under this light intensity (Figs. 6, 7). Concerning the mean mobility, a dose-dependent reduction ($p = 0.03$) was observed under the dark condition before 2200 lx with a significant effect only in embryos exposed to 1 mg/L ($p = 0.04$; Fig. 7) as even for the mean velocity ($p = 0.03$; Fig. 6).

In this case, the measurement of the turn angle (Fig. 8) was negatively affected ($p = 0.02$) at 4400 lx light phase in the embryos exposed to the intermediate concentration of PEG (0.5 mg/L) and a dose-dependent change in rotation ($p = 0.04$) of embryos was observed at 300 lx light phase. Also the parameter of the change in distance moved for embryos exposed to the lowest concentrations of PEG 0.001 mg/L ($p = 0.001$), 0.5 mg/L ($p = 0.01$) was significantly affected under the light phase at 4400 lx, showing a lower dose-dependent responsivity ($p = 0.03$) of the exposed organisms under the dark phase before 300 lx (Fig. 9).

The exposure to the lowest concentration of PEG (0.001 mg/L) also affected the parameter of the cumulative duration, increasing significantly ($p = 0.03$) the tendency of the embryos to stay in the centre zone under the condition at 4400 lx (Fig. 10).

4. Discussion

This study aimed to face a new challenge based on the possible impact that a new class of emerging contaminants, the so-called "liquid plastics" or more properly WSPs, can determine on aquatic organisms and consequently on the ecosystems (Gross and Kalra, 2002). Our study evaluated and compared the effects of low concentrations of three of the most used WSPs (PAA, PEG and PVP) on the swimming behaviour of zebrafish embryos, which is a signature of high ecological relevance as it assesses an organisms' level effect and it is now considered as a useful methodological approach for the (eco)toxicity assessment.

Due to the poor investigations performed so far on aquatic biological models, our first objective was to verify the correct set up of the experimental design. Thus, since the aim of the study was to evaluate a sub-lethal toxicity, we initially carried out a check on possible lethal effects, which would have prevented to carry out the exposure to the selected concentrations. In addition, we also checked that the zebrafish embryos exposed to the three WSPs did not present morphological anomalies that could have altered the following swimming performance. The results showed that the exposure to all the concentrations of WSPs did not induce both significant lethal and sub-lethal morphological anomalies, that are in line with previous studies reporting that WSPs did not determine any acute effects on aquatic organisms (neotropical tadpole, mussels and crustaceans; Buczek et al., 2017; Nascimento et al., 2021; Mondellini et al., 2022).

Moving to the discussion of the main results obtained after the exposure tests, Table 2 summarizes the significant effects and/or dose/response significant relationships ($p \leq 0.05$) observed by measuring different behavioural endpoints for the three concentrations of each WSP and for different light conditions. From the general point of view, Table 2 shows that the parameters related to locomotion (distance moved, velocity, mobility, variations in distance moved during the moment of illumination

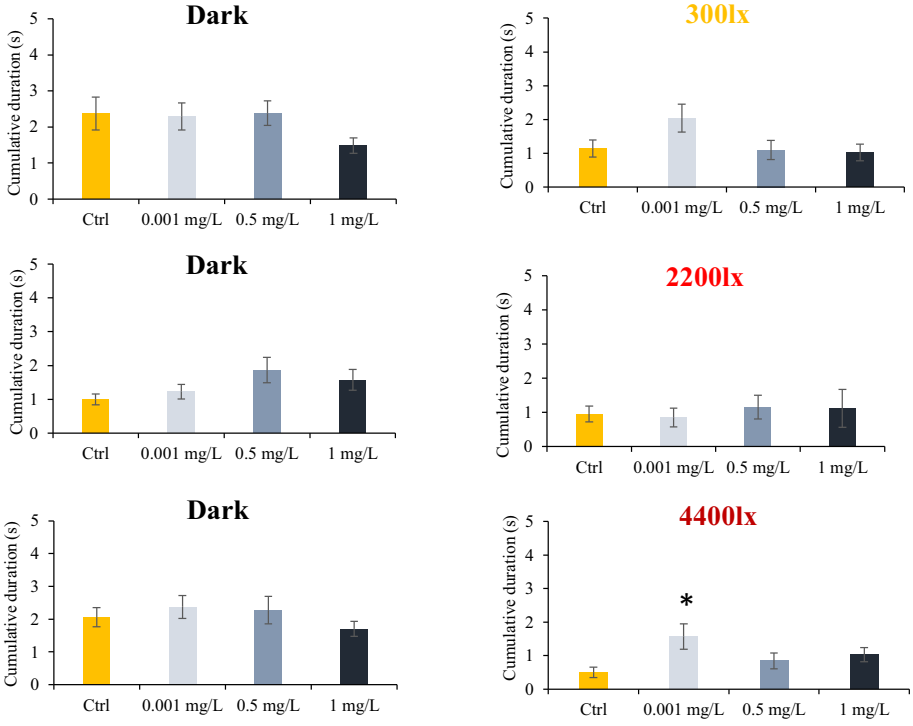

Fig. 10. Cumulative duration assessed on zebrafish exposed to three different concentrations of PEG. Exposure in triplicate, N = 54 specimens *per* treatment. Data are expressed as mean ± SE. Asterisks indicate the significant differences (*$p < 0.05$) compared to control (one-way ANOVA, Bonferroni post-hoc test).

transition) seem to have been more sensitive than the evaluation of thigmotaxis and directionality (turn angle) as they showed a greater number of significantly different WSPs' effects.

4.1. PAA effects

Going into more detail of the comparison among the three different WSPs tested, we can clearly highlight how there is a scale of behavioural effects, with the PAA exclusively showing only a slight, but significant increase in the cumulative duration of time spent by the embryos in the center of the well due to the intermediate concentration (0.5 mg/L) only in the darkness preceding the 2200 lx light phase (Fig. 2 and Table 2). This parameter, which was observed also for the lowest concentration (0.001 mg/L) of PEG in the most intense light phase (4400 lx; Table 2), measures the so-called thigmotaxis behaviour which, from the ethological point of view, indicates the tendency to avoid the open field in a new environment and to stay and move near the more protected boundaries. It is a phylogenetically ancient and energetically inexpensive exploratory strategy, evolutionarily conserved in a wide range of species, including fish (Peitsaro et al., 2003; Lopez-Patino et al., 2008; Sharma et al., 2009; Champagne et al., 2010; Colwill and Creton, 2011a) and is a well-known behaviour also in humans (Kallai et al., 2005, 2007) for which it is considered a consolidated and validated index of anxiety (Lopez-Patino et al., 2008; Champagne et al., 2010; Buske and Gerlai, 2012), as demonstrated by being attenuated by anxiolytics (diazepam) and significantly increased by anxiogenic drugs, such as caffeine (Schnörr et al., 2012). Thigmotaxis is a fundamental ecological behaviour because delays or inhibits the exploration of the central zone of a novel space until the organism identifies salient or relevant cues that are then used as elements of a spatial or cognitive map (Besson and Martin, 2005). Thus, this “bold behaviour” of zebrafish embryos observed both for PAA and PEG could point to a danger in wildlife as it would lead to an increased possibility of being preyed upon. The fact that this thigmotactic effect has been lost both during darkness (PAA) and in the phase of highest light (PEG; Table 2) would seem to suggest that it does not depend on the presence or absence of light, considering that normal behaviour of zebrafish embryos is characterized by low basal activity in light and a substantial increase of activity upon the switch to darkness (Haigis et al., 2022).

Unfortunately, there are very few ecotoxicological data to compare our results on PAA, as polyacrylates are generally considered *non*-toxic because they do not contain acrylamide monomers (Dell'Ambrogio et al., 2019). The only data available are related to two studies on *Daphnia magna*: Hayes et al. (1993) showed an acute toxicity for dissolved polyacrylate only at very high concentration ($_{48h}LC_{50}$ = 175 mg/L), confirming the low acute toxicity of this compound, while Mondellini et al. (2022) showed how a treatment with 10 mg/L of PAA determined a reduced amount of *D. magna* reproductive cycles.

In conclusion, our study highlights that the PAA determined a slight behavioural effect only for one of the endpoints measured and for concentration close to the predicted environmental ones, at least under the experimental conditions and exposure times we used in the present study, supporting the consideration about the low toxicity of this WSP. However, it should also be considered that the PAA used has the highest MW of the three WSPs (~450,000 Da) which could result in lower capability to enter embryos, consequently decreasing its adverse effects.

4.2. PVP effects

Another picture than PAA and PEG arose after exposure to PVP, which affected most parameters in many dark/light phases (Table 2). In detail, a significant decrease was observed for all the parameters involved in locomotion, excluding variations in the distance moved during the moment of illumination transition, already from the lowest concentration of PVP administered (Figs. 3, 4, 5 and Table 2). The concentration of 1 mg/L was the one that caused the greatest changes in the swimming behaviour of zebrafish embryos, as the distance moved, velocity and mobility were significantly lower in both dark, and low (300 lx) and intermediate (2200 lx) light phases (Figs. 3, 4 and Table 2), while surprisingly there were no significant changes for the phases at 4400 lx.

Table 2 shows that even no significant effects have ever been detected in the phases at the highest light intensity not only for PVP, but also for the other two WSPs tested, apart from two parameters for the PEG. This result deserves an in-depth discussion because the high light intensities are normally used in (eco)toxicological studies based on light/dark transition (LDT), as they represent an illumination able to produce, at least in this stage of development of zebrafish, an anxiogenic condition that implies a low basal activity (MacPhail et al., 2008; Faught and Vijayan, 2022) or even the blocking of motility (freezing; Best et al., 2017). However, in our study the greatest effects, for all the three WSPs administered, were observed for the two lowest light intensities, which represent a natural condition, as explained above. A possible explanation for this particular result could be due to the fact that the movement response depends strictly on the magnitude-change in lighting, as postulated by Burgess and Granato (2007). Indeed, if we compare the decrease in distance moved by control embryos from darkness to the different light phases, we measured how in the transition from darkness to light at 300 lx and 2200 lx the mean distance moved by the embryos decreased of only 6.6 % and 7.8 %, respectively (Fig. 5). In contrast, the decrease in distance travelled by embryos in the change of dark/light at 4400 lx was about 21 % (Fig. 5). This shows that zebrafish embryos undergo a high anxiogenic effect only when a high light/dark change occurs, while light intensities similar to the environmental one produce a lower movement response. Thus, this induced behaviour can hide the effects of contaminants on the swimming performances, which instead become manifest only at the lowest light intensities, when this interfering factor is missing. Obviously, more in-depth analyses of this unexpected effect are needed, but surely our data suggest that it is more appropriate to test the environmental contaminants at different light intensities because if we had performed a classic LDT test at 4400 lx we would not have appreciated any effect on the swimming behaviour of the WSPs tested.

Another consideration to make is that the observed effects are not due to an external effect determined by the characteristics of PVP, such as the increase in the viscosity of water that could have determined the hypoactivity observed, since the viscosity of the low molecular weight PVP used (p-value = 13–19) is very low and not such as to determine an appreciable change in the aqueous matrix. In addition, we must remember that, during the tests performed by the DanioVision™ video tracking system, the embryos were kept in “zebrafish water” without the presence of contaminants (see Section 2.4), precisely to avoid this possible interfering effect.

Having said that, since Klüver et al. (2015) showed that some neuroactive substances exhibit effects on behaviour in zebrafish embryos at concentrations well below the lethal range, the hypoactivity observed after our exposures could be an indicator for neuroactivity of PVP, even if further investigation is needed to understand in detail the possible mode of action (MoA), as neuroactivity may be due to different factors. One of the causes of neuroactivity is due to the direct interaction of a substance with the nervous system that leads to an evident structural change, such as axonal deformation or neuritis outgrowth inhibition (Ogungbemi et al., 2019). The lack of sub-lethal morphological anomalies, as explained above, would seem to exclude this type of MoA. Another hypothesis could be related to the ability of PVP to modify specific nervous receptors in a functional way or to alter the neurotransmission (Bugel and Tanguay, 2018). For this reason, we will carry out many analyses of several biomarkers, some of which are specifically linked to neurotransmission, to investigate in more detail this possible MoA of PVP.

The fact that PVP was the tested WSP that determined the greatest effects at the organism level in a biological model used also in studies on pharmacology and human diseases is certainly not reassuring considering the many applications in which it is used. PVP was initially used as a blood plasma expander for trauma victims (Aravamudhan et al., 2014), while now it is present in many applications of common use, such as in various personal care products (shampoos, toothpastes, hair sprays and gels,

Table 2
Behavioural parameters analysed on *Danio rerio* after exposure with three different concentration (0.001 mg/L, 0.5 mg/L and 1 mg/L) of PAA, PEG and PVP. Asterisks indicate the significant differences (*p < 0.05) compared to control (one-way ANOVA, Bonferroni post-hoc test). Arrows indicate significant correlation between concentrations.

			THIGMOTAXIS	MEAN MOBILITY	MEAN VELOCITY	DISTANCE MOVED	ILLUMINATION TRANSITION		TURN ANGLE
mg/L			CENTER				LIGHT-DARK	DARK-LIGHT	
PAA	0.001	Dark-300lx							
		300lx							
		Dark-2200lx							
		2200lx							
		Dark-4400lx							
		4400lx							
	0.5	Dark-300lx							
		300lx							
		Dark-2200lx	*						
		2200lx							
		Dark-4400lx							
		4400lx							
	1	Dark-300lx							
		300lx							
		Dark-2200lx							
		2200lx							
		Dark-4400lx							
		4400lx							
PEG	0.001	Dark-300lx					↙		
		300lx							↙
		Dark-2200lx		↙					
		2200lx				↙			
		Dark-4400lx							
		4400lx	*					*	
	0.5	Dark-300lx					↙		
		300lx							↙
		Dark-2200lx		↙					
		2200lx				↙			
		Dark-4400lx							
		4400lx						*	*
	1	Dark-300lx					↙		
		300lx							↙
		Dark-2200lx		↙*	*	*			
		2200lx		*	*	↙			
		Dark-4400lx							
		4400lx							
PVP	0.001	Dark-300lx		*	*	*	↙		
		300lx		*	*	*			
		Dark-2200lx							
		2200lx		↙*	*				
		Dark-4400lx							
		4400lx		↙					
	0.5	Dark-300lx					↙		
		300lx							
		Dark-2200lx							
		2200lx		↙*	*	*			
		Dark-4400lx							
		4400lx		↙					
	1	Dark-300lx		*			↙		
		300lx		*	*	*			
		Dark-2200lx		*	*	*			
		2200lx		↙*	*	*			
		Dark-4400lx							
		4400lx		↙					

contact lens solutions), as a binder in pharmaceutical tablets, as disinfectant in liquid soap and surgical scrubs (Kariduraganavar et al., 2014; Panda, 2015; Teodorescu et al., 2019; Kurakula and Rao, 2020). PVP is also used as food additive as stabilizer and is known by the code E 1201, so much so that the European Food Safety Authority (EFSA) has recently re-evaluated positively its safety as a food additive and considered the extension of its use in food for special medical purposes in tablet and coated tablet forms (EU Commission Regulation 2022/1038). Our data, which it should be remembered were obtained by testing concentrations comparable to the few available environmental ones and were measured at the organismic level, would seem instead to suggest the need for an in-depth investigation of the possible deleterious effects of PVP at least in respect of aquatic organisms. On the other hand, the last revised datasheet on PVP of the American Polymer Standards Corporation (APSC, 2022) indicates in the section about "Ecological Information" that no data are available for toxicity, bio-accumulative potential, persistence and degradability, mobility on soil.

4.3. PEG effects

The effects of PEG were much higher than of the PAA, as already at the lowest concentrations we detected changes in the behaviour of embryos exposed to this compound (Figs. 6, 7, 8, 9, 10 and Table 2). As expected, it was the higher concentration (1 mg/L) that had the greatest effects, solely for locomotion parameters and, more surprisingly, for the two phases of darkness and the intermediate light intensity at 2200 lx (Fig. 6 and Table 2). From the comparison of the three WSPs we can see that PEG was the one that showed the most heterogeneous effects, as we observed statistically significant decreases for all the endpoints measured (Table 2). In particular, it was the only WSP that resulted in a significant decrease in the distance moved by embryos during the transition from darkness to light (4400 lx) for the two lowest concentrations (Fig. 9 and Table 2). We added this new parameter to evaluate the startle effect represented by the direct response to the sudden visual stimulus (Haigis et al., 2022) which can be buffered in the only use of the mean values of the other locomotion parameters. This endpoint was suggested by Thomas et al. (2019) as a complementary parameter especially to the mean distance moved, as it demonstrated a lack of correlation between them, indicating how they can represent two distinct signatures of zebrafish behaviour and perhaps associated with different MoAs. Our results confirm this evidence, as zebrafish embryos exposed to PEG showed different behaviour only during illumination transition from darkness to light, while no significant effect was observed for the other locomotion parameters (Table 2).

Since among the three WSPs tested, the PEG is the one that presents more data on its ecotoxicological effects, we can highlight how the impact shown on the swimming behaviour we detected in zebrafish embryos is confirmed by several previous studies. The explanation for the hypoactivity observed may be suggested in a recent study in which was demonstrated how a short exposure to 5 and 10 mg/L of PEG induced neurotoxicity in the tadpole *Physalaemus cuvieri*, in which an increased activity of acetylcholinesterase and butyrylcholinesterase, as well as a reduction of superficial neuromasts, have been observed, that could produce locomotor changes, anti-predatory defensive response deficit and failures in the perception of aquatic stimuli (Nascimento et al., 2021). Nephrotoxicity, damage to the central nervous system and heart (Webster et al., 2009) and decrease in the amount of new-borns (Mondellini et al., 2022) are other effects observed in some model organisms exposed to PEG. The negative impact of this WSP seems to be linked to its ability to act as a surfactant and thus to play an active role in increasing the permeability of membranes, facilitating their decomposition and accelerating the processes of lipid peroxidation (Hatami et al., 2019) and lysis (Noudeh et al., 2008).

5. Final remarks

Although the scientific community has mainly focused to plastics as solid and insoluble contaminants, our results clearly showed that these WSPs could have negative effects on organisms and therefore ecosystems and that many in-depth studies are needed, considering also the few information available not only about their potential (eco)toxicity, but also their degradation in the environment. Indeed, due to the continuous release mainly in aquatic ecosystems based on their wide use in large consumer products, WSPs can be considered pseudo-persistent contaminants, such as pharmaceuticals and drugs of abuse (Bu et al., 2016; Chen et al., 2021), as they expose aquatic organisms throughout their entire life cycle. Consequently, it is necessary to define better the fate and the degradation of these polymers in the environment, all factors that could influence their toxicity towards the organisms. This situation is also reflected in the comparison of the effects observed with the three WSPs used in the present study, as the (eco)toxicological behaviour could depend not only by their polymeric structure but also by their MWs, which is very different for the WSPs available on sale. The different MWs certainly did not determine a difference in terms of solubility, due to the low concentrations used, but may affect polymer bioavailability, as explained above (Section 4.1), with direct consequences on uptake and then toxicity. Bearing in mind all these considerations, which make it even more complex to understand the fate and environmental hazard of these emerging contaminants, we can say that, from our dataset obtained by using the three WSPs with their specific chemical-physical characteristics, it is possible to suggest a preliminary toxicity scale that can drive future research in this context: PVP > PEG ≫ PAA.

One last consideration, however, must be emphasized, as our results refer exclusively to possible effects on behaviour due to specific standards of the three WSPs tested, even if it would be appropriate not only to investigate the other polymeric forms characterized by different MWs, but especially the consumer products that contain these WSPs, as the possible presence of additives could modify their toxicity. An example of this crucial point is the result obtained in our previous study (Nigro et al., 2022) comparing the behavioural effects of both the solubilized polyvinyl alcohol (PVA) powder and PVA-based commercial bags for carp-fishing by using both zebrafish embryos and *Daphnia magna*. A new data elaboration, not present in the old article, points out a significant decrease in the distance moved of zebrafish embryos exposed to bags, but not for the PVA standard (Fig. S10). Since the gas-chromatographic analysis showed the presence, only in the bags, of three additives derived from ethylene glycol, namely the triethylene glycol (TEG), tetraethylene glycol (TetraEG) and pentaethylene glycol (PentaEG), probably used as plasticizer in the PVA-based bag production, it is likely that the effects on swimming behaviour observed only after exposure to the bags are due to these additives. On the other hand, several adverse effects caused by these compounds are known: Liu et al. (2017) showed cytotoxicity on cells exposed to TEG, LeBlanc and Surprenant (1983) observed a low aquatic toxicity by prolongated exposure of TEG, Schladt et al. (1998) reported TetraEG as a mild skin and eye irritant on rabbits, while pentaEG causes serious eye and skin irritation and may cause respiratory irritation (ECHA (VI)).

6. Conclusions

This study is the first to investigate the potential ecotoxicological effect of the widely used WSPs on *D. rerio* embryos by using concentrations closed to the expected environmental ones and measuring many endpoints related to eventual changes in swimming behaviour. The results showed a significant toxicity induced by PVP > PEG ≫ PAA on many parameters with considerable differences, however, between the three WSPs.

The most important result obtained is perhaps linked to the fact that it has been highlighted how these emerging contaminants, considered until now completely harmless both for the environment and for humans, have determined apical effects on a biological model used in ecotoxicology, as well as in human toxicology and pharmacology, suggesting that perhaps it would be appropriate to perform more in-depth studies on the presence, fate and environmental hazard for this kind of polimers that are present in numerous products not only for industrial uses, but that we use, often unconsciously, in many daily activities.

Credit author statement

Lara Nigro: Methodology; Investigation; Formal analysis; Validation; Data Curation; Writing - Original Draft; Software; - **Stefano Magni:** Conceptualization; Methodology; Writing - Review & Editing; Funding acquisition; Project administration; Software - **Marco Aldo Ortenzi:** Methodology; Formal analysis; Writing - Review & Editing - **Stefano Gazzotti:** Methodology; Formal analysis; Writing - Review & Editing - **Silvia Signorini:** Formal analysis - **Riccardo Sbarberi:** Formal analysis - **Camilla Della Torre:** Conceptualization; Writing - Review & Editing - **Andrea Binelli:** Conceptualization; Writing - Original Draft; Software; Supervision.

Data availability

Data will be made available on request.

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.

Acknowledgements

This study was funded by Line 2 grant of the Research Support Plan (PSR) 2021 (PSR2021_DIP_002) assigned to Dr. Stefano Magni by Department of Biosciences of the University of Milan.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.164843.

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Science of the Total Environment 906 (2024) 167699

Contents lists available at ScienceDirect

Science of the Total Environment

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

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

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HIGHLIGHTS

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

GRAPHICAL ABSTRACT

ARTICLE INFO

Editor: Henner Hollert

Keywords:
Water-soluble polymers
Liquid plastics
Adverse effects
Freshwaters
Ecotoxicology

ABSTRACT

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

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https://doi.org/10.1016/j.scitotenv.2023.167699
Received 27 June 2023; Received in revised form 26 September 2023; Accepted 7 October 2023
Available online 11 October 2023

1. Introduction

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

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

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

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

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

2. Methods and materials

2.1. Preparation of WSP testing solutions

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

2.2. Zebrafish exposures

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

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

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

2.3. Gel free proteomics

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

2.4. Biomarker suite

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

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

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

2.5. Statistical approach

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

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

3. Results

3.1. Proteomics

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

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

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

3.2. Biomarkers

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

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

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

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

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

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

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

4. Discussion

4.1. Proteomics

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

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

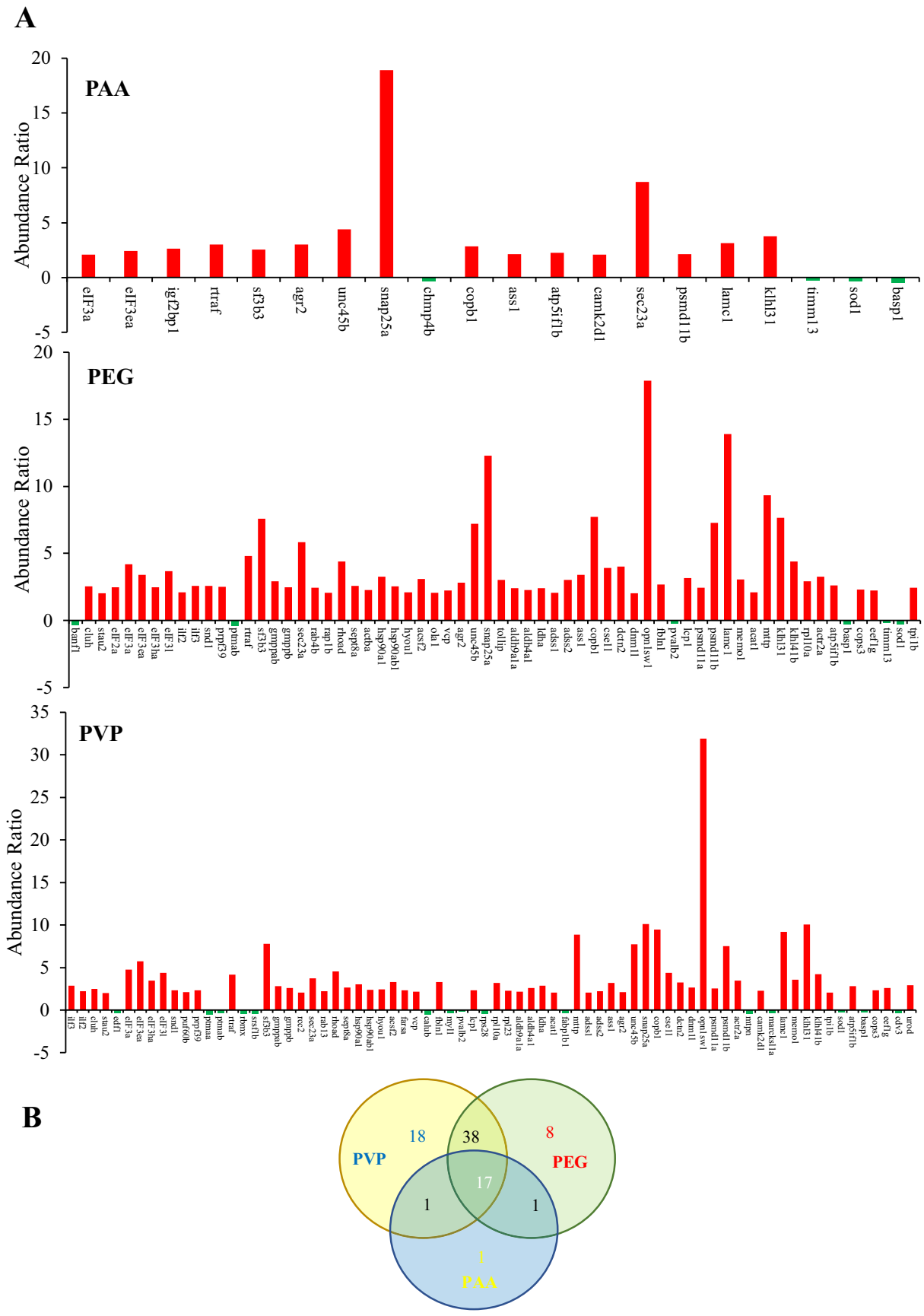

Fig. 1. Gel-free proteomics analysis results; (A) Abundance ratio of modulated proteins by 1 mg/L of the three WSPs compared to controls (red = up-regulation; green = down-regulation). (B) Venn's diagram showing proteins modulated by individual WSPs and in common with each other.

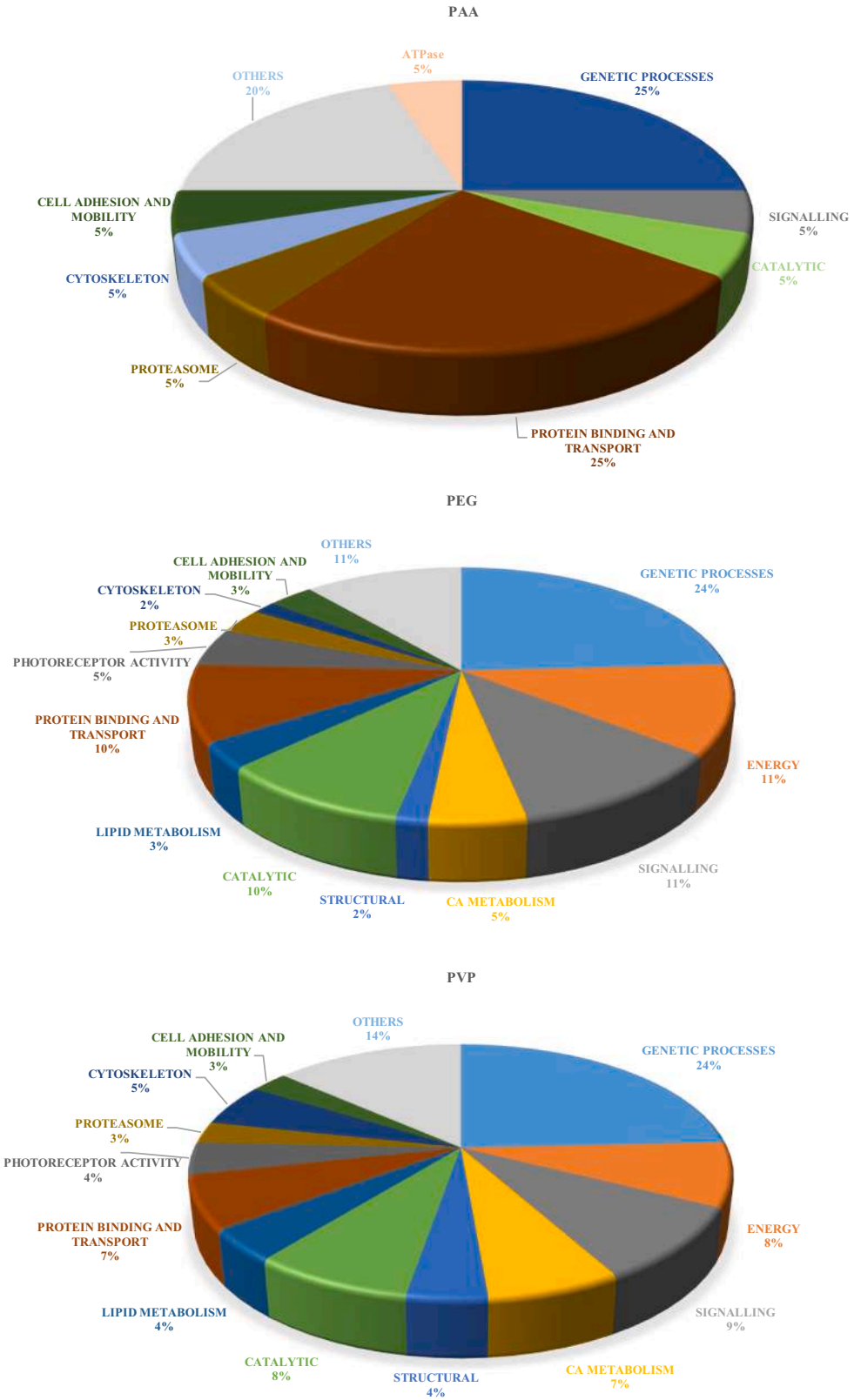

Fig. 2. Protein classes modulated by the three WSPs tested (1 mg/L) with their relative percentages.

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

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

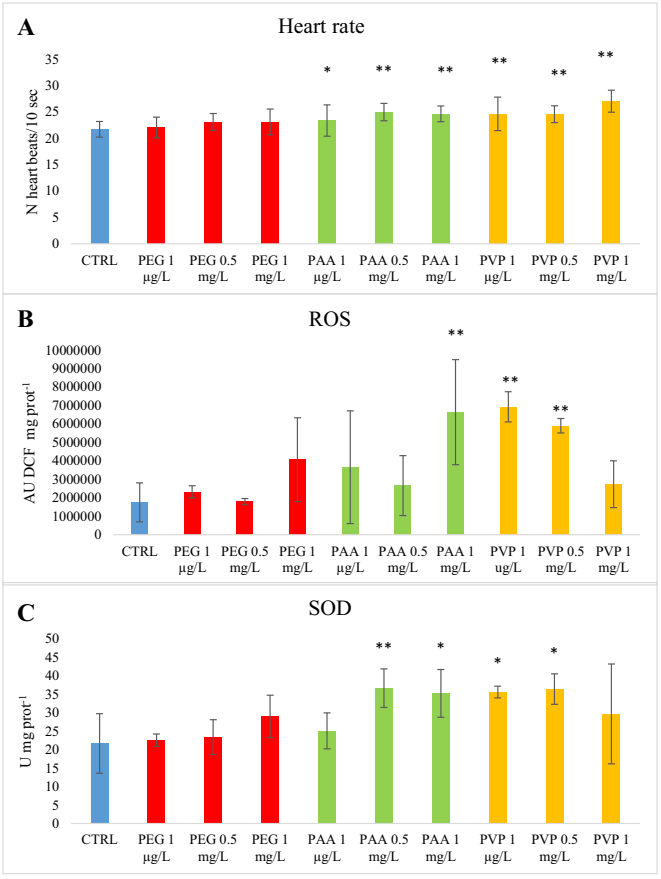

Fig. 3. Effects of different concentrations of the three WSPs on heart rate and oxidative status of *Danio rerio*, data are expressed as mean ± standard deviation, * means significant differences between treated and controls ($p < 0.05$), while ** means significant differences between treated and controls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

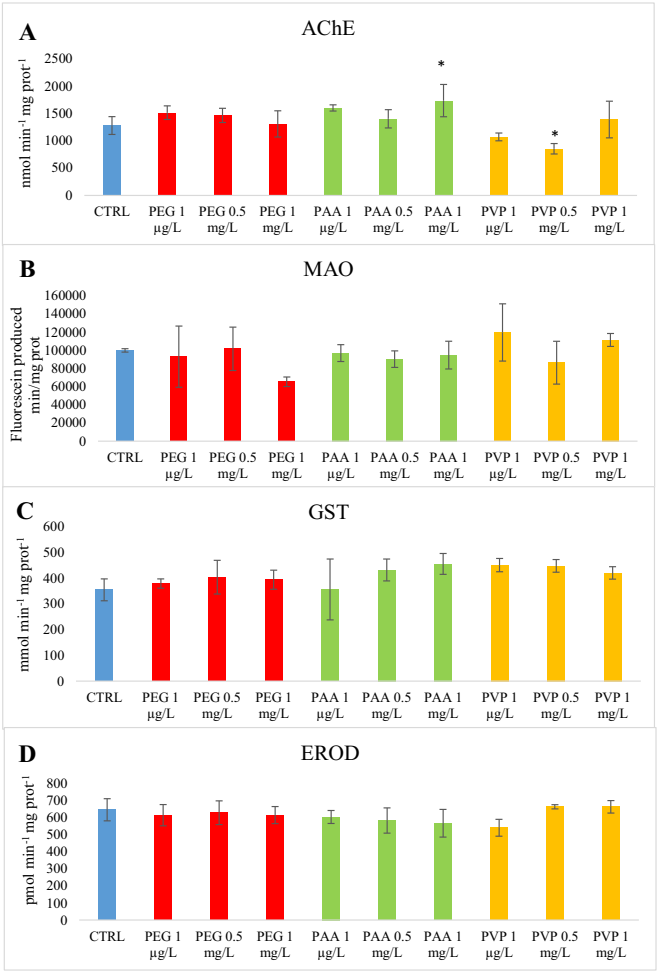

Fig. 4. Evaluation of the WSPs neurotoxicity and of their effects on the detoxifying enzymes, data are expressed as mean ± standard deviation, * means significant differences between treated and controls ($p < 0.05$). (A) AChE activity, (B) MAO activity, (C) GST activity, (D) EROD.

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

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

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

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

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

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

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

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

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

4.2. Biomarkers

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

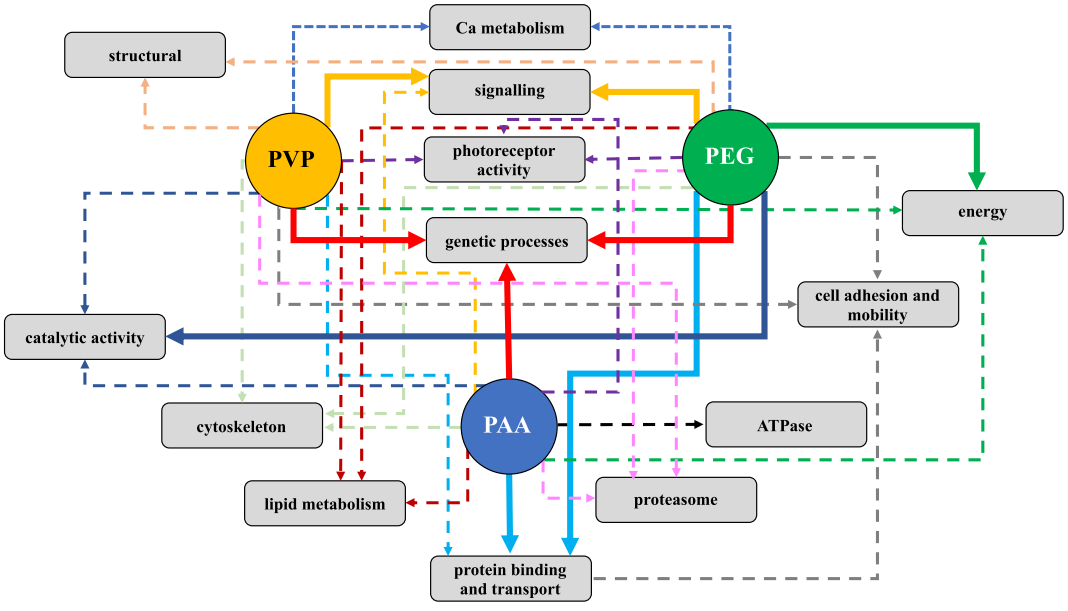

Fig. 5. Network of the different cellular pathways affected by protein modulation due to the 3 WSPs (1 mg/L). Same color = common effect on a specific cellular pathway. Bold lines = modulation of >10 % of the proteins involved in this pathway by each individual WSP. Dotted lines: modulation of <10 %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

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

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

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

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

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

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

4.3. Final remarks

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

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

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

5. Conclusions

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

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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.167699.

CRediT authorship contribution statement

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

Declaration of competing interest

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

Data availability

Data will be made available on request.

Acknowledgements

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

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Journal of Hazardous Materials 469 (2024) 134000

Contents lists available at ScienceDirect

Journal of Hazardous Materials

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

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

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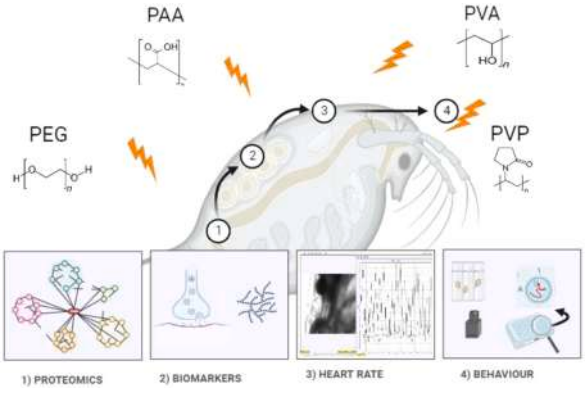
HIGHLIGHTS

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

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Synthetic polymers
Freshwaters
Chronic toxicity
Ecotoxicology
Ecology

ABSTRACT

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

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

1. Introduction

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

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

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

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

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

2. Materials and methods

2.1. Preparation of WSP testing solutions

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

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

2.2. *D. magna* exposures

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

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

2.3. Polymer quantification

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

2.4. Gel free proteomics

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

Protein characterization was conducted at the UNITECH OMICs facility of the University of Milan. For analysis, 5 μL of each sample was injected in triplicate into a Dionex Ultimate 3000 nano-LC system (Sunnyvale CA, USA), which was connected to a Thermo Scientific Orbitrap Fusion™ Tribrid™ Mass Spectrometer equipped with a nano-electrospray ion source. Peptides were initially concentrated onto a Thermo Scientific Acclaim PepMap 100 - 100 μm x 2 cm C18 column, followed by separation on an EASY-Spray column ES802A, which measured 15 cm × 75 μm ID and was packed with Thermo Scientific Acclaim PepMap RSLC C18 particles (3 μm, 100 Å). The separation was accomplished using mobile phase A (0.1% formic acid) and mobile phase B (0.1% formic acid in acetonitrile, *v/v*) at a flow rate of 0.300 μL/min. To prevent any sample contamination, a blank run was performed between each sample. Spectra were acquired in positive polarity mode across a mass-to-charge ratio (*m/z*) range of 375–1500 Da, with at 120,000 resolutions. The instrument operated in data-dependent mode with a cycle time of 3 s between master scans and a collision energy of 35 eV. Data processing was conducted using Thermo Scientific Proteome Discoverer Software 2.5, utilizing the *Daphnia magna* database and trypsin as the enzyme.

2.5. Biomarkers

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

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

2.5.1. Biomarker of neurotoxicity

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

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

2.5.2. Biomarker of energetic metabolism

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

2.6. Heart rate assay

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

2.7. Behavioural alteration on D. magna

2.7.1. Horizontal swimming

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

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

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

2.7.2. Vertical migration

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

2.8. Statistical analysis

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

3. Results

3.1. Acute toxicity

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

3.2. Proteomics

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

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

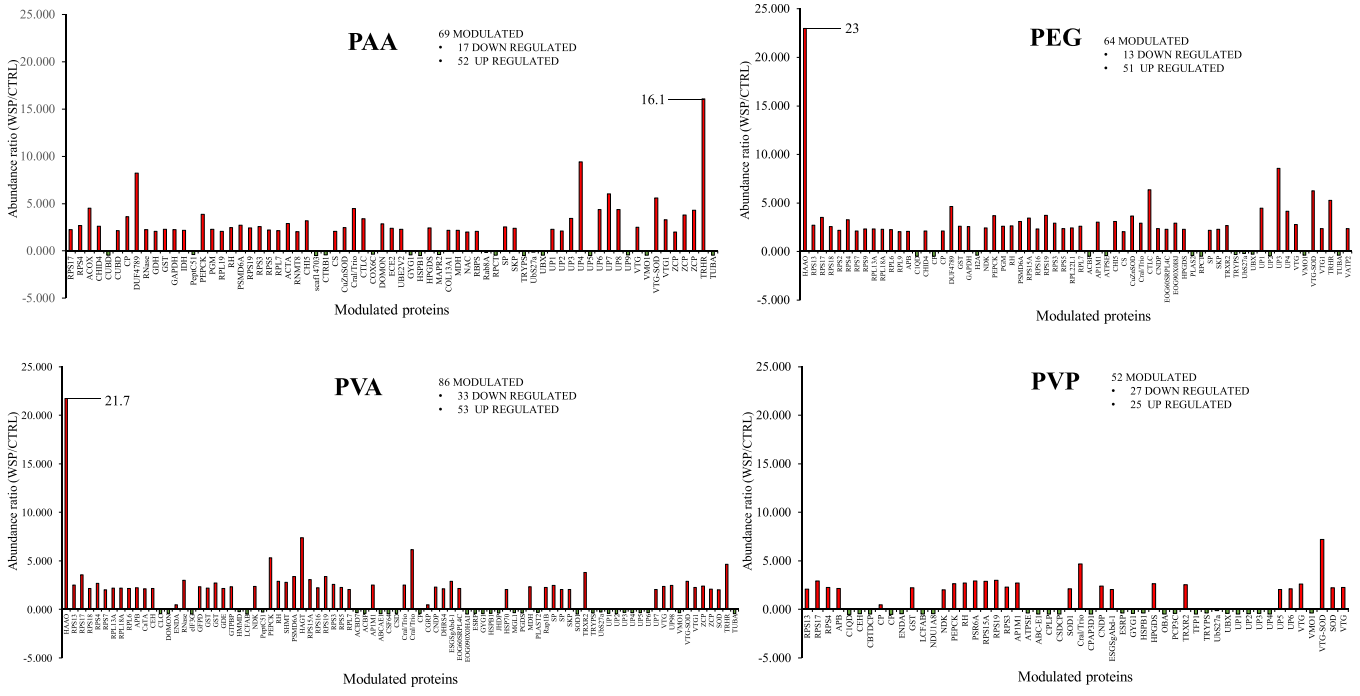

Fig. 1. Gel-free proteomics analysis results, Abundance ratio of modulated proteins by 1 mg/L of polyacrylic acid (PAA), polyethylene glycol (PEG), polyvinyl alcohol (PVA) and polyvinylpyrrolidone (PVP) compared to controls (red=up-regulation; green=down-regulation). Acronymous and the corresponding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

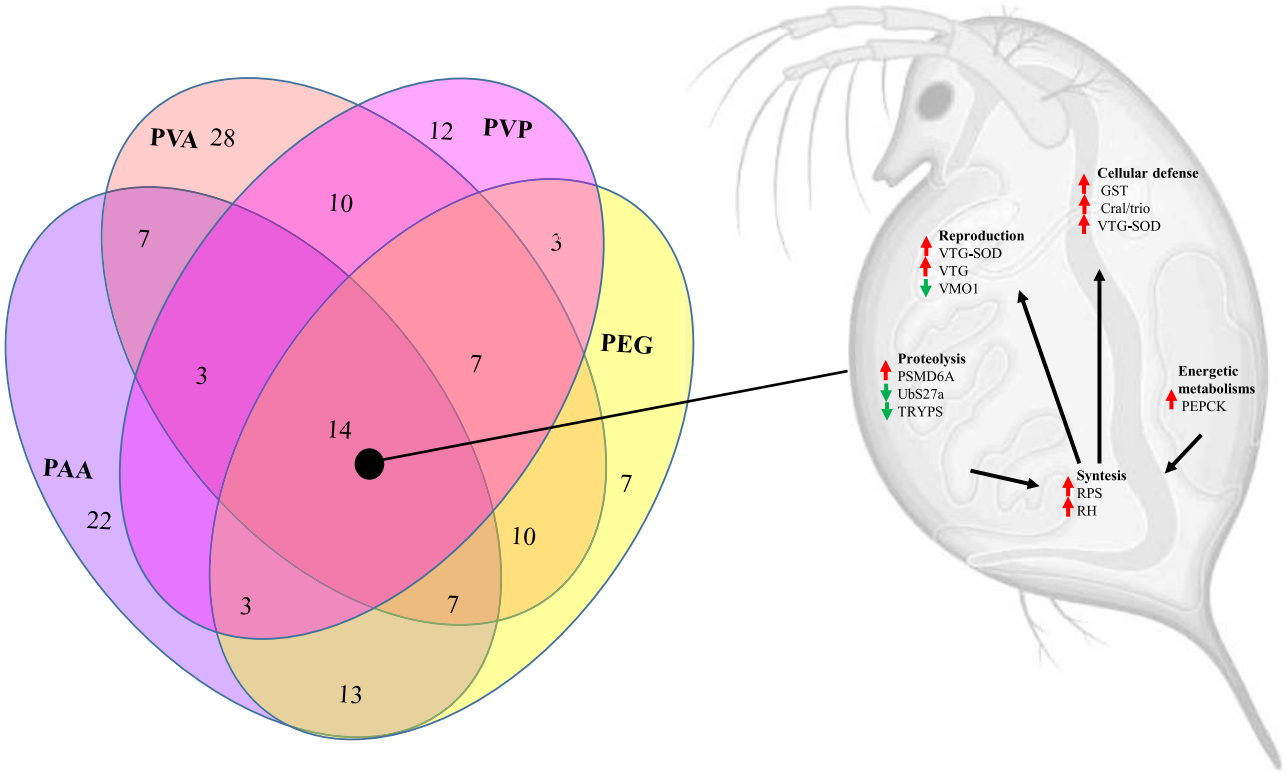

Fig. 2. Venns' diagram showing proteins modulated by individual Water-soluble polymer (WSP) at 1 mg/L of polyacrylic acid (PAA), polyethylene glycol (PEG), polyvinyl alcohol (PVA) and polyvinylpyrrolidone (PVP) in common with each other. On the right a representation of the common modulated proteins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

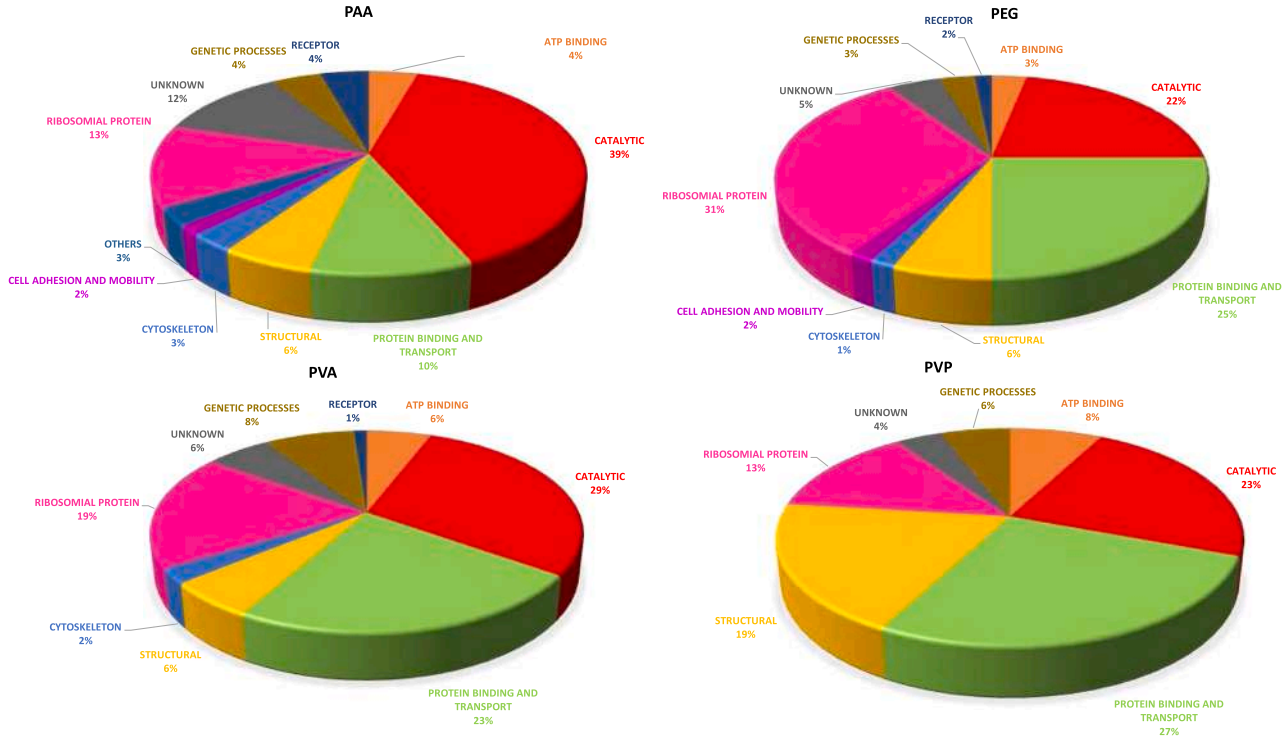

Fig. 3. Protein classes modulated by the four WSPs tested, polyacrylic acid (PAA), polyethylene glycol (PEG), polyvinyl alcohol (PVA) and polyvinylpyrrolidone (PVP), at 1 mg/L with their relative percentages.

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

3.3. Biomarkers

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

3.4. Heart rate

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

3.5. Behavioural alteration

3.5.1. Horizontal swimming

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

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

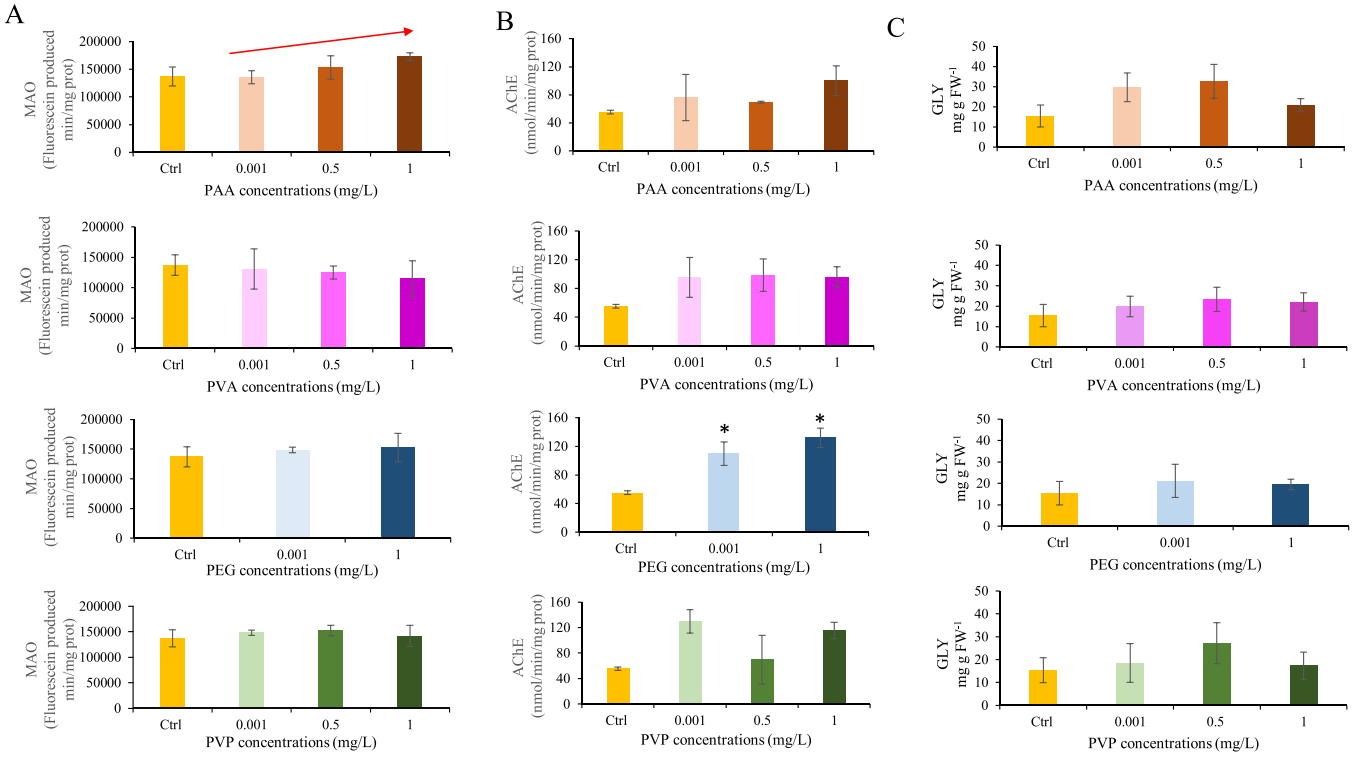

Fig. 4. Effects of different concentrations of polyacrylic acid (PAA), polyvinyl alcohol (PVA), polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP) on monoamine oxidase activity (MAO) **(A)**, acetylcholinesterase activity (AChE) **(B)**, and glycogen content (GLY) **(C)**. Exposure in triplicate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

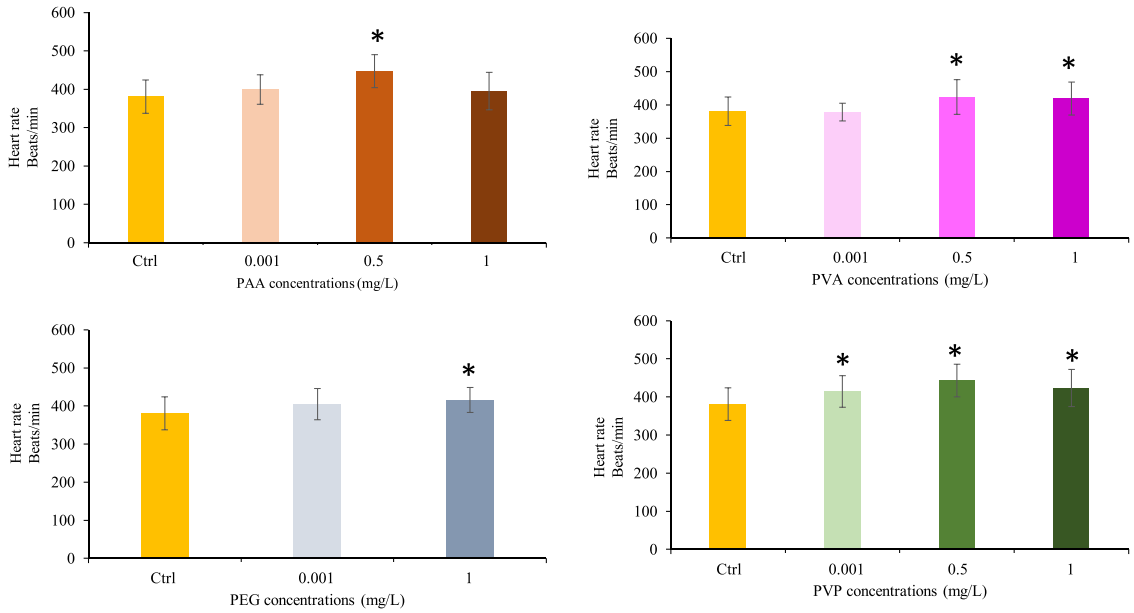

Fig. 5. Heart rate every min. *D. magna* exposed to different concentrations of polyacrylic acid (PAA), polyvinyl alcohol (PVA), polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP). Exposure in triplicate N = 30 specimens *per* treatment. Data are expressed as mean ± SD. * means significant difference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

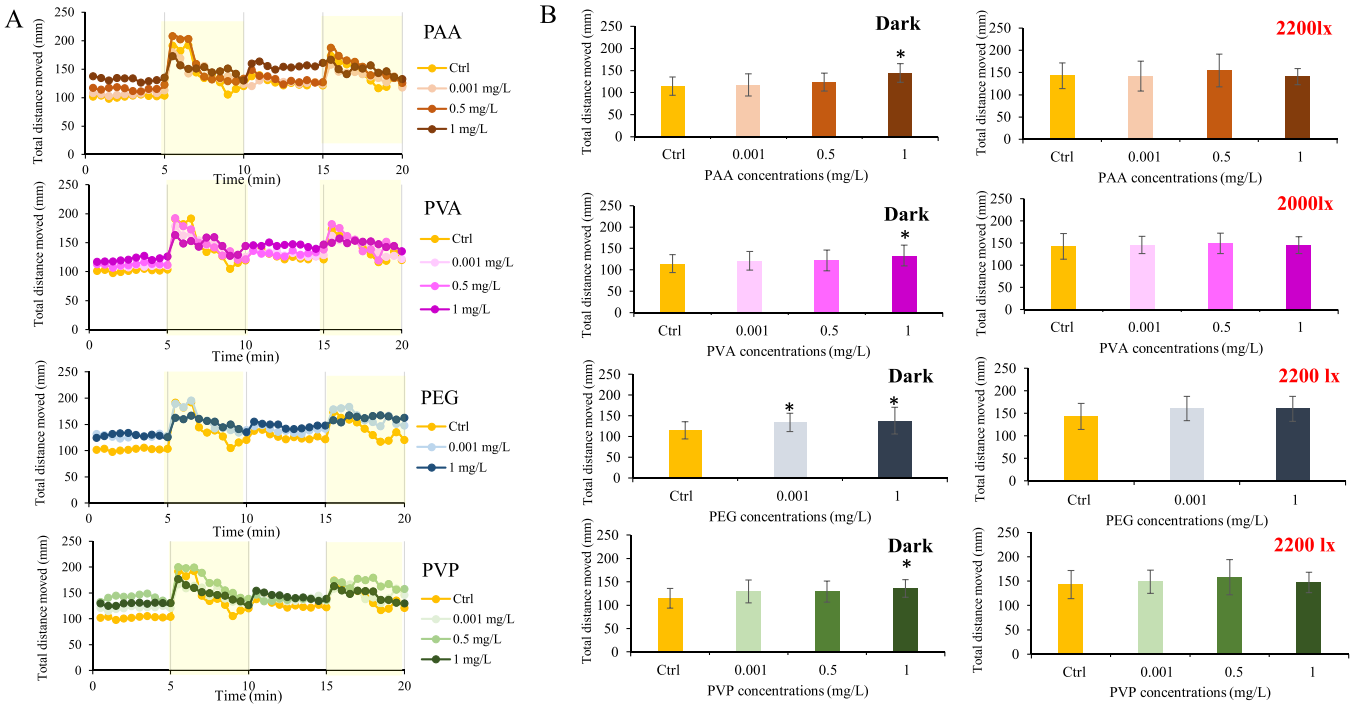

Fig. 6. Distance moved every 30 s **(A)** by *D. magna* exposed to different concentrations of polyacrylic acid (PAA), polyvinyl alcohol (PVA), polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP). Organisms undergone to two consecutive cycles of light/dark phases whose duration was 5 min each one.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

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

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

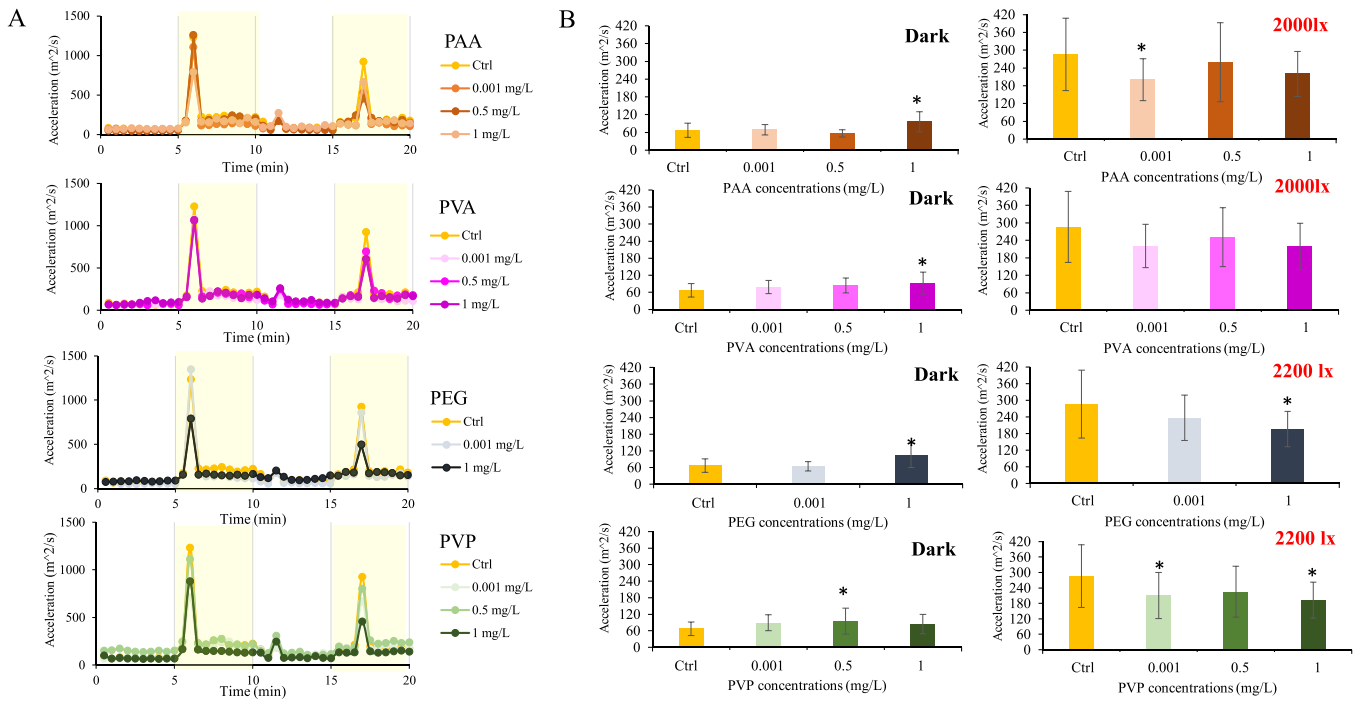

Fig. 7. Acceleration every 30 s **(A)** by *D. magna* exposed to different concentrations of polyacrylic acid (PAA), polyvinyl alcohol (PVA), polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP). Organisms undergone to two consecutive cycles of light/dark phases whose duration was 5 min each one.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

3.5.2. *Vertical migration*

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

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

4. Discussion

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

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

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

4.1. *Proteomics*

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

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

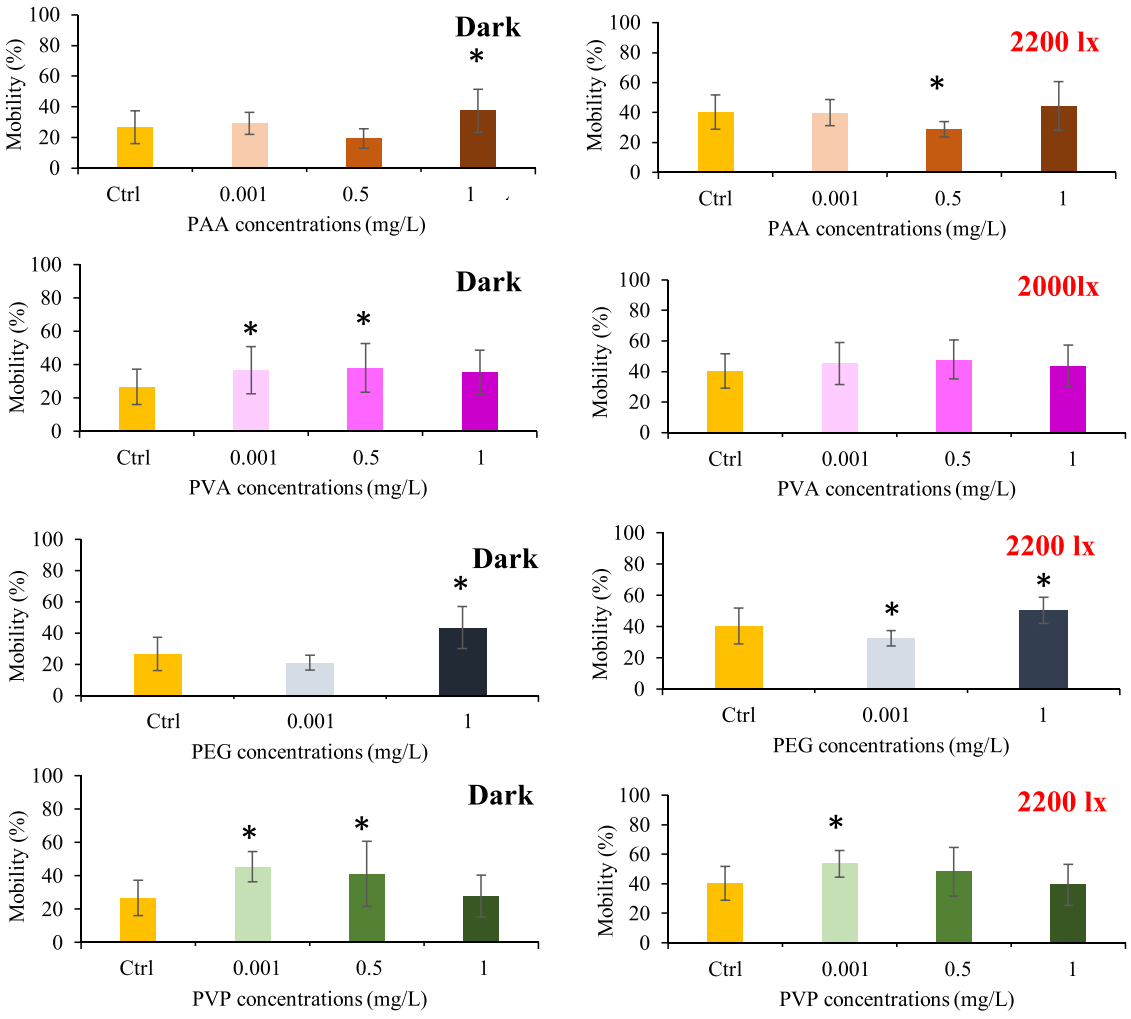

Fig. 8. Mobility of *D. magna* exposed to different concentrations of polyacrylic acid (PAA), polyvinyl alcohol (PVA), polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP) under both Dark and Light (2200 lx) conditions. Exposure in triplicate N = 30 specimens *per* treatment. Data are expressed as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

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

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

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

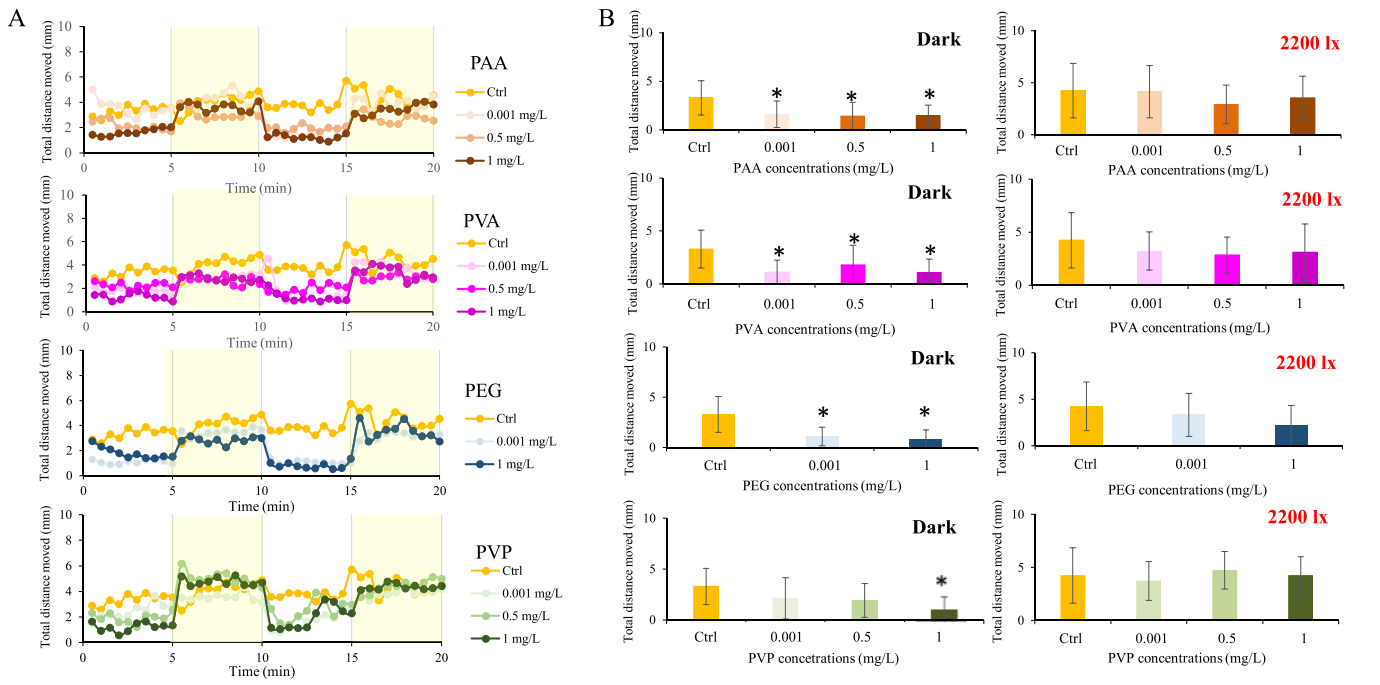

Fig. 9. Vertical migration every 30 s **(A)** by *D. magna* exposed to three different concentrations of polyacrylic acid (PAA), polyvinyl alcohol (PVA), polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP). Organisms undergone to two consecutive cycles of light/dark phases whose duration was 5 min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

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

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

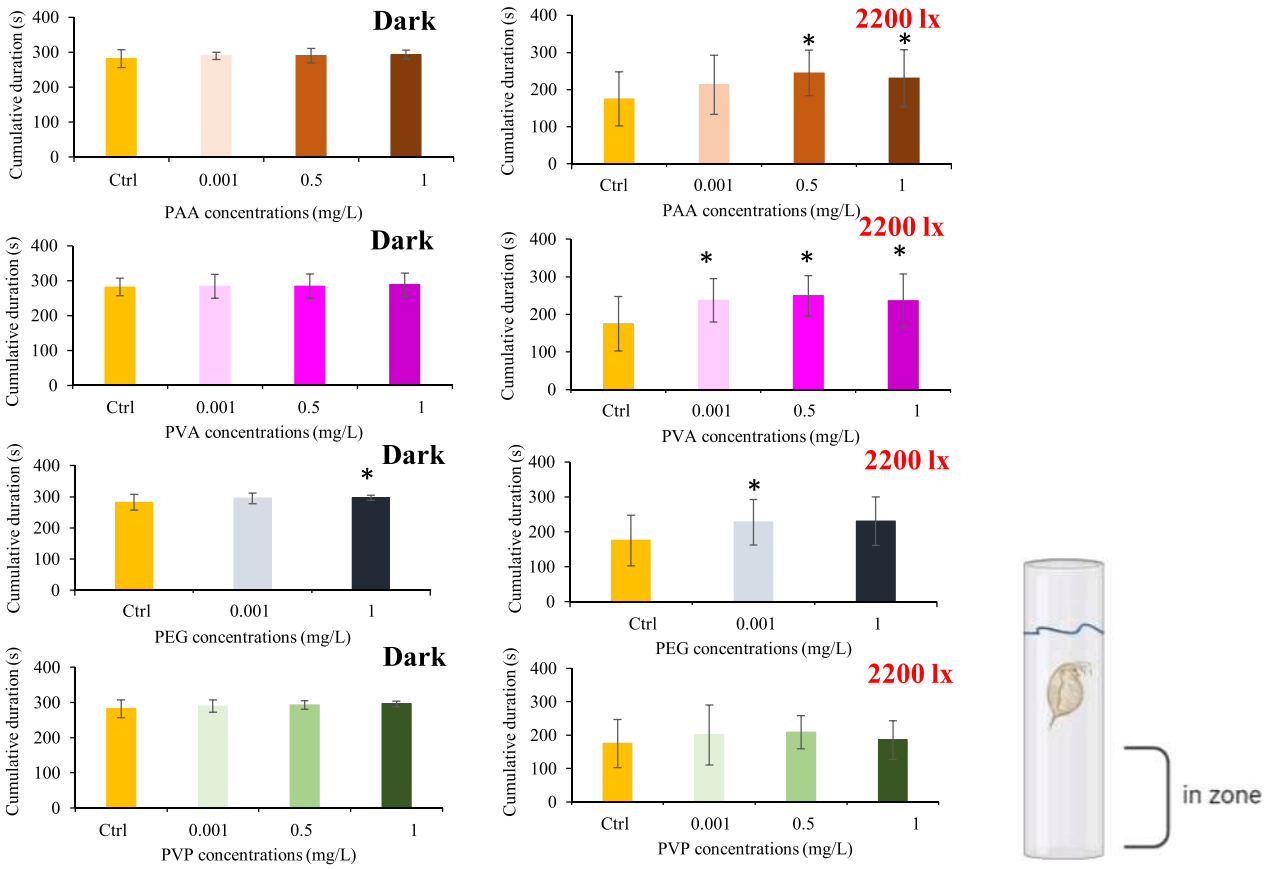

Fig. 10. Negative Phototaxis, expressed as the time spent (s) by the specimens in the lower zone of the vial, of *D.magna* exposed to three different concentrations of polyacrylic acid (PAA), polyvinyl alcohol (PVA), polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP) under both Dark and Light (2200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

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

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

4.2. Biomarkers and heart rate

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

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

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

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

4.3. Behavioural effects

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

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

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

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

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

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

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

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

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

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

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

4.4. Final remarks

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

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

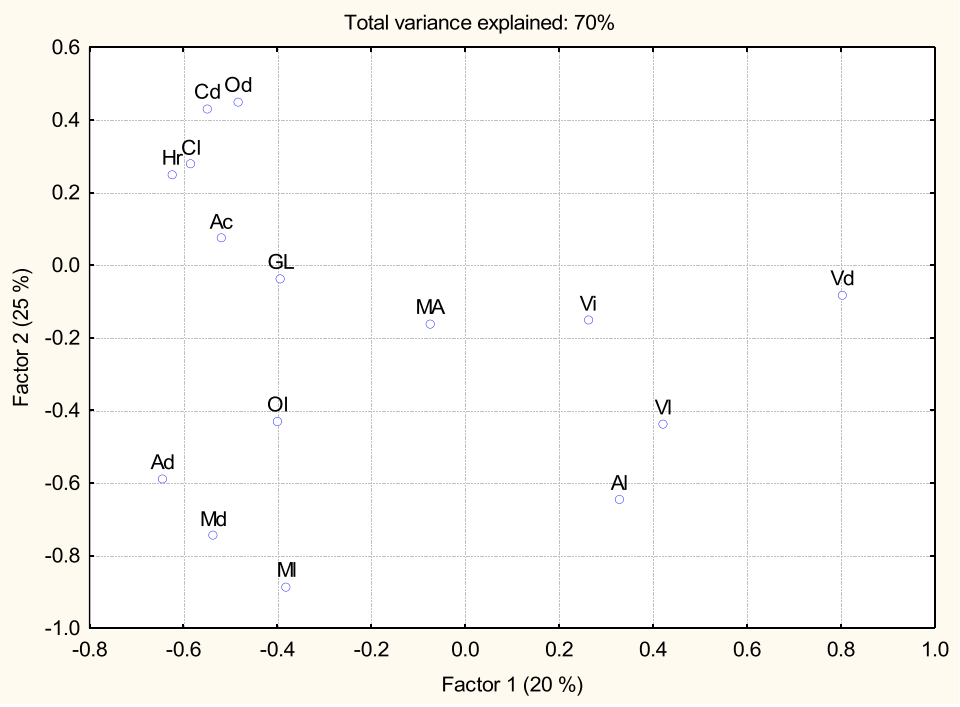

Fig. 11. Factorial analysis using all biomarkers, behavioural endpoints and vitality: AChE (Ac), MAO (MA), GLY (GL), Heart rate (Hr), Mobility (dark; Md), Mobility (light; Ml), Orizontal distance moved (dark; Od), Orizontal distance moved (light; Ol), Acceleration (dark; Ad), Acceleration (light; Al),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

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

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

5. Conclusions

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

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

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

Environmental implications

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

CRediT authorship contribution statement

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

Declaration of Competing Interest

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

Data Availability

Data will be made available on request.

Acknowledgements

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

Appendix A. Supporting information

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

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Transgenerational Effects of Water-Soluble Polymers on *Daphnia magna* at Environmentally Relevant Concentrations: the role of transgenerational plasticity.

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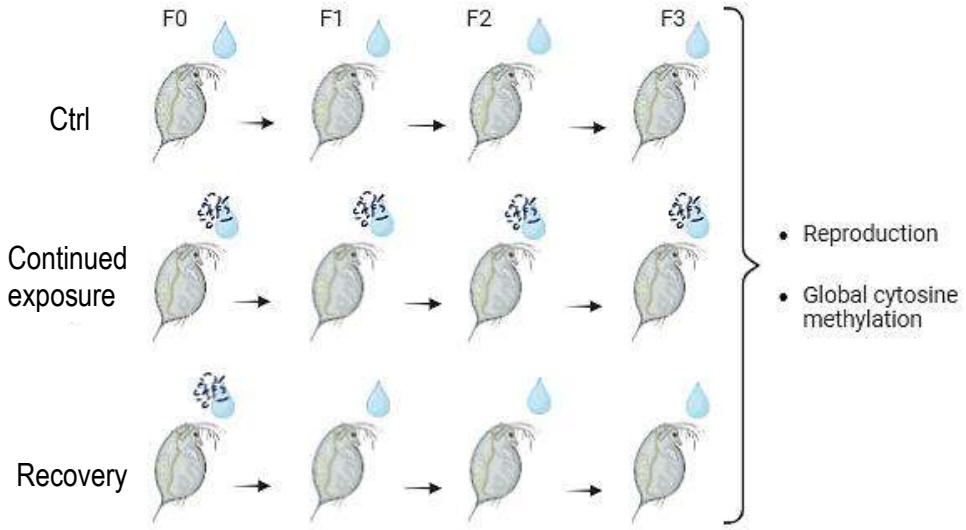
GRAPHICAL ABSTRACT



The widespread use of water-soluble polymers (WSPs) like polyethylene glycol (PEG) and polyvinyl alcohol (PVA) across multiple industrial and household uses has recently raised concerns about their environmental persistence and potential toxicity to aquatic organisms. Despite being excluded from regulatory oversight, recent studies suggest possible ecological risks associated with sub-lethal exposures to these polymers. In this context, this study investigates the transgenerational effects of PEG and PVA on *Daphnia magna*, focusing on both life-history parameters and epigenetic modifications at the environmentally relevant concentration of 1 µg/L. Through continuous exposure experiments, spanning three generations (from F_0 to F_3), and "recovery" groups, where only the parental generation (F_0) was exposed, our results reveal significant reductions in the number of newborns and reproductive parameters in the F_0 generation exposed to PEG but not in subsequent generations. This suggests a multigenerational plasticity in *Daphnia* through a compensatory or acclimation reproductive response over time. Global cytosine methylation patterns also showed a significant initial increase in the F_0 generation exposed to PEG, which decreased in later generations,

indicating a possible epigenetic mechanism underlying observed reproductive effects. In contrast, PVA exhibited no significant changes in both life history parameters and methylation but showed a global methylation trend suggesting its likely epigenetic influence. These findings underscore the need for comprehensive risk assessments of WSPs, particularly their potential for inducing long-term (epigenetic) effects, influencing reproductive functions across generations and how increased plasticity may affect responses against novel other stressors.

Keywords: Synthetic polymers, Transgenerational effects, Freshwaters, Ecology

1. Introduction

Water soluble polymers (WSPs) have become a significant category of the global polymer market (ARC Industry, 2019; Wang et al., 2021), with extensive applications in a wide range of products, including those for personal care, pharmaceuticals, fertilizers, flocculants, and in various other industrial applications (Duis et al., 2021). Despite their widespread use, the WSPs have not been subject to regulatory oversight, as being excluded from the Registration, Evaluation, Authorisation, and Restriction of Chemicals (REACH) due to their perceived low environmental impact. This is attributed to their high molecular weight (Huppertsberg et al., 2020) and solubility in water, falling outside the conventional definition of "plastic." As a result, WSPs have been released into the environment without significant restrictions, probably leading to their ubiquitous presence in ecosystems (Huppersberg et al., 2020; Wang et al., 2023). Although a reassessment of their exemption from REACH registration is currently underway, detailed information on their production volumes remains elusive since their estimates rely only on data from the production of their educts (Pauelsen et al., 2023).

Among the wide class of WSPs, polyethylene glycol (PEG) and polyvinyl alcohol (PVA) are particularly outstanding due to their solubility and widespread use (Falqi et al., 2018, Mohammadi et al., 2022, D'souza et al., 2016, O¨zdemir et al., 2023), including multiple applications in the medical and pharmaceutical fields. PEG, known for its biocompatibility, high hydrophilicity, and low immunogenicity, has found extensive applications in drug delivery systems, tissue engineering, and as an excipient in many pharmaceutical formulations (D'souza et al., 2016, O¨zdemir et al., 2023). Its ability to modify drug pharmacokinetics has revolutionized medicine, enabling controlled and sustained release of therapeutics (Ezike et al., 2023). Thanks to its unique properties, PEG has also received approval from the Food and Drug Administration (FDA) for being the constituent of medical

and cosmetic devices (Kolate et al., 2014; Ozdemir et al., 2023). Moreover, PVA is valued for its biodegradability and film-forming properties, making it integral to various biomedical applications such as wound dressings, drug delivery carriers, and tissue scaffolds (Nagarkar and Patel, 2019; Chen et al., 2011; Fernandes et al., 2013; Salunkhe et al., 2013). PVA-based hydrogels are also essential in the production of soft contact lenses, providing comfort and hydration due to their high-water content and biocompatibility (Solaro et al., 2000). Like PEG, PVA has also been approved by the FDA for clinical use in humans (Chong et al., 2013; Nagarkar and Patel, 2019).

Despite the significant contributions of these versatile polymers to medical and pharmaceutical advancements, recent studies have raised concerns about their environmental persistence and potential toxicity to aquatic organisms (Wang et al., 2021; Menzies et al., 2023). While classic (eco)toxicological tests have evinced a negligible toxicity of such compounds, there remains a notable gap in our understanding of the chronic and long-term ecological impacts of WSPs, particularly at environmentally relevant concentrations (Nigro et al., 2024). Existing studies have largely focused on apical effects based on the No Observed Effect Concentration/Lowest Observed Effect Concentration (NOEC/LOEC) and Effect Concentration at 50% (EC_{50}) that often overlook the sub-organismic effects which have recently been observed in several model organisms exposed to some WSPs (Hatami et al., 2019; Nascimento et al., 2021; Mondellini et al., 2022; Nigro et al., 2023; 2024; Binelli et al., 2024).

To address this gap, our study emphasizes the need for a comprehensive assessment of sublethal effects of these polymers at realistic environmental concentrations, to better understand also their multigenerational impacts and implications on ecological traits. In fact, it is crucial to advance beyond traditional apical tests to fully elucidate the risks posed by WSPs, including their broader ecological impacts over extended exposure periods (Silva et al., 2017). This study, therefore, investigates the multigenerational effects of PVA and PEG on *Daphnia magna* at the environmentally relevant concentration of 1 µg/L, with exposure spanning up to the F_3 generation. The experimental design includes continuous exposure to PVA or PEG across all the three generations, as well as "recovery groups" where only the parental generation (F_0) was exposed to either PVA or PEG, followed by subsequent generations (F_1, F_2, F_3) reared solely in reconstituted water. The primary objective was to assess the heritability of epigenetic modifications, specifically global cytosine methylation patterns, and to determine whether these induced changes persist even after the contaminant is removed. Additionally, the number of juveniles was counted for every generation in each treatment to evaluate the possible modification of this crucial functional trait. By integrating the epigenetic perspective, this study aims to contribute crucial insights into the long-term ecological implications of PVA and PEG, addressing the need for a deeper understanding of their inherited effects on freshwater

organisms. Building upon foundational studies, such as Dietrich et al. (2010), Coutellec and Barata (2013), Barata et al. (2017), Silva et al. (2017), and Straub et al. (2020), this work seeks to improve future environmental conservation strategies by advancing our knowledge of the multigenerational impacts induced by WSPs.

2. Materials and methods

2.1. Preparation of WSP testing solutions

The WSP standard powders of PEG (CAS number: 25322-68-3) and PVA (CAS number: 9002-89-5) were purchased from Sigma-Aldrich (Merck Life Science, Milan, Italy). The molecular weights (MWs) of these powders were approximately, 1,900–2,200 Da for PEG and 89,000 - 98,000 Da for PVA. To prepare the four WSP stock solutions (10 mg/L), COMBO water was used modifying the protocol outlined by Kilham et al. (1998; Table S1). Solutions were heated to ensure complete solubilization of the WSPs, following the procedure detailed by Nigro et al. (2024). These stock solutions were then diluted to achieve the two exposure concentrations at 0.001 mg/L based on previous research (Nigro et al., 2024). Before use, the solutions were aerated and maintained at 20 °C.

2.2 Quantification of polymers

The concentration of WSPs in MilliQ water solutions was determined through a two-step process. The analysis was performed on 2 L of the stock solution. It was conducted twice, at the initial time point (t_0) and at the end of each exposure (t_{17}). First, a rotary evaporator was used to completely evaporate the water, allowing for the collection of the solute and determining its weight. The second step involved confirming the structure of the recovered WSP using ^{1}H NMR analysis, with spectra recorded on a Bruker Ultrashield 400. The samples were prepared by dissolving the recovered WSP in 0.7 mL of DMSO-d_6. The amount of recovered compounds at the start and the end of exposures was found to be 9.72 mg/L for PVA and 9.93 mg/L for PEG, respectively, closely matching the expected value (10 mg/L), thus validating the accuracy of the method used. The chemical structure of the recovered PVA was confirmed through ^{1}H NMR analysis (see Fig. S1,2).

2.3 D. magna rearing

Monoclonal cultures of *Daphnia magna* have been reared in the Ghent University laboratory for more than 50 generations (De Coen and Janssen, 1998). Daphnids were cultured in COMBO medium as described in the SI (Kilham et al., 1998). Cultures were maintained under a temperature of 20±2 °C and a 16 h/8 h light/dark photoperiod (provided by cool fluorescent white lights). Culture medium

was renewed, and organisms were fed three times a week, with concentrated suspensions of *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii*.

2.4 Trangenerational exposures

Experiments were conducted under the previously described temperature and photoperiod conditions. The experimental design is shown in Fig. 1. In detail, there were five treatment groups: the Control group (Ctrl), two continuous exposure groups (PEG and PVA) and two recovery groups (PEGrec and PVArec). For all the treatments (without control) the parent generation (F_0) was exposed to 0.001 mg/L of PVA or PEG. In the continuous exposure groups, this exposure persisted through all subsequent generations (F_1, F_2, F_3). In the recovery groups, only the parent generation (F_0) was exposed, while the following generations were placed in fresh culture medium without further exposure.

In the toxicity tests, daphnids (less than 24 hours old) were exposed under semi-static conditions, with the medium being renewed three times per week. The exposure continued until the initial daphnids matured and produced their third brood. Each subsequent generation began with neonates from the third brood (less than 24 hours old) and were kept in the same medium until they released their own third brood. After this, the mothers of daphnids were removed, and new generations were initiated with the released neonates. This process was repeated for all four generations included in the experimental design (see Fig. 1).

Specimens were housed in 500 mL glass vessels filled with pre-aerated reconstituted COMBO water (oxygenation >3 mg/L and pH maintained in the range 6–9), in accordance with OECD guideline 211. Each treatment included 45 daphnids (15 specimens *per* glass vessels) maintained at 20 °C, under a photoperiod of 16 h of light (1500 lx) and 8 h of darkness. The exposure was conducted in triplicate, with viability, immobilization and offspring number data recorded daily. Throughout the exposure period the food for *Daphnia magna* specimens consisted of a mixture of two algae species: *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii*, mixed in an 3/1 proportion. The amount of food given to the Daphnids is 0.1-0.2 mg C/Daphnia/day.

After the exposure for every generation, mothers were harvested, pooled per replicate and stored at −80 °C for DNA extraction, immediately after releasing the third brood. Always it was ensured under the microscope that their brood pouch was empty to avoid extracting DNA originating from eggs.

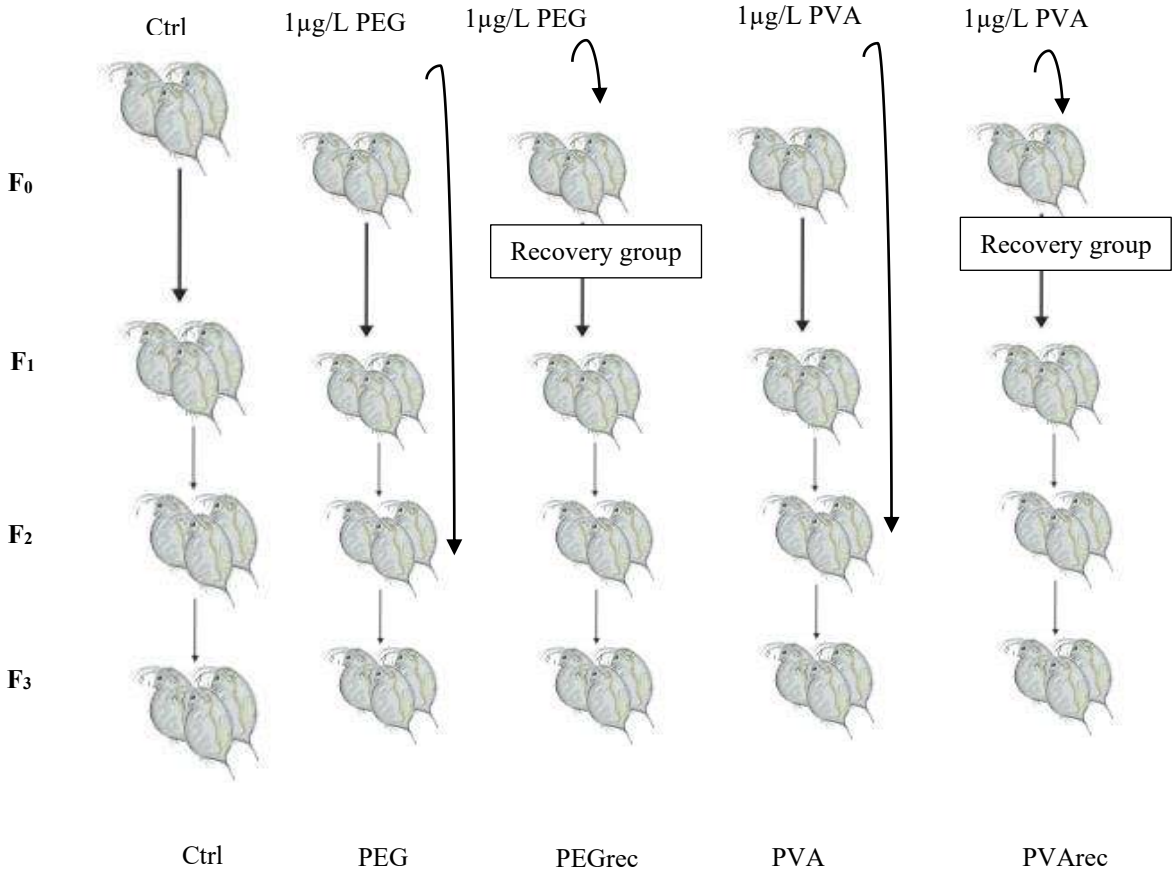


Fig. 1 Experimental design of the transgenerational experiment. *D. magna* was exposed to 1 µg/L of polyvinyl alcohol (PVA) and polyethylene glycol (PEG) for all the subsequent generations F_0, F_1, F_2 and F_3 in the "PEG" and "PVA" treatments. While the treatment exposed to PVA and PEG only in the parental generations ("PEGrec" and "PVArec") from the F_1 generation returned to freshwater culture medium for the recovery.

2.5 Assessment of the global cytosine methylation

Total genomic DNA of all samples was extracted using the Cetyltrimethylammonium Bromide (CTAB) DNA isolation protocol (Chen and Ronald, 1999) with opportune modifications. CTAB buffer was pre-warmed to 65 °C and 300 µl was added to the thawed samples (10 pooled organisms). Samples were then grinded using an autoclaved pestle and incubated for 1 h at 65 °C with vortexing every 20 min. After adding 300 µl of phenol-choroform-isoamyl alcohol (25:24:1) to the samples, the mixture was mixed and centrifuged for 20 min at 15,000 rcf (relative centrifugal force) and 4 °C after which the organic phase was removed. Then 0.5 µl of RNase A (20 µg/mL) was added to each sample and incubated for 20 min at room temperature. To this solution, 500 µl of chloroform/isoamyl alcohol (24:1) mixture was added to separate additional contaminants. The liquids were mixed and centrifuged at 15,000 rcf for 15 min. This step was repeated twice.

The aqueous phase in the supernatant was transferred into a new clean tube. DNA was precipitated by adding 27 µl of 3 M Na acetate and 500 µl of 2-propanol. The mixture was gently mixed and incubated for 10 min at room temperature and DNA was pelleted by spinning the tube at 4 °C, 10,000 rcf for 30 min. The supernatant was removed, and the pellet was washed with 70% ethanol, air dried, and resuspended in 50 µl of TE buffer.

NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used for a preliminary screening of DNA purity. Samples with a 260/230 ratio above 1.7 and a 260/280 ratio between 1.8 and 2.1 were stored at -80 °C for the quantification of total 5-mC methylation, using the MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit (Colorimetric) following the manufacturers' protocol. The DNA input was 100 ng per assay, with a detection sensitivity as low as 0.05% methylation. Reagents are prepared according to the kit instructions, including dilution steps and preparation of a standard curve using the provided controls. The intensity of the colour, which correlates with the level of DNA methylation, is measured using a microplate reader (Tecan i-control, infinite 200) at 450 nm. The percentage of methylated DNA in the sample is calculated based on the optical density (OD) values obtained, using the standard curve for reference. Results were expressed in total 5-mC percentage per total DNA content.

2.6 Statistical Analysis

Statistical analyses were conducted using R version 4.3 (R Core Team 2024). Data collected during the experiment (number of newborns and vitality) were utilized to calculate the sum of the juveniles and the life history parameters: the age at first reproduction (AFR), net reproductive rate (R_0), and the per capita rate of population increase (r), the latter being derived from the Euler-Lotka equation (Jeremias et al., 2022).

To evaluate the number of newborns among treatments the one-way ANOVA and Tukey *post-hoc* test were conducted. The assumptions of normality and homogeneity of variances were evaluated using the Shapiro-Wilk test and Levene's test, respectively. Also to compare the trends in the number of newborns daily among treatments two-way ANOVA test was utilized followed Linear Mixed Model (Table S2).

The effects of different treatments on the life-history parameters, where the data did not meet the normality assumption, a Kruskal-Wallis test was applied, followed by Dunn's *post-hoc* test for multiple comparisons. The same statistical procedures were employed for the analysis of the global cytosine methylation data, while for the analyses of the average of the total methylation across different treatments two-way ANOVA test was performed. A three-parameter model (LL.3) was used to analyse the trends in the global cytosine methylation of treatments across generations.

3. Results

3.1. Multigenerational life-history effects

Regarding the vitality of individuals, no effects were observed across all generations in all treatments. However, we observed that the sum of juveniles was significantly different in the first generation in all treatments compared to the other generations ($p<0.05$) (Figs. 2, 3) and the life-history parameters between the treatments (AFR, R_0 and r, Table 1).

	AFR	χ2	df	p-value	**R0**	Chi-squared	df	p-value	**r**	χ2	df	p-value
Comparison between treatments in the same generation	F_0	4	4	0.406	F_0	14.797	4	**0.005142**	F_0	15.843	4	**0.003237**
	F_1	0	4	1	F_1	4.5999	4	0.3309	F_1	2.9714	4	0.5626
	F_2	5.9608	4	0.2021	F_2	2.5269	4	0.6398	F_2	2.7286	4	0.6042
	F_3	NaN	4	NA	F_3	6.3631	4	0.1736	F_3	11.429	4	**0.02215**
Trend of the same treatment along the generations	Ctrl	4.2308	3	0.2376	Ctrl	5.0423	3	0.1687	Ctrl	5.5809	3	0.1339
	PVArec	3	3	0.3916	PVArec	4.4183	3	0.2197	PVArec	6.8162	3	0.07799
	PVA	2.1429	3	0.5433	PVA	4.9482	3	0.1756	PVA	2.2059	3	0.5308
	PEGrec	2.1429	3	0.5433	PEGrec	10.301	3	**0.0167**	PEGrec	9.9926	3	**0.01863**
	PEG	3	3	0.3916	PEG	11.846	3	**0.007931**	PEG	1.2574	3	**0.005656**

Table 1. Data analyzed regarding the per capita rate of population increase (r), the net reproductive rate (R0), the age at first reproduction (AFR) between treatments in the same generations and between the same treatment along the generations. Df= freedom degree.

Specifically concerning the number of the newborns (Fig. 2), both treatments involving PEG exposure in the F_0 (PEGrec and PEG), showed significantly lower values than controls of the same generation ($F_{4,15} = 15.6$, $p<0.05$). However, when comparing the treatments to their respective control groups, over the subsequent generations (F_1, F_2, and F_3), no significant differences were observed (Fig. 2). Another picture arose when examining the sum of the juveniles for the same treatment across the four generations (Fig. 3). In detail, PEG and PEGrec treatments exhibited a similar pattern, showing a higher number of newborns in the intermediate generations (F_1 and F_2) compared to the F_0, with a subsequent return to values similar to F_0 in the last generation considered (F_3). Finally, concerning the daily number of newborns, the ANOVA analysis revealed that the interactions between treatment and day for every generation are not significant (Table S2).

Moving to the other life-history parameters, when comparing treatments within the same generation, no significant differences were observed for the AFR in the F_0 generation (Table 1). However, significant differences were found for the R_0 parameter and the r parameter in the F_0 (Table 1, Fig S3,4). In detail, for the R_0 parameter a significant difference ($p<0.05$) was observed between PEGrec and controls (Fig. S3) while concerning the r parameters the differences regarded both PEGrec and PEG (Fig. S4). In the F_1 and F_2 generations, no significant differences were observed for AFR, R_0, and r (Table 1), while in the F_3 generation significant differences ($p<0.05$) were instead found for the r parameter (Table 1). Examining the trend of the same treatment across different generations, no significant differences were observed for the controls in AFR, R_0 and r. The AFR parameter was not different along the generations for all the treatments, while significant differences were found for R_0 and r (Table 1; Fig. S5,6). Specifically, both PEG and PEGrec treatments showed a significant increment of r in the generations F_1 and F_2 in comparison to the controls (Fig. S5). Considering the R_0 parameter, the treatment PEGrec showed an increased value only in the generation F_2 with respect to controls, while for the PEG group both generations F_1 and F_2 showed a significant increment ($p<0.05$) of this parameter (Fig. S6).

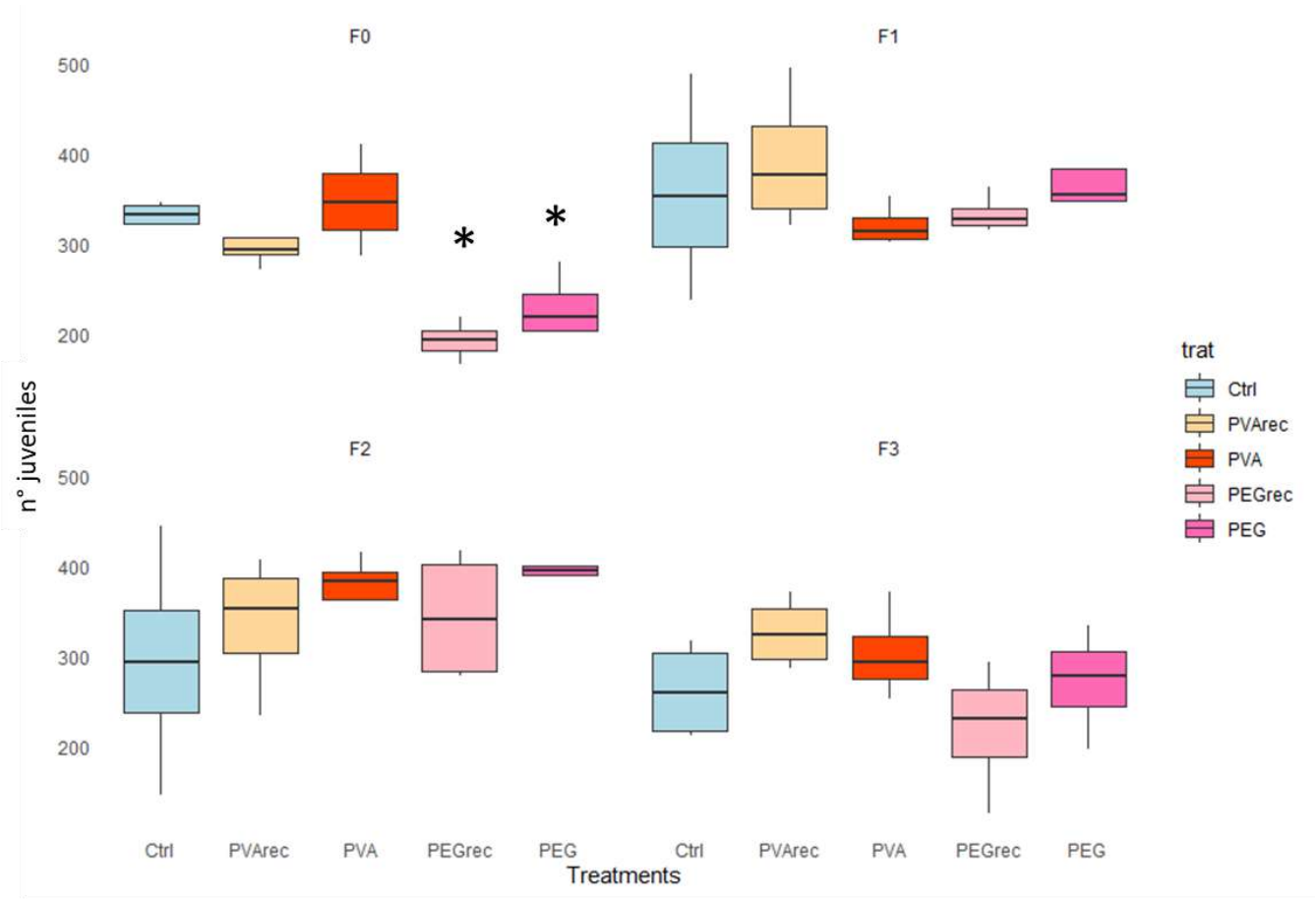


Fig. 2 Sum of the juveniles in the F_0, F_1,F_2, F_3 generations of *D. magna* exposed to 1 µg/L of polyvinyl alcohol (PVA) and polyethylene glycol (PEG) over all the generations (PEG and PVA treatments), exposed to PVA and PEG only in the parental generations (F_0) (PEGrec and PVArec) and the control organisms (Ctrl). Error bars correspond to standard deviation of the mean values,* rapresent statistically differences between the exposed and control organisms of the same generation. Data from F_0 performed throught the one-way ANOVA, Turkey.

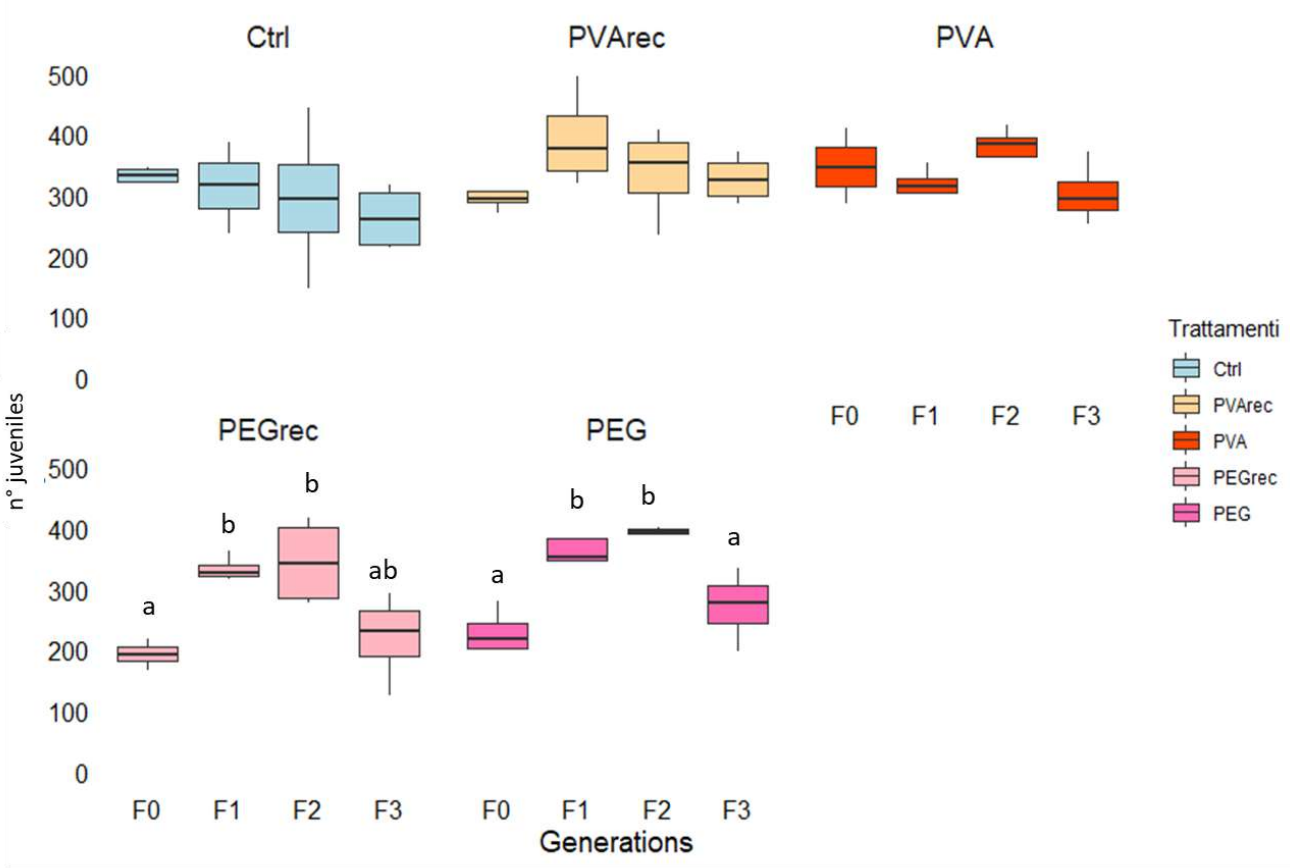


Fig. 3 Sum of the juveniles in the F_0, F_1,F_2, F_3 generations of *D. magna* exposed to 1 µg/L of polyvinyl alcohol (PVA) and polyethylene glycol (PEG) over all the generations (PEG and PVA treatments), exposed to PVA and PEG only in the parental generations (F_0) (PEGrec and PVArec) and the control organisms (Ctrl). Error bars correspond to standard deviation of the mean values, the letters rapresent statistically differences between the same treatment along the generations. PEGrec analysed thoriught the ANOVA test. Turkey. PEG trhought the Krustkal Wallis test, Dunn

3.2. Global cytosine methylation

The analysis of global cytosine methylation levels revealed a similar percentage of methylated cytosines in the controls over all generations with an average of 0.65% (Fig. 4, Fig S7).
Comparing treatments across the four generations, it is notable that in the PEGrec treatment, where only the parental generation was exposed to PEG, there is a general decrease of methylation in the subsequent generations with a significant ($p<0.05$) hypomethylation in F_2 and F_3 (Fig 4). In contrast, no significant effect was observed in the other treatments. Comparing the global cytosine methylation among the treatments at the initial (F_0) and final generation (F_3), it is interesting to highlight that both PEG treatments in the F_0 generation exhibited significant (χ^2 = df 2, 7.4231, $p<0.05$) higher global methylation levels compared to the controls of the same generation (Fig. 5). In contrast, for both treatments exposed to PVA in the F_0 (PVA and PVArec), no significant variations were observed, although there appears to be a general increase in the global cytosine methylation in F_0, which returns to levels similar to the control in the F_3 generation (Fig. 5).

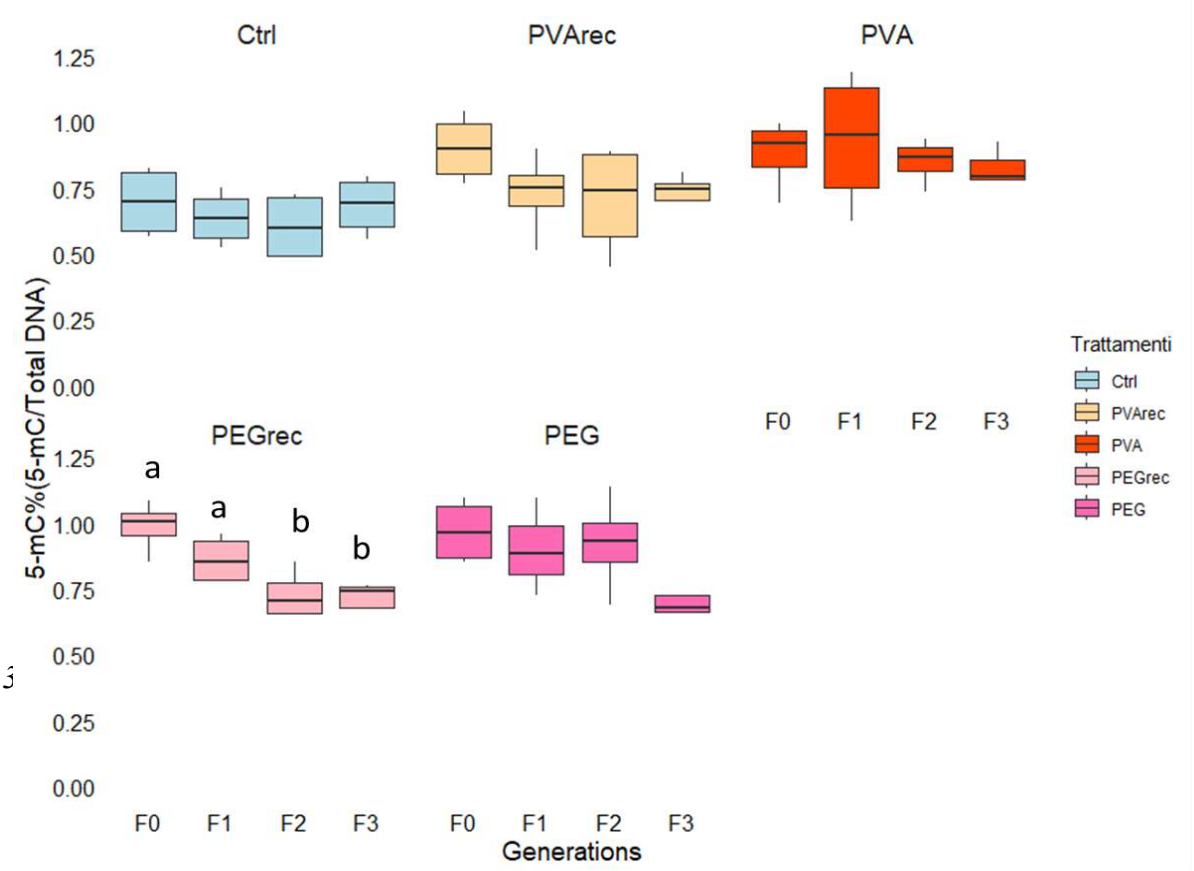


Fig. 4 Total 5-methylcytosine (5-mC) DNA methylation levels in the F_0, F_1, F_2, F_3 generations of *D. magna* exposed to 1 µg/L of polyvinyl alcohol (PVA) and polyethylene glycol (PEG) over all the generations (PEG and PVA treatments), exposed to PVA and PEG only in the parental generations (F_0) (PEGrec and PVArec) and the control organisms (Ctrl). Error bars correspond to standard deviation of the mean values, the letters rapresent statistically differences (one-way ANOVA, $p < 0.05$) of the same treatment along the generations.

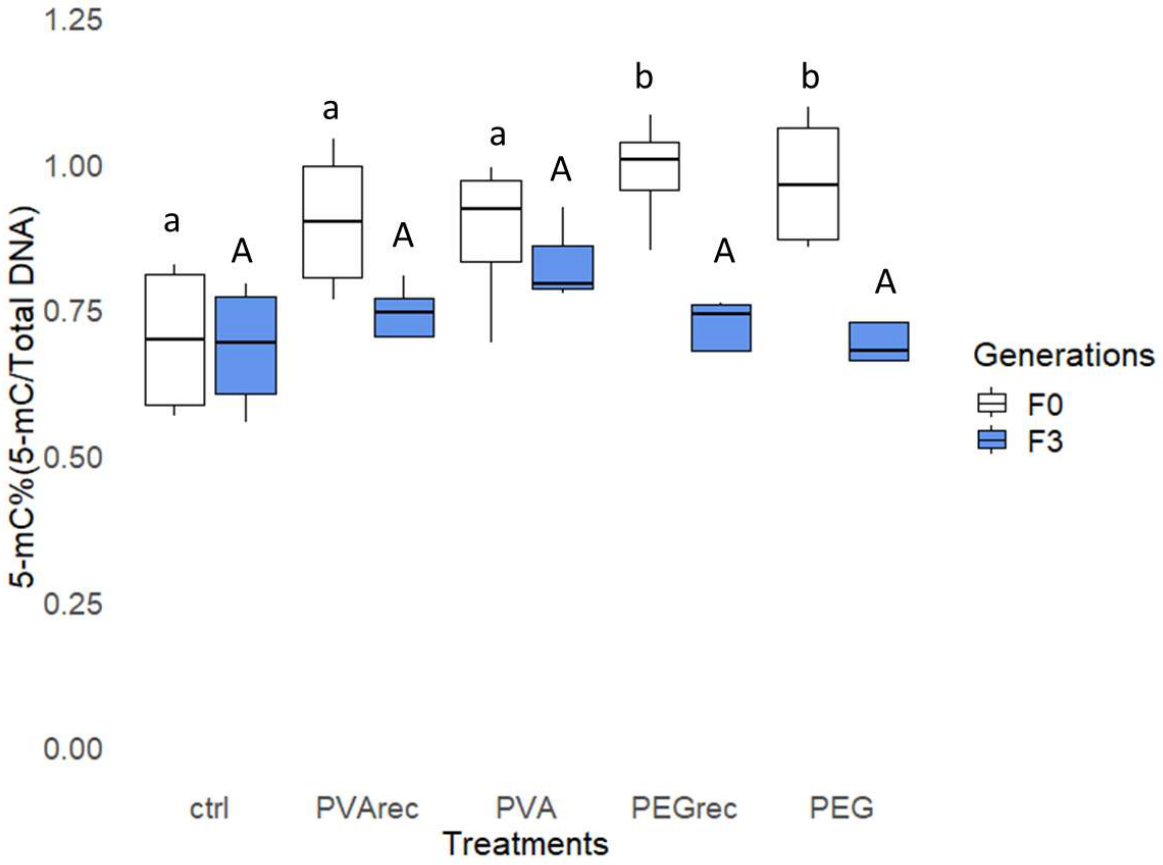


Fig. 5 Total 5-methylcytosine (5-mC) DNA methylation levels in F_0 and F_3 generations of *D. magna* exposed to 1 µg of polyvinyl alcohol (PVA) and polyethylene glycol (PEG) in both generations F_0 and F_3 (PEG and PVA treatments), the treatment exposed to PVA and PEG only in the parental generations (PEGrec and PVArec) and the control organisms (Ctrl). The white color indicate F_0 generation while the blue color indicate the F_3 generation. Error bars correspond to standard deviation of the mean values . The letters represent statistically significant differences (Kruskal-Wallis test, $p < 0.05$) in the sum of juveniles between the treatments and the control for the same generations. Lowercase letters indicate differences for the F_0 generation, while uppercase letters indicate differences for the F_3 generation

Trend of global cytosine Methylation in treatments across the generations

Figure 7 shows the trend of the total 5-mC DNA methylation levels across the four generations for the five treatments. The slopes were obtained by a three-parameter logistic model (Table 2) in which the parameters (b), (d), and (e) represent different aspects of the percentage of the global cytosine methylation in response to the treatments. As we can see, the baseline DNA methylation (Ctrl) is the lowest, while the PEG treatment shows a higher starting value, which was maintained for both F_1 and F_2, with a final decline in F_3, however not significantly different with previous values (Fig. 6; Table 2). The PEGrec treatment exhibits a linear negative trend, as expected, with a starting value exactly overlapping with PEG treatment and a return to baseline values in F_3 (Fig. 6; Table 2). The PVA treatment has a constat trend, with a slightly higher value at F_3 (Fig. 6; Table 2). Finally, the PVArec treatment shows a similar starting value than PEG treatment with a complete recovery of methylation levels at F_3, as stated also for PEGrec (Fig. 6; Table 2).

Moreover, a linear model was fitted to the control group data to assess the relationship between generations and DNA methylation. The model showed no significant relationship between generations and DNA methylation in the controls, suggesting that the observed differences in treated groups are likely due to the treatments themselves rather than generational effects (Table S3). Additionally, statistical analyses (two-way ANOVA; *post-hoc* Tukey's HSD test) provided insights into the differences in the slopes of the global cytosine methylation changes across generations for different treatments (Table 3). The comparison between controls with PEG and PEGrec treatments revealed a statistically significant difference ($p<0.05$) (Table 3). Similarly, the comparison between controls and the PVA treatment group showed a highly significant difference ($p<0.01$) in the global cytosine methylation. Other comparisons between treatments did not yield statistically significant results.

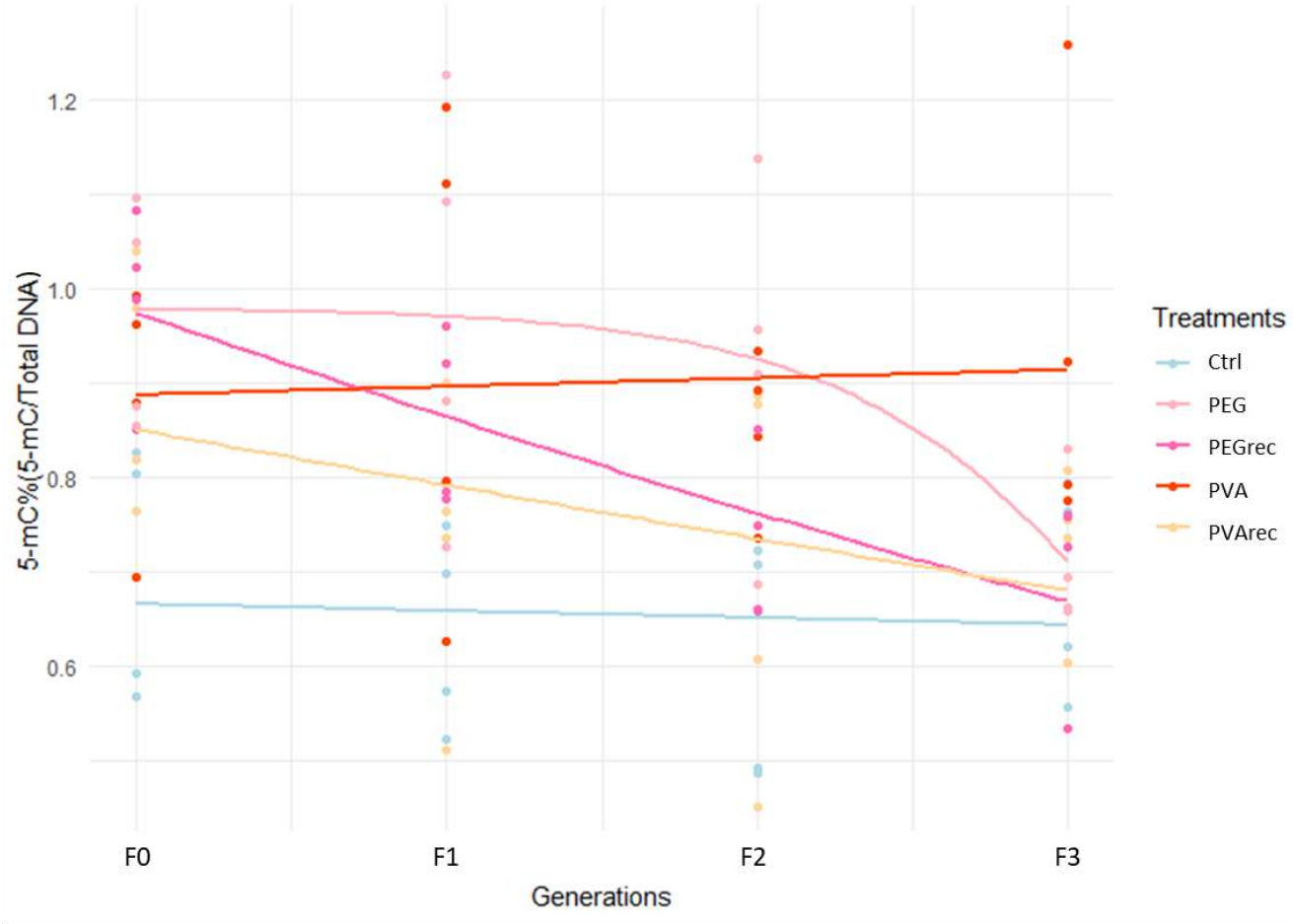


Fig. 6 Trend of the total 5-methylcytosine (5-mC) DNA methylation levels in the F_0, F_1,F_2, F_3 generations of *D. magna* exposed to 1 µg/L of polyvinyl alcohol (PVA) and polyethylene glycol (PEG) over all the generations (PEG and PVA treatments), and exposed to PVA and PEG only in the parental generations (F_0) (PEGrec and PVArec) compared to the control organisms (Ctrl). Data were analyzed using a three-parameter logistic model (LL.3).

Treatment	Estimate	Std. Error	t-value	p-value
Ctrl				
b:(Intercept)	0.08579843	NaN	NaN	NaN
d:(Intercept)	130.974.387	NaN	NaN	NaN
e:(Intercept)	221.509.652	NaN	NaN	NaN
PEG				
b:(Intercept)	0.25523615	0.1752258	14.566.126	0.1689495
d:(Intercept)	251.726.980	30.870.878	0.8154189	0.4295196
e:(Intercept)	0.08478012	0.7213060	0.1175370	0.9082311
PEGrec				
b:(Intercept)	-0.8031440	56.853.153	-0.1412664	0.8898251032
d:(Intercept)	0.9207004	0.2081367	44.235.367	**0.0006872383**
e:(Intercept)	0.0179372	0.4136664	0.0433615	0.9660723183
PVA				
b:(Intercept)	0.6601088	1.202.514	0.5489407	0.5923501
d:(Intercept)	14.080.719	1.638.882	0.8591659	0.4058134
e:(Intercept)	36.859.971	12.767.748	0.2886959	0.7773661
PVArec				
b:(Intercept)	65.404.879	650.401.537	1.005.608	3,33E+05
d:(Intercept)	0.9763203	0.05945979	16.419.839	4,50E-04
e:(Intercept)	46.504.950	0.74148977	6.271.826	2,87E+01

Table 2. Data analyzed using a three-parameter logistic model (LL.3). The parameter (b) is the lower asymptote, (d) is the upper asymptote and (e) is the inflection point. The estimate, standard error (std. error), t-value and p-value were analysed throught R version 4.3.

	diff	lwr	upr	p adj
PEG-Ctrl	2,41E+03	0.0009729443	3,85E+03	**0.0001223**
PEGrec-Ctrl	1,63E+03	0.0001912489	3,06E+03	**0.0184573**
PVA-Ctrl	2,45E+03	0.0010163014	3,89E+03	**0.0000895**
PVArec-Ctrl	1,10E+03	-0.0003368658	2,54E+03	0.2136566
PEGrec-PEG	-7,82E+02	-0.0022184901	6,55E+02	0.5509936
PVA-PEG	4,34E+01	-0.0013934375	1,48E+03	0.9999882
PVArec-PEG	-1,31E+03	-0.0027466048	1,27E+02	0.0907641
PVA-PEGrec	8,25E+02	-0.0006117420	2,26E+03	0.4974526
PVArec-PEGrec	-5,28E+02	-0.0019649093	9,09E+02	0.8409920
PVArec-PVA	-1,35E+03	-0.0027899619	8,36E+01	0.0745111

Table 3. Results of the two-way ANOVA test followed by the Tukey post-hoc test to compare the average5-methylcytosine (5-mC) DNA methylation levels in D. magna exposed to 1 µg/L of polyvinyl alcohol (PVA) and polyethylene glycol (PEG) over all the generations (PEG and PVA treatments), exposed to PVA and PEG only in the parental generations (F_0) (PEGrec and PVArec) and the control organisms (Ctrl). The reported values include the lower (lwr) and upper (upr) confidence interval limits, as well as the adjusted p-values (p adj) for each comparison. The treatments were compared both among themselves and against the control to evaluate significant differences in the measured parameters.

4. Discussion

This study provides significant insights into the multigenerational effects of PVA and PEG on *Daphnia magna*, particularly focusing on life-history parameters and global cytosine methylation. The observed significant reduction *($p<0.05$*) in the number of newborns and in the life history parameters (r, R_0) in the parental generation (F_0) exposed to PEG (PEG and PEGrec) pointed out an adverse effect of PEG on the reproductive capabilities of *D. magna* (Table 1). However, the lack of significant differences in these parameters for both PEG and PEGrec treatments in subsequent generations (F_1, F_2, and F_3) suggested that the effect on reproduction was not multigenerational (Table 1, Fig. 2,3). While such result can be expected for PEGrec treatment, in which this WSP was not administered to *D. magna* specimens after F_0, more controversial is the result obtained in the treatment in which PEG was continuously administered in all generations. This pattern could indicate a compensatory or acclimative reproductive response to initial stress, a phenomenon observed also in other studies where organisms initially exposed to sub-lethal contaminants even exhibited increased reproductive output in subsequent generations. For instance, Vandegehuchte et al. (2009) showed how reproduction in *D. magna* was affected in the daphnids of F_0 exposed to the anti-cancer genistein, but how this effect was not transferred to the F_1 recovery treatments. Furthermore, Vandegehuchte et al. (2010) also showed how the exposure to 388 g/L Zn significantly reduced the *D. magna* reproduction in the F_0 generation without affecting the reproduction of the F_1 and F_2 recovery treatments. One of the hypotheses suggested by the authors was that the induction of vitellogenin as an oxidative stress response in the F_0 generation might lead to an immediate adverse effect on reproduction, but to an over-compensation in the subsequent generations. This could explain also the reproductive changes observed in our study, corroborated by results obtained in our previous research (Nigro et al., 2024) in which an up-regulation of vitellogenin domain-containing protein was observed after the exposure of 1 mg/L PEG to *D. magna,* as well as a shift of energy allocation and a modulation of proteins involved in reproduction.

Examining the long-term effects, it is possible to conclude that the exposure to this polymer led to immediate significant effects on reproduction of *D. magna*, both in continuous and in recovery exposures, but this was followed by a potential recovery mechanism (in the recovery treatments) and acclimation mechanism (in the continuous exposed treatments), which surely requires further investigation into the underlying biological processes. This observation aligns with evidence from Muyssen and Janssen (2004), who showed that *D. magna* could develop increased tolerance to cadmium over several generations, albeit with diminishing benefits over time. Their findings emphasized that while acute and chronic tolerance mechanisms can emerge after short-term exposure, extended exposure often results in trade-offs, including altered energy reserves and reduced

reproduction. This supports the hypothesis that the recovery in reproductive parameters observed in this study could be linked to acclimative plasticity within *D. magna* populations under chronic PEG exposure. Similarly, Im et al. (2020) highlighted multigenerational responses in *D. magna* under thermal stress, where reproductive output and oxidative stress metrics initially showed significant alterations but normalized in later generations (F_6–F_9). This pattern suggests a non-adaptive response in early generations transitioning to acclimation, possibly driven by epigenetic modifications or trade-offs between reproduction and maintenance costs. This aligns with the transgenerational effects observed in this study. Overall, our results indicate a transgenerational plasticity in *D. magna* in both recovery and continuous exposure treatments. However, the observed acclimation to PEG in continuous treatments raises further questions about the associated cost-of-tolerance to future stressors, as proposed by Shaw et al. (2019).

In line with these effects on some life-history parameters, the significant increase *($p<0.05$)* in global cytosine methylation observed in the F_0 generation of both PEG and PEGrec treatments (Fig.5, Fig. 6) suggested an initial epigenetic response to PEG exposure, which diminished over time (Fig.4, 6). This decreasing trend in methylation levels across generations could suggest an epigenetic mechanism underlying the reproductive effects previously described. On the other hand, previous studies have shown how the natural DNA methylation can be altered in response to many environmental stressors (Harris et al., 2012) and how the (re)establishment of DNA methylation marks upon exposure cessation requires a significant increase of energy budget, potentially impairing physiological parameters such as growth, heart rate, reproduction, and lifespan (Jeremias et al., 2022). Other studies supported the role of epigenetic inheritance in trans-generational reproductive effects, particularly through DNA methylation at cytosine residues. For instance, Yu et al. (2021) demonstrated that single maternal NPs exposure (F_0) causes reproductive effects through germline toxicity and these effects are associated with DNA methylation and histone modification. All these evidences are coherent with our findings, where the initial exposure to PEG induced significant methylation changes (Fig. 6), potentially influencing the reproductive functions in the F_0 generation (Table 1). However, the return to methylation levels of controls in subsequent generations suggested that the epigenetic changes induced by PEG in F_0 were not stable enough to be inherited, or that a reprogramming mechanism restored the methylation status in the progeny. This transient nature of methylation changes and their impact on reproduction could be part of the hypotized broader recovery and acclimation mechanism in *D. magna* suggesting transgenerational plasticity to WSPs. The initial stress response in the F_0 generation, characterized by increased methylation and reduced reproductive output, might trigger compensatory mechanisms that normalize these parameters in subsequent generations. It should be highlighted, however, that the overall methylation status gives no

information about the methylation of specific genes or about other epigenetic mechanisms possibly influencing certain genes.

Concerning PVA, despite the treatments not exhibiting significant variations in global cytosine methylation, a different trend can be observed in the treatments always exposed to PVA and their respective recovery treatments (Fig. 6). Additionally, the value of global methylation calculated without considering the generations showed a significant increase in all treatments (except for PVArec; Table 3), suggesting also a possible epigenetic effect of PVA, although less strong than PEG, which certainly should be investigated further in future to define whether this WSP can also actually result in these kinds of DNA modifications.

As final remark, the observed increase in total 5-mC DNA methylation may be explained by changes in DNA methyltransferases activity and/or alterations in the methionine cycle affecting the availability of methyl donors, similar to changes in the methylome caused by other environmental contaminants (Athanasio et al., 2018; Lindeman et al., 2019; Srut, 2021; Huang et al., 2022; Cuiping et al., 2023, Pinto et al., 2024). Changes in DNA methyltransferases activity may be a direct result of oxidative stress that inhibits the expression and biological activity of these enzymes, critical to maintaining existing and establishing *de novo* DNA methylation marks (Donkena et al., 2010; Srut, 2021; Huang et al., 2022).

5. Conclusions

This is the first study conducted to assess possible epigenetic and transgenerational effects due to two of the most widely used WSPs.. The different effects of PEG and PVA on a typical ecotoxicological model as *D. magna* highlight the importance of considering both immediate and long-term impacts of WSPs on aquatic organisms, bearing in mind also the numerous studies that are demonstrating several sub-organism level effects due to these new environmental contaminants. Nevertheless, this work highlights a clear role for transgenerational plasticity in both acclimation and recovery to PEG. Overall, the significant reproductive and epigenetic changes induced by PEG underscore its potential as a more hazardous contaminant compared to PVA. These findings are crucial for environmental risk assessment and management, as they suggest that PEG, even at low concentrations, can have significant effects particularly in the early generations of aquatic organisms, both at the molecular level through epigenetic effects, and at the population level through reproductive-related impact. The possible relationship between these two types of effect detected at very different levels of biological organization is certainly something to be investigated, as the observed epigenetic modifications raise crucial questions about the potential for transgenerational inheritance of stress responses due to WSPs, which could have broader ecological implications. Additionally, a potential cost-of-tolerance

to future stress associated with this increased transgenerational plasticity needs to be further investigated. Further research is needed to elucidate the mechanisms underlying these epigenetic changes and their potential impact on population dynamics and ecosystem traits. Understanding these mechanisms is essential to evaluate the hazard of WSPs to the aquatic biocenosis and eventually to develop effective strategies to mitigate their ecological risks.

H I G H L I G H T S

• *D. magna* was exposed for subsequent generations (F_0-F_3) to two different water-soluble polymers (WSPs).

• Transgenerational effects on reproduction and methylation were applied to evaluate the WSP toxicity.

• PEG induced effects at F_0 generation on both reproduction and methylation.

• Further investigations on the risk assessment of these WSPs are needed.

CRediT authorship contribution statement

Andrea Binelli: Writing – Supervision, Software, Funding acquisition, Conceptualization.

Lara Nigro: Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Iene Herman: Methodology - Formal analysis

Jana Asselman: Writing – review & editing, Methodology, Formal analysis, Project administration, Supervision, Software, Funding acquisition, Conceptualization.

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

Acknowledgements We thank Blue Growth Research Lab for the opportunity to work there with the ERASMUS traineeship grant assigned to Lara Nigro by Department of Biosciences of the University of Milan. An acknowledgment also to Dr. Luca Ihlaiane, Dr. Corrado Alessandrini and Dr. Susan Helen Mckinlay, for the help with statistical analyses.

Supporting information

	conc. In stock (g/L)	mL/L medium
Majors	Allemaal afzonderlijk	
NaHCO3	12,6	1
NaSiO3.5H2O	28,42	1
MgSO4.7H2O	55,45	1
CaCl.2H2O	55,14	1
H3BO3	1	1
KCl	7,45	1
Fe + EDTA		1
Na2EDTA.2H2O	4,36	
FeCl3.6H2O	0,77	
Animate trace		1
LiCl	0,31	
KI	0,0033	
NaBr	0,016	
RbCl	0,07	
SrCl2.6H2O	0,15	
Vitamin stock		1
B12	0,00055	
Biotin	0,0005	
Thiamin	0,1	
Algal trace		1
MnCl2.4H2O	0,18	
CuSO4.5H2O	0,001	
ZnSO4.7H2O	0,022	
CoCl2.6H2O	0,012	
NaMoO4.2H2O	0,022	
H2SeO3	0,0016	
Na3VO3	0,0018	

Table S1. COMBO medium formula

Error.Source	Df	Sum.Sq	Mean.Sq	F.value	Pr..F.
gen					
Residuals	3	88912	29637		
gen:day					
day	1	1441675	1441675	63.67	0.00411
Residuals	3	67933	22644		
Within					
trat	4	197758	49439	1.450	0.223024
trat:day	4	687501	171875	5.039	0.000942
Residuals	104	3547108	34107		

Effect	Chisq	Df	Pr(>Chisq)
(Intercept)	19.787	1	0.159526
trat	91.084	4	0.058446
day	12.985	1	0.254479
trat:day	204.174	4	0.000413

Table S2 This table presents the results of the ANOVA and Linear Mixed Model (LMM) analysis for the response variable daily number of juveniles between all the treatments. The ANOVA section shows the degrees of freedom (Df), sum of squares (Sum Sq), mean squares (Mean Sq), F value (F value), and p-value (Pr(>F)) for the effects of generation (gen), day (day), and treatment (trat), as well as their interactions. LMM Analysis: The LMM section reports the chi-square values (Chisq), degrees of freedom (Df), and p-values (Pr(>Chisq)) for the fixed effects in the model.

treatments	generations	DNA
Ctrl :16	00:20	Min. :0.4521
PVArec:16	01:20	1st Qu.:0.6944
PVA :16	02:20	Median :0.7888
PEGrec:16	03:20	Mean :0.8067
PEG :16		3rd Qu.:0.9121
		Max. :1.2587

Table S3 The linear model fitted to the control group data likely used the generations variable as the predictor and DNA as the response variable. The summary shows that the control group ("Ctrl") has 16 observations, with DNA concentrations ranging from 0.4521 to 1.2587.

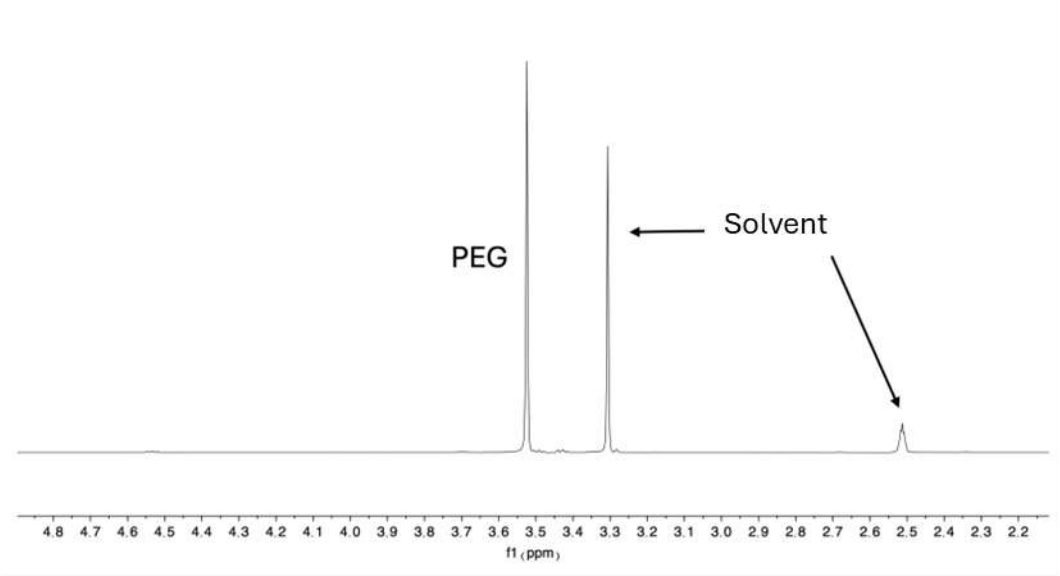


Fig. S1 The nominal concentration of PEG stock solution. It was conducted twice, at the initial time point (T0) and at time point T17, corresponding to the duration of exposure for each generation. The amount of recovered PEG was 9.93 mg/L. The chemical structure of the recovered PEG was confirmed through 1H NMR analysis.

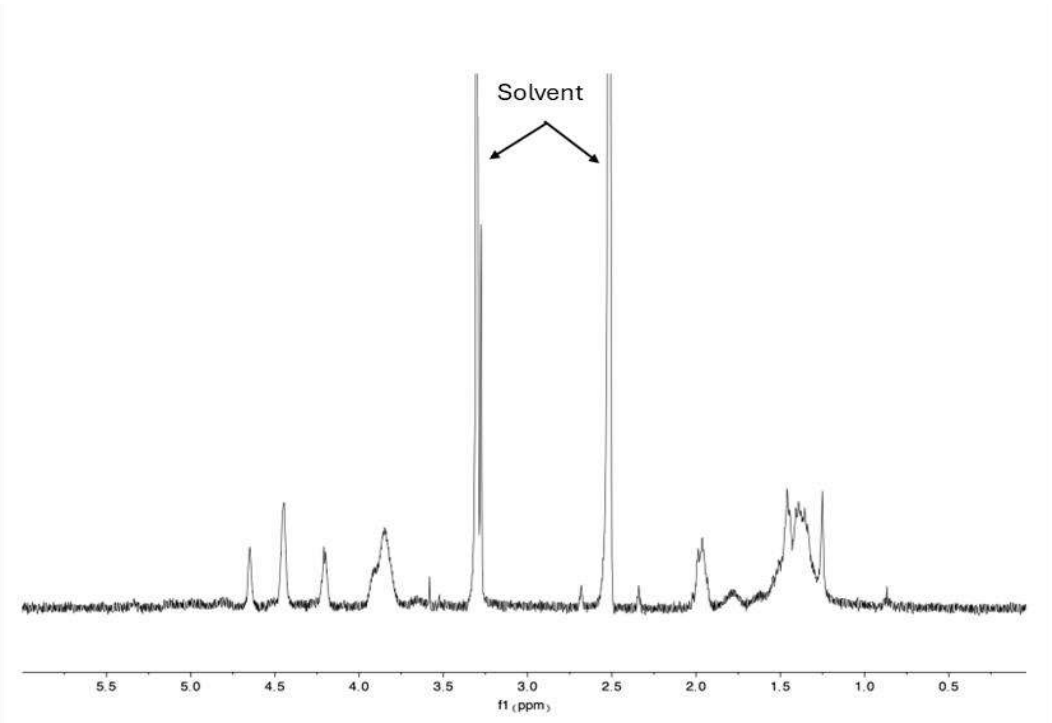


Fig. S2 The nominal concentration of PVA stock solution. It was conducted twice, at the initial time point (T0) and at time point T17, corresponding to the duration of exposure for each generation. The amount of recovered PVA was found to be 9.72 mg. The chemical structure of the recovered PVA was confirmed through 1H NMR analysis

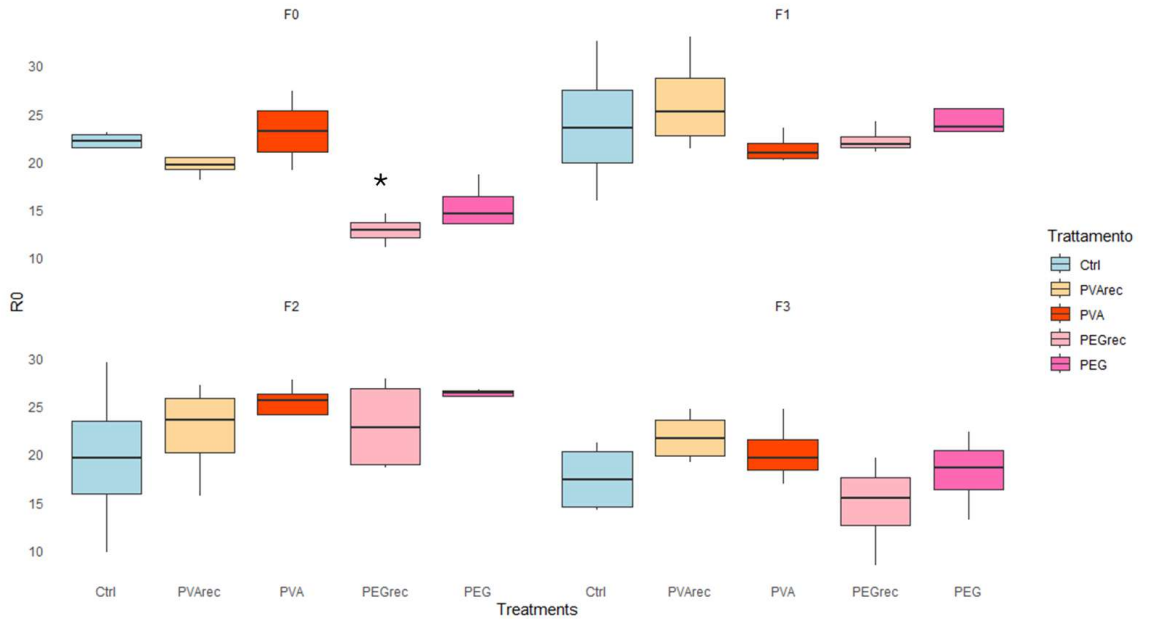


Fig. S3 net reproduction rate in the F0, F1,F2, F3 generations of *D. magna* exposed to 1 µg/L of polyvinyl alcohol (PVA) and polyethylene glycol (PEG) over all the generations (PEG and PVA treatments), exposed to PVA and PEG only in the parental generations (F0) (PEGrec and PVArec) and the control organisms (Ctrl). Error bars correspond to standard deviation of the mean values,* rapresent statistically differences (one-way ANOVA/ Krustkal Wallis test, $p < 0.05$) between the exposed and control organisms of the same generation.

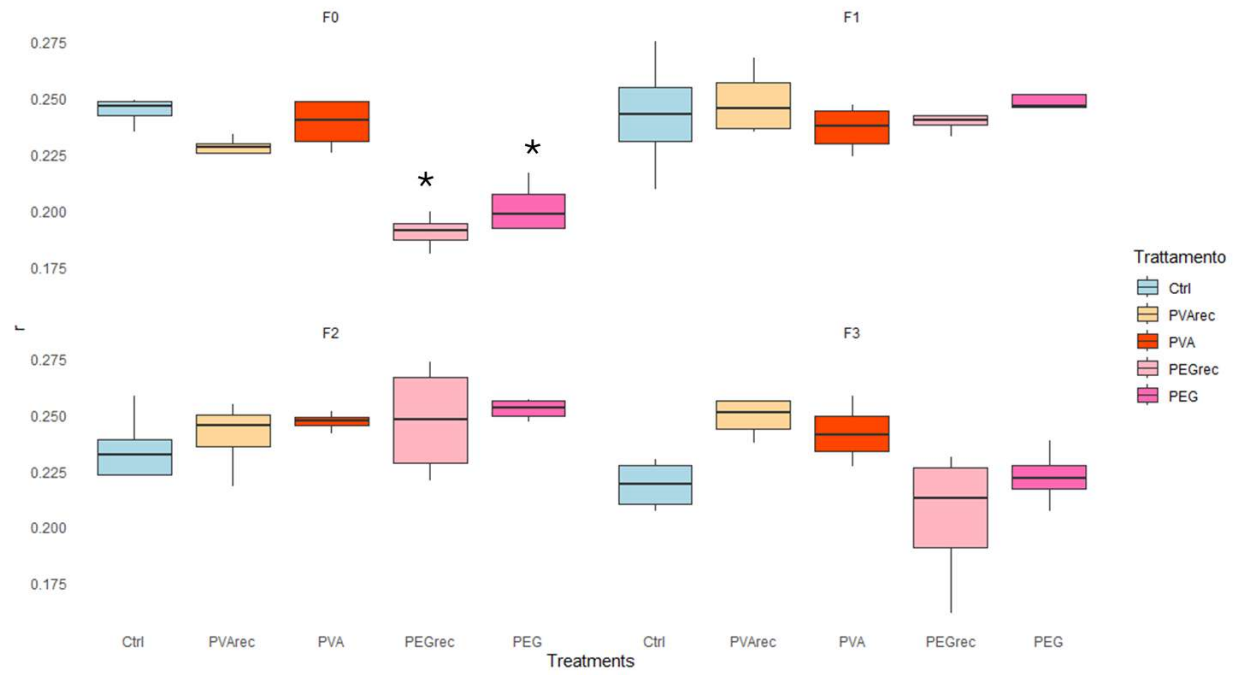


Fig. S4 Per capita rate of population increase (r) derived from the Euler-Lotka equation in the F0, F1,F2, F3 generations of *D. magna* exposed to 1 µg/L of polyvinyl alcohol (PVA) and polyethylene glycol (PEG) over all the generations (PEG and PVA treatments), exposed to PVA and PEG only in the parental generations (F0) (PEGrec and PVArec) and the control organisms (Ctrl). Error bars correspond to standard deviation of the mean values,* rapresent statistically differences (Krustkal Wallis test, $p < 0.05$) between the exposed and control organisms of the same generation.

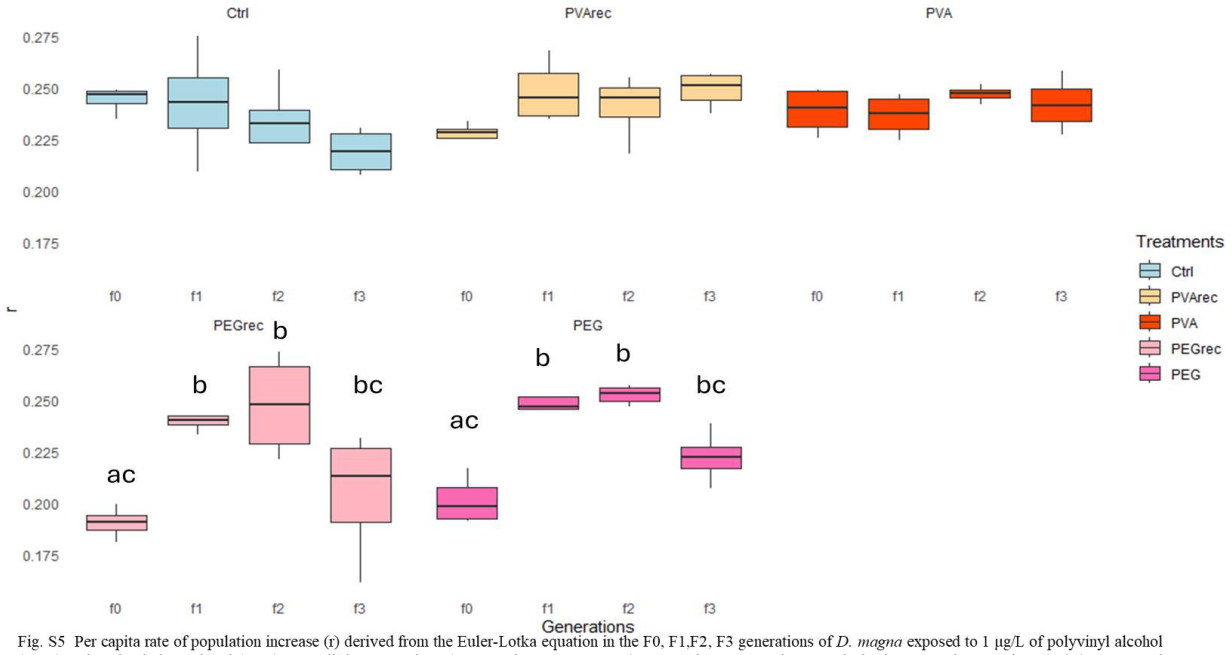


Fig. S5 Per capita rate of population increase (r) derived from the Euler-Lotka equation in the F0, F1,F2, F3 generations of *D. magna* exposed to 1 µg/L of polyvinyl alcohol (PVA) and polyethylene glycol (PEG) over all the generations (PEG and PVA treatments), exposed to PVA and PEG only in the parental generations (F0) (PEGrec and PVArec) and the control organisms (Ctrl). Error bars correspond to standard deviation of the mean values,* rapresent statistically differences (Krustkal Wallis test, $p < 0.05$) between the exposed and control organisms of the same generation.

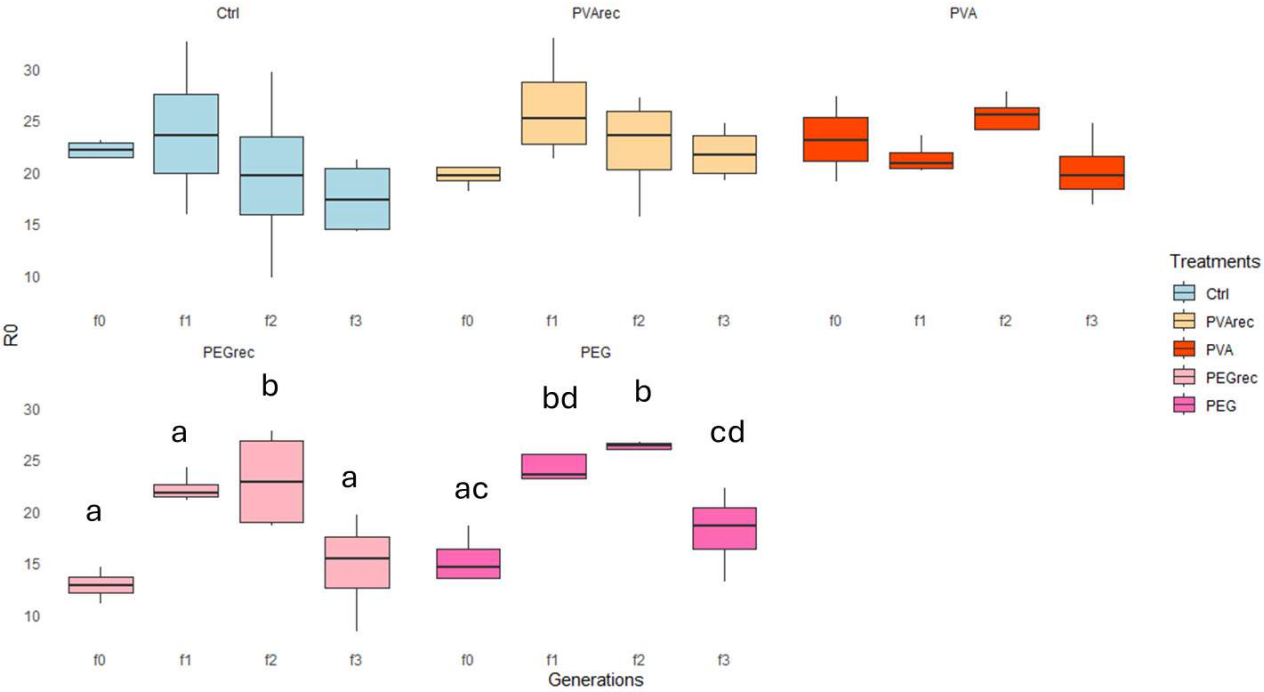


Fig. S6 net reproduction rate in the F0, F1,F2, F3 generations of *D. magna* exposed to 1 µg/L of polyvinyl alcohol (PVA) and polyethylene glycol (PEG) over all the generations (PEG and PVA treatments), exposed to PVA and PEG only in the parental generations (F0) (PEGrec and PVArec) and the control organisms (Ctrl). Error bars correspond to standard deviation of the mean values,* rapresent statistically differences (one-way ANOVA/ Krustkal Wallis test, $p < 0.05$) between the exposed and control organisms of the same generation.

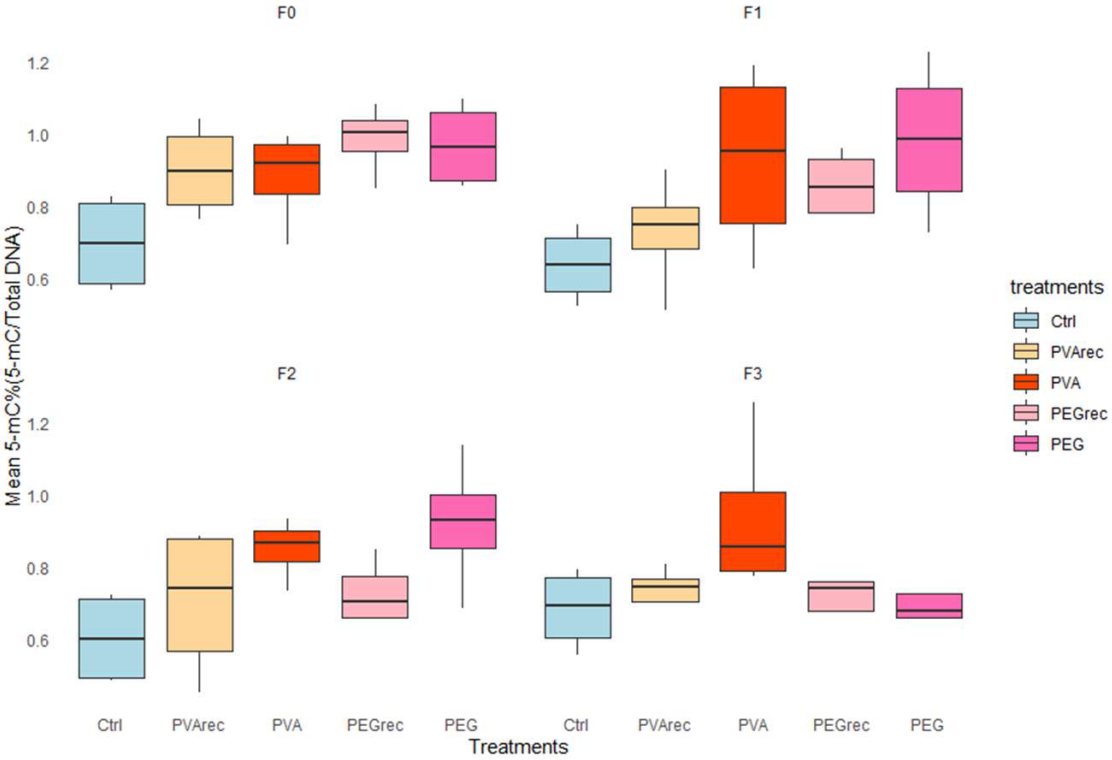


Fig S7 Total 5-methylcytosine (5-mC) DNA methylation levels in the F0, F1,F2, F3 generations of *D. magna* exposed to 1 µg/L of polyvinyl alcohol (PVA) and polyethylene glycol (PEG) over all the generations (PEG and PVA treatments), exposed to PVA and PEG only in the parental generations (F0) (PEGrec and PVArec) and the control organisms (Ctrl). Error bars correspond to standard deviation of the mean values.

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