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

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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 hours 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.

Keywords: *water-soluble polymers*; *freshwaters*, *behaviour*, *neurotoxicity*

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 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 hours 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 x 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.

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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 *per* second (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; Gonzàlez et a., 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).

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 x 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 *per* second (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 (Figure 1A and B, respectively) are characterized by a peak centred at 1.96 ppm, which can be attributed to the methyl group (Figure 1A and B, in red) of the 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® (Figure 1A), while it is well visible in the PVA-based bag (Figure 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 Figure 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 Figure 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 Figure 2A, no peaks are detectable in the Mowiol® extract, while three main peaks are detectable in the chromatogram of the PVA-based bag (Figure 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 Figure 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 ≥ 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 ≥ 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 (Figures 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 (Figure 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 (Figure 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 (Figures 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 (Figure 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 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 (Figures 3 C and D). Interestingly, zebrafish specimens did not show the natural freezing 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 (Figure 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 (Figure 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 (Figure 3C and D). To deeply investigate the effects of selected 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 (Figure 6). In detail, *D. magna* showed an increase of MAO activity at 1 μg/L and 1 mg/L of PVA powder (Figure 6A), while zebrafish at 1 μg/L of PVA powder (Figure 6C) and at all tested concentrations of PVA-based bag (Figure 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.

5 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.

6 AUTHOR STATEMENT

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

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Captions

Figure 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.

Figure 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).

Figure 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.

Figure 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.

Figure 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.

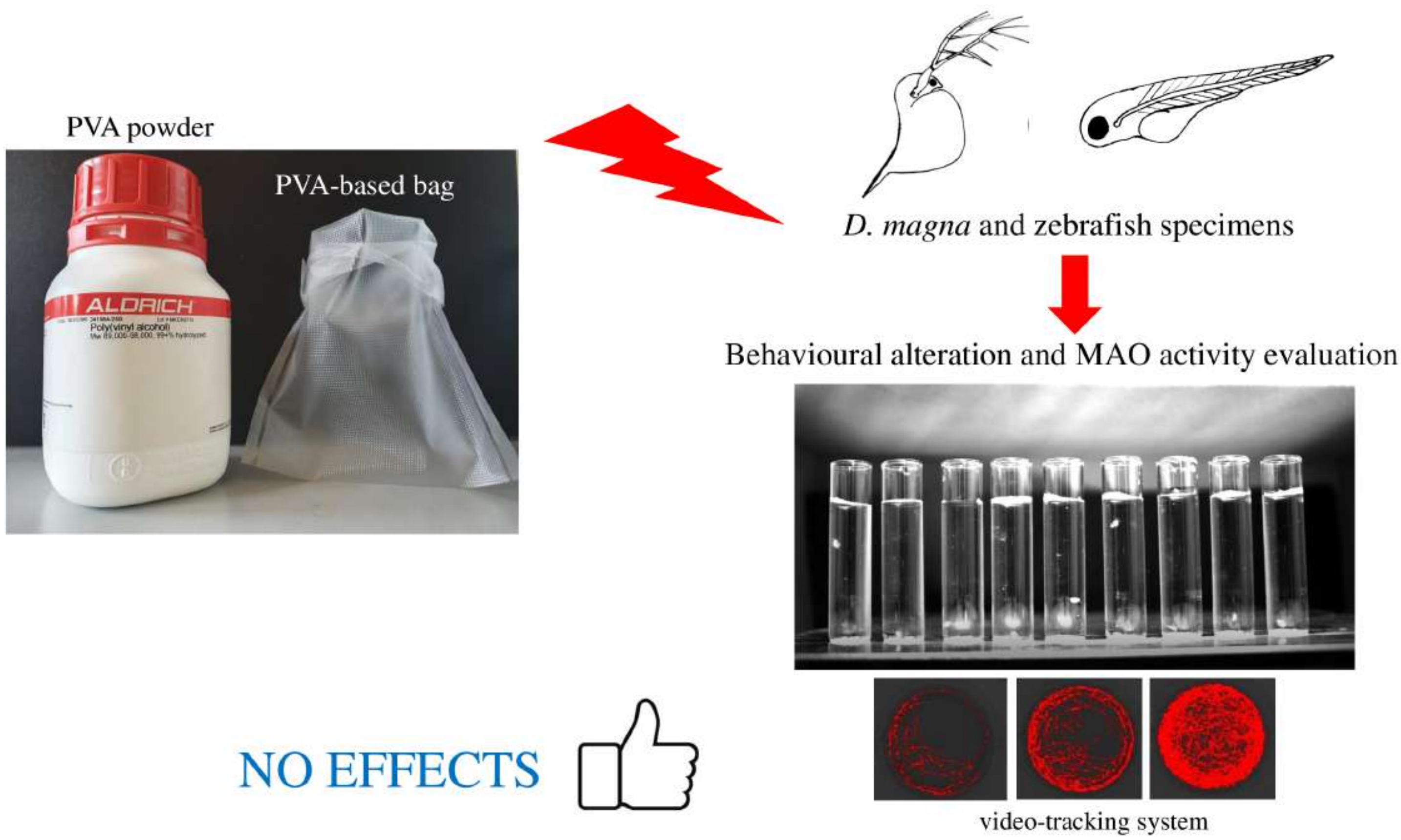
Figure 6: MAO activity (mean ± standard deviation; SD) in *D. magna* (A, B) and zebrafish embryos (C, D) at the end of exposure (14 days and 120 hpf, respectively) to PVA and PVA-based bag (for *D. magna*: exposure in triplicate with 4 beakers for each treatment - 5 specimens *per* beakers, n = 3 pools 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.

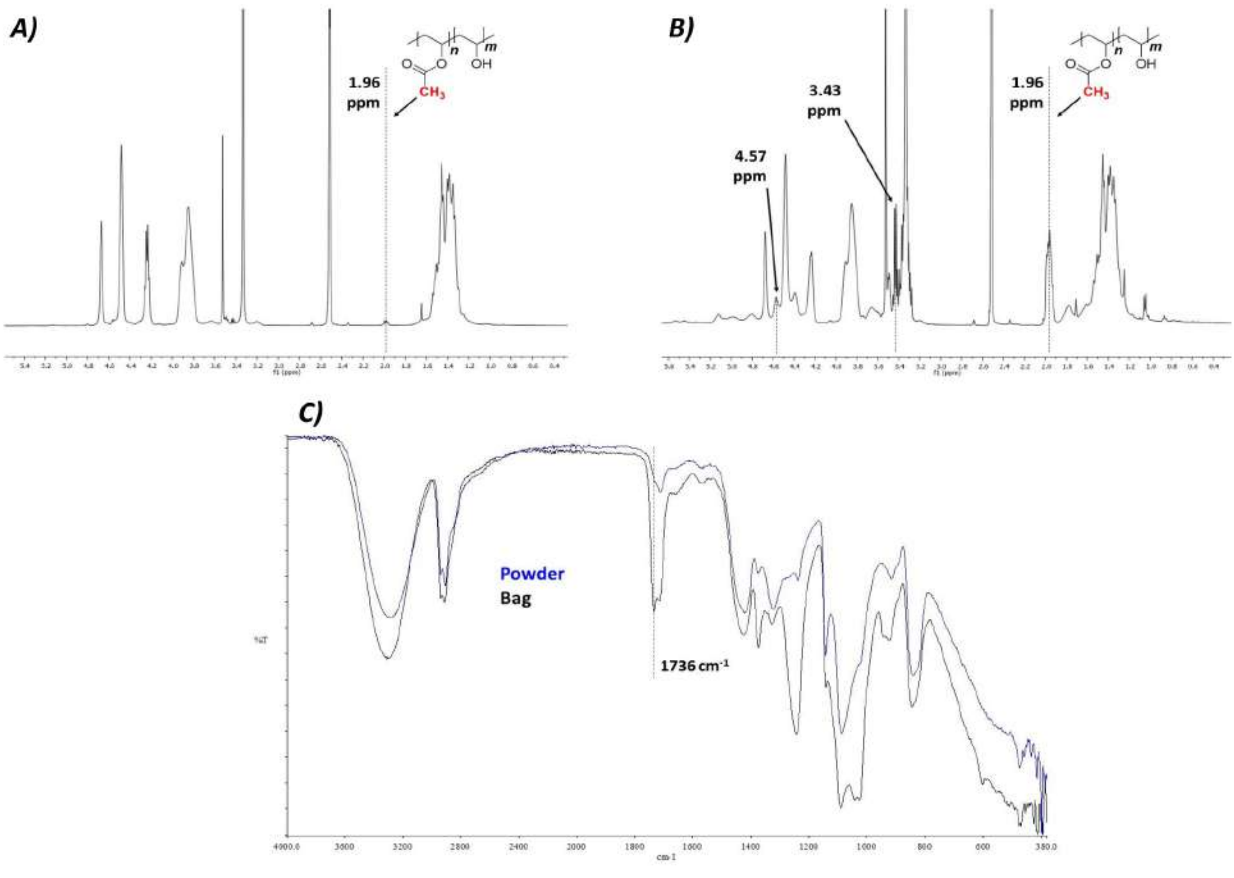
-Water-soluble polymers represent an overlooked global issue

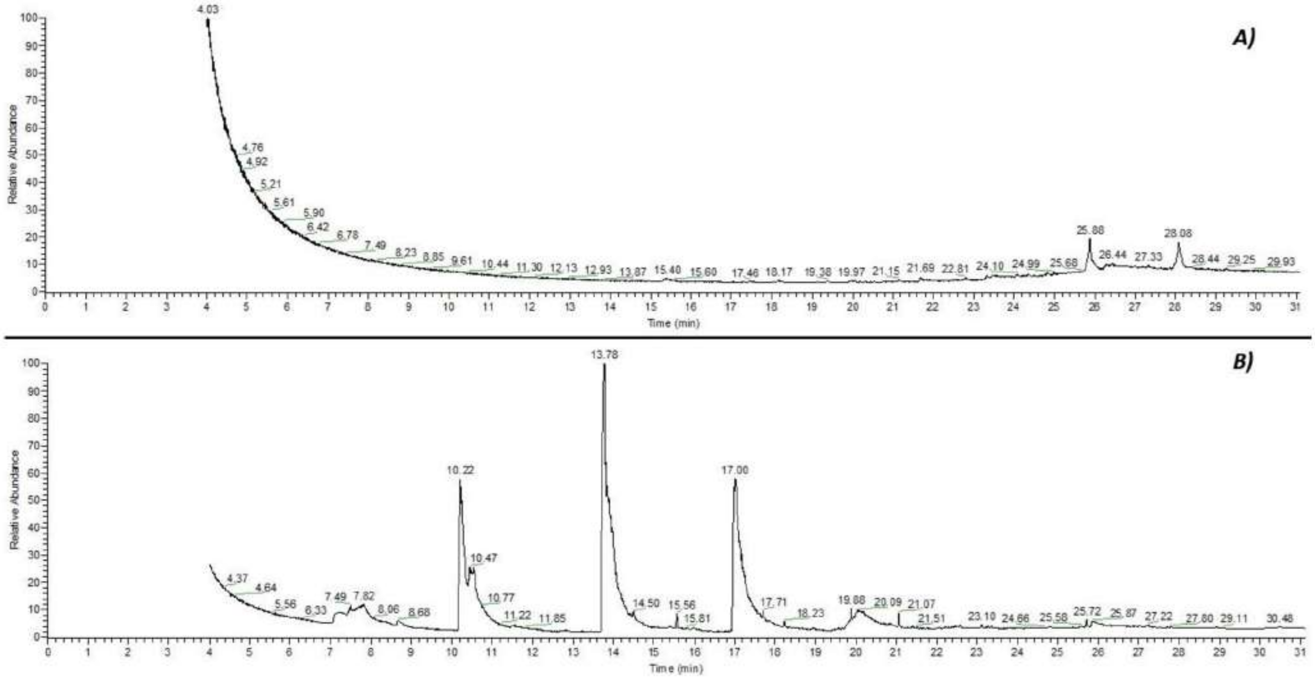
-The effects of polyvinyl alcohol were investigated on freshwater organisms

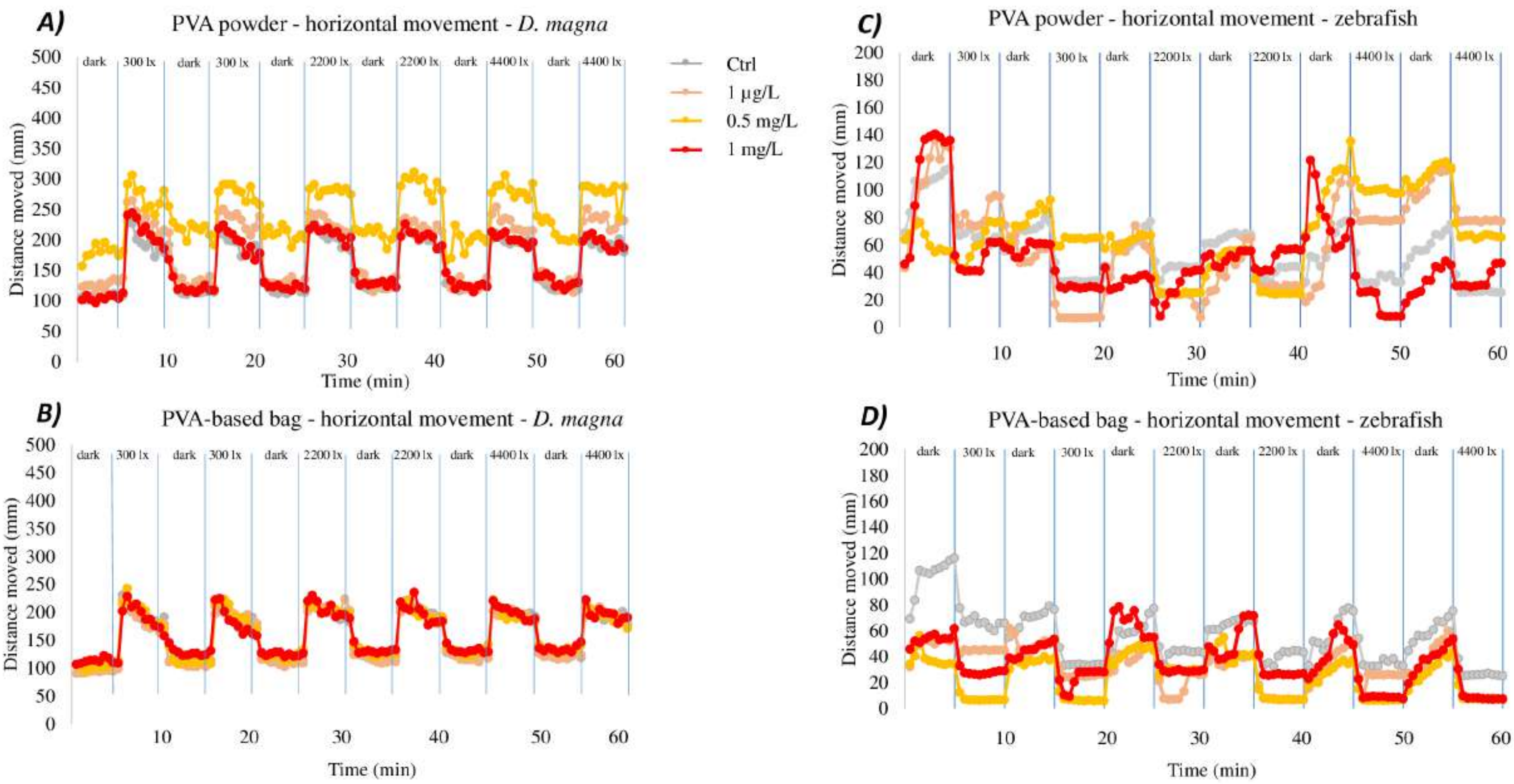
-Chronic toxicity was evaluated through behavioural and neurotoxicity biomarkers

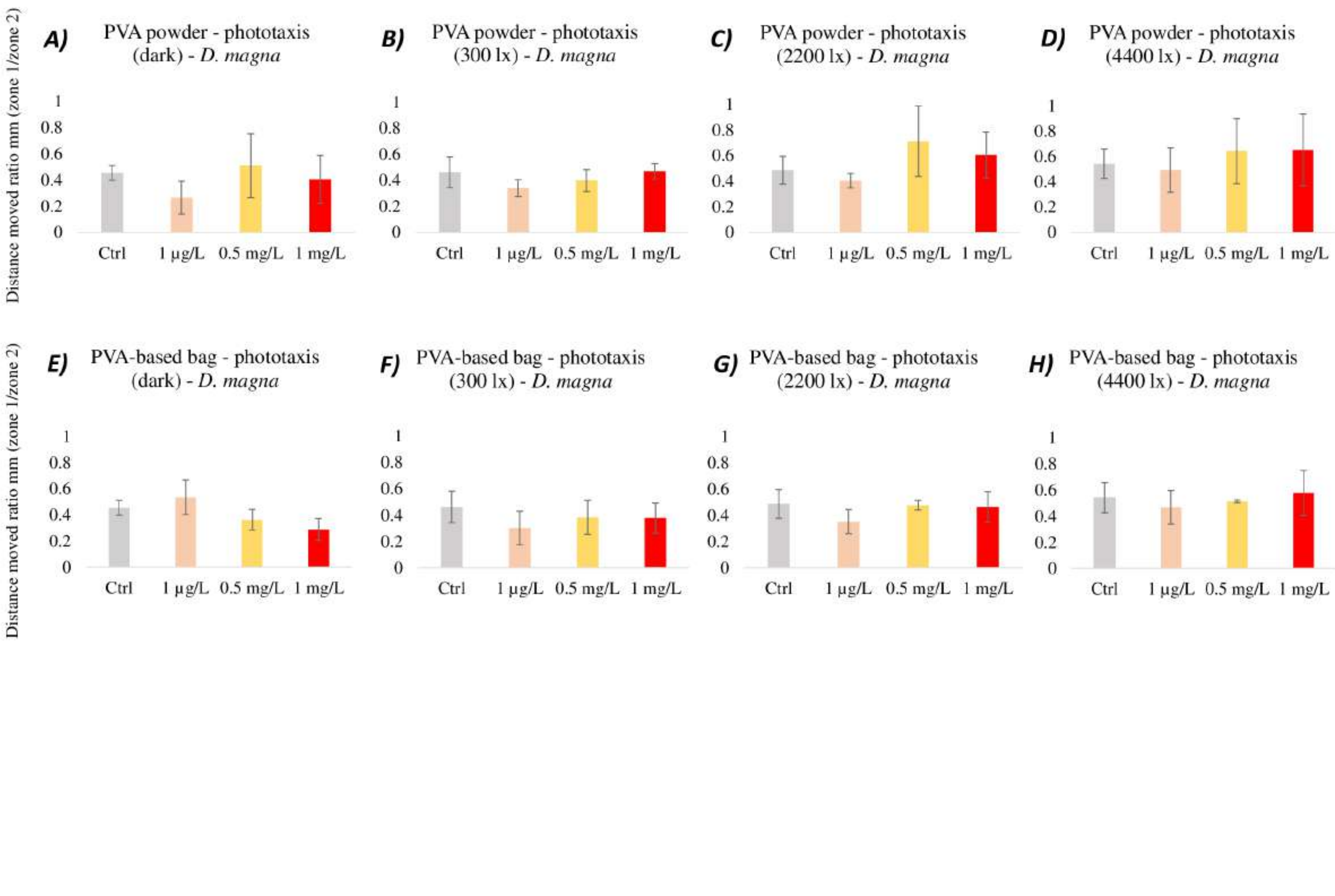
-Polyvinyl alcohol did not induce significant effects on exposed organisms


PVA powder
PVA-based bag
ALDRICH
Poly(vinyl alcohol)
D. magna and zebrafish specimens
Behavioural alteration and MAO activity evaluation
NO EFFECTS
video-tracking system

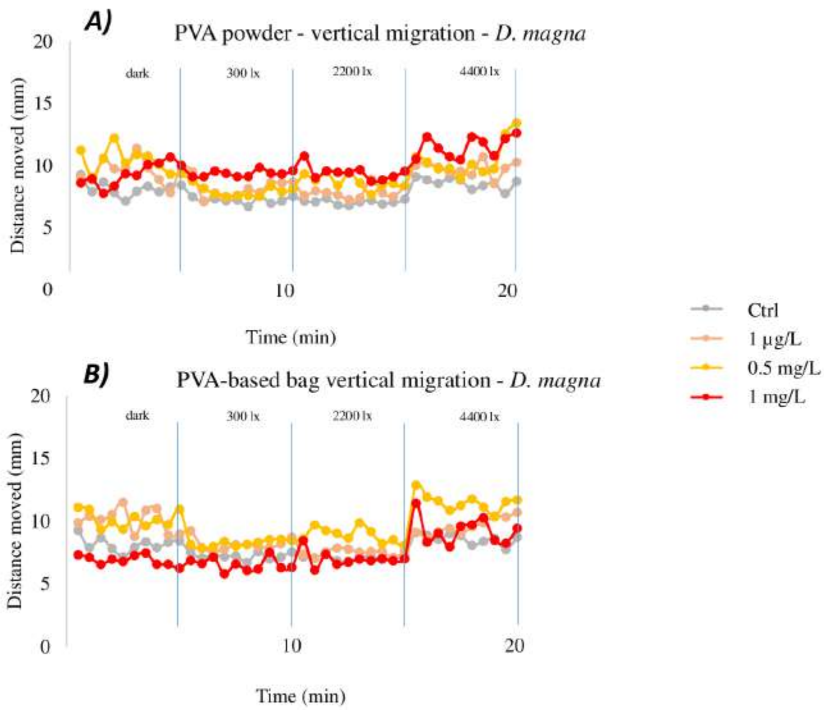

A)
1.96
ppm
n
m
OH
CH3
B)
3.43
ppm
4.57
ppm
1.96
ppm
n
m
OH
CH3
C)
Powder
Bag
%T
1736 cm-1
cm-1
4000.0
380.0

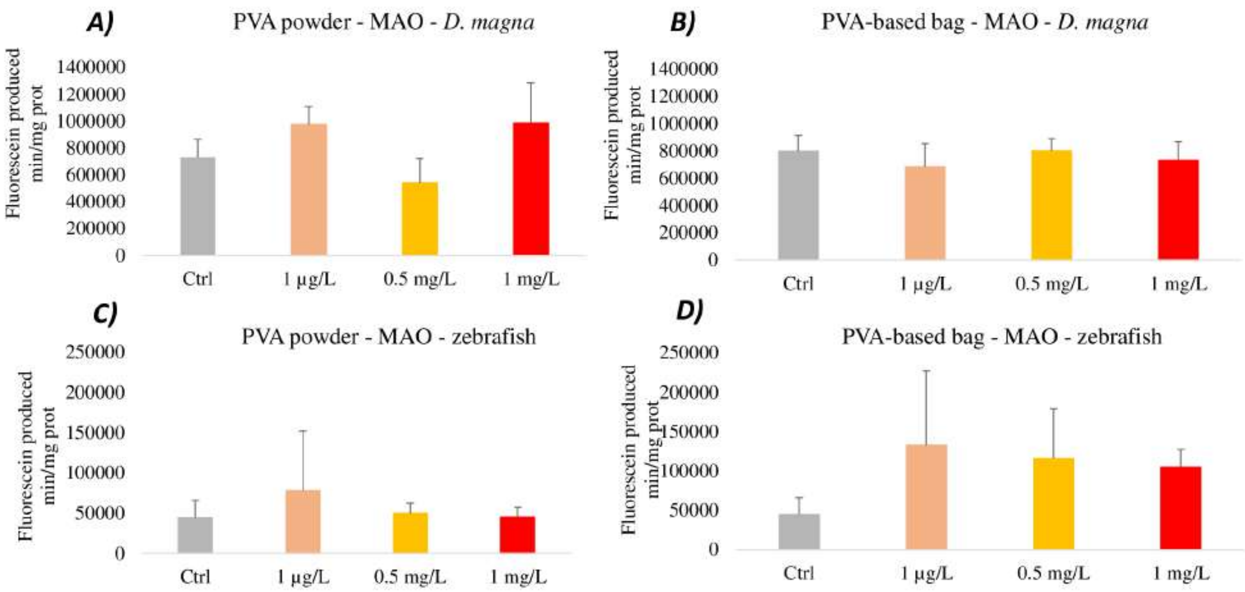

A)
B)
Relative Abundance
Time (min)
4.03
4,76
4,92
5,21
5,61
5,90
6,42
6,78
7,49
8,23
8,85
9,61
10,44
11,30
12,13
12,93
13,87
15,40
15,60
17,46
18,17
19,38
19,97
21,15
21,69
22,81
24,10
24,99
25,68
25.88
26,44
27,33
28.08
28,44
29,25
29,93
13.78
10.22
10,47
17.00
4,37
4,64
5,56
6,33
7,49
7,82
8,06
8,68
10,77
11,22
11,85
14,50
15,56
15,81
17,71
18,23
19,88
20,09
21,07
21,51
23,10
24,66
25,58
25,72
25,87
27,22
27,80
29,11
30,48


A)
PVA powder - horizontal movement - D. magna
B)
PVA-based bag - horizontal movement - D. magna
C)
PVA powder - horizontal movement - zebrafish
D)
PVA-based bag - horizontal movement - zebrafish
Ctrl
1 µg/L
0.5 mg/L
1 mg/L
dark
300 lx
2200 lx
4400 lx
Distance moved (mm)
Time (min)



Distance moved ratio mm (zone 1/zone 2)
A) PVA powder - phototaxis (dark) - D. magna
B) PVA powder - phototaxis (300 lx) - D. magna
C) PVA powder - phototaxis (2200 lx) - D. magna
D) PVA powder - phototaxis (4400 lx) - D. magna
E) PVA-based bag - phototaxis (dark) - D. magna
F) PVA-based bag - phototaxis (300 lx) - D. magna
G) PVA-based bag - phototaxis (2200 lx) - D. magna
H) PVA-based bag - phototaxis (4400 lx) - D. magna
0
0.2
0.4
0.6
0.8
1
Ctrl
1 µg/L
0.5 mg/L
1 mg/L


A)
PVA powder - vertical migration - D. magna
Distance moved (mm)
dark
300 lx
2200 lx
4400 lx
Time (min)
B)
PVA-based bag vertical migration - D. magna
Distance moved (mm)
dark
300 lx
2200 lx
4400 lx
Time (min)
Ctrl
1 µg/L
0.5 mg/L
1 mg/L



A)
PVA powder - MAO - D. magna
Fluorescein produced min/mg prot
1400000
1200000
1000000
800000
600000
400000
200000
0
Ctrl
1 µg/L
0.5 mg/L
1 mg/L
B)
PVA-based bag - MAO - D. magna
Fluorescein produced min/mg prot
1400000
1200000
1000000
800000
600000
400000
200000
0
Ctrl
1 µg/L
0.5 mg/L
1 mg/L
C)
PVA powder - MAO - zebrafish
Fluorescein produced min/mg prot
250000
200000
150000
100000
50000
0
Ctrl
1 µg/L
0.5 mg/L
1 mg/L
D)
PVA-based bag - MAO - zebrafish
Fluorescein produced min/mg prot
250000
200000
150000
100000
50000
0
Ctrl
1 µg/L
0.5 mg/L
1 mg/L

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

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