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Transgenerational effects of water-soluble polymers on *Daphnia magna* at environmentally relevant concentrations: The role of multigenerational plasticity

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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.

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 (Huppertsberg 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). In addition, very little data exist regarding their environmental concentrations, as their analytical determination is by no means simple and standardized methods are completely lacking (ECETOC, 2020). The only available environmental data refer to a recent study (Sainju et al., 2023) that found polyethylene glycol (PEG) concentrations between 2.1 μg/L

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and 33.5 μg/L in some UK water courses, while Wang et al. (2021) found PEG concentrations of 21.9 μg/L in fresh falling snow in Montreal (Canada). Levels ranging from 0.9 mg/L to 7.1 mg/L of polyvinylpyrrolidone (PVP) have been detected in the municipal wastewater treatment plants (WWTPs) of Aachen and Duren (Germany), and in the industrial wastewater canal of Pancevo (Serbia), as well as a concentration of 0.1 mg/L was detected in the Rur River (Germany) near municipal WWTP of Duren (Antic et al., 2011).

Among the wide class of WSPs, PEG and polyvinyl alcohol (PVA) are particularly outstanding due to their solubility and widespread use (Falqi et al., 2018, Mohammadi and Babaei, 2022; D'souza and Shegokar, 2016, Özdemir 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 and Shegokar, 2016, Özdemir 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; Özdemir 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., 2022, 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 concentration of 1 μg/L, with exposure spanning up to the F_3 generation. This concentration was selected for two reasons: first, it is comparable to or lower than the levels of WSPs detected in various aquatic environments, as described above; and second, it represents the lowest concentration used in previous experiments, where we observed adverse effects on the two biological models *Daphnia magna* and *Danio rerio* (Nigro et al., 2022, 2023, 2024; Binelli et al., 2024). Given that PEG and PVA are WSPs widely used in the medical field, this conservative choice was deemed the most appropriate to prevent effects arising solely from excessively high concentrations that lack ecological relevance.

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 (Mn) 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 (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 Figs. S1 and 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 h 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 h 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 (OECD, 2012). 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.

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. The supernatant was then carefully transferred into a new tube, leaving the debris and organic layer behind. 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

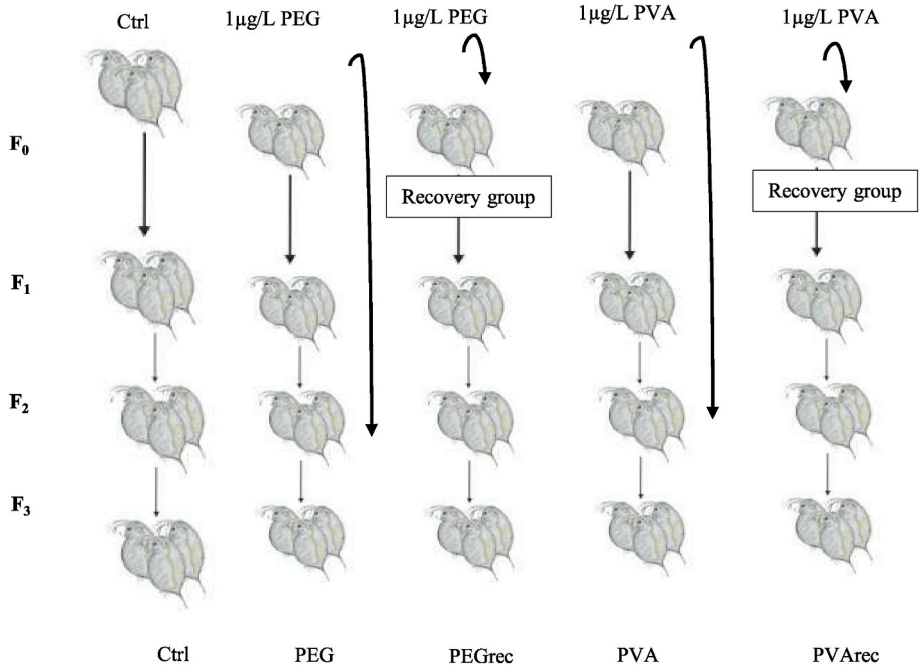

Fig. 1. Experimental design of the transgenerational experiment. *D. magna* was exposed to 1 µg/L of polyvinyl alcohol (PVA) and polyethylene glycol (PEG) for all the subsequent generations F_0, F_1, F_2 and F_3 in the "PEG" and "PVA" treatments. While the treatment exposed to PVA and PEG only in the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

(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), the net reproductive rate (R_0), and the per capita rate of population increase (r) 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 and 3) and the life-history parameters between the treatments (AFR, R_0 and r, Table 1). 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 (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, Figs. S3 and 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; Figs. S5 and 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).

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

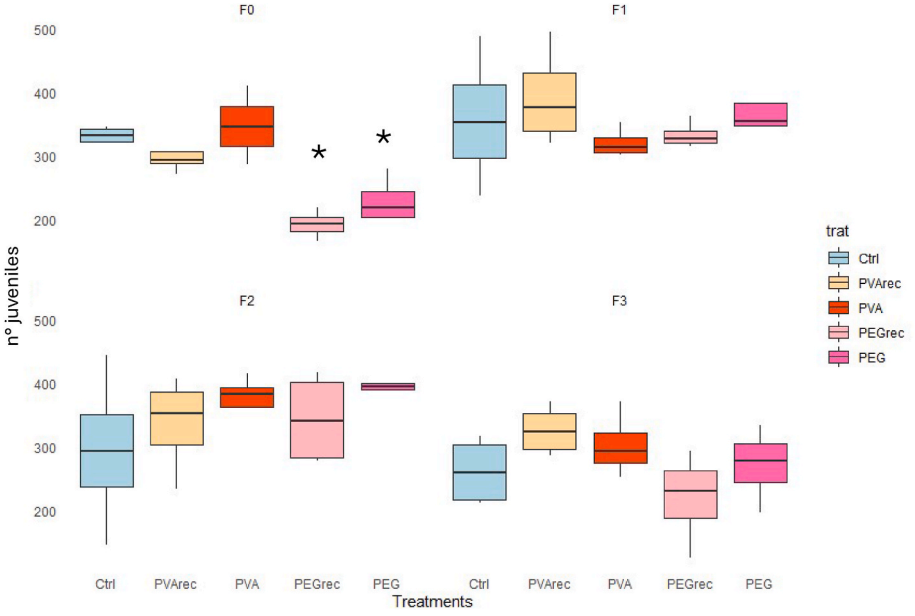

Fig. 2. Sum of the juveniles in the F_0, F_1,F_2, F_3 generations of *D. magna* exposed to 1 μg/L of polyvinyl alcohol (PVA) and polyethylene glycol (PEG) over all the generations (PEG and PVA treatments), exposed to PVA and PEG only in the parental generations (F_0) (PEGrec and PVArec) and the control 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.

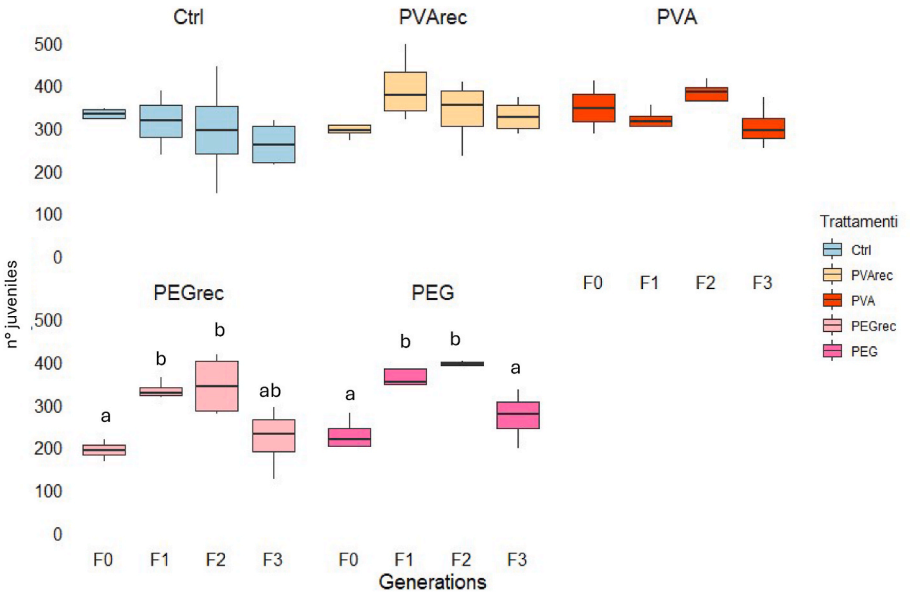

Fig. 3. Sum of the juveniles in the F_0, F_1,F_2, F_3 generations of *D. magna* exposed to 1 μg/L of polyvinyl alcohol (PVA) and polyethylene glycol (PEG) over all the generations (PEG and PVA treatments), exposed to PVA and PEG only in the parental generations (F_0) (PEGrec and PVArec) and the control 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 analyzed thoriught the ANOVA test. Turkey. PEG trhought the Krustkal Wallis test, Dunn.

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

	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**

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 ($\chi^2 =$ 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).

3.3. Trend of global cytosine methylation in treatments across the generations

Fig. 6 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 is 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.

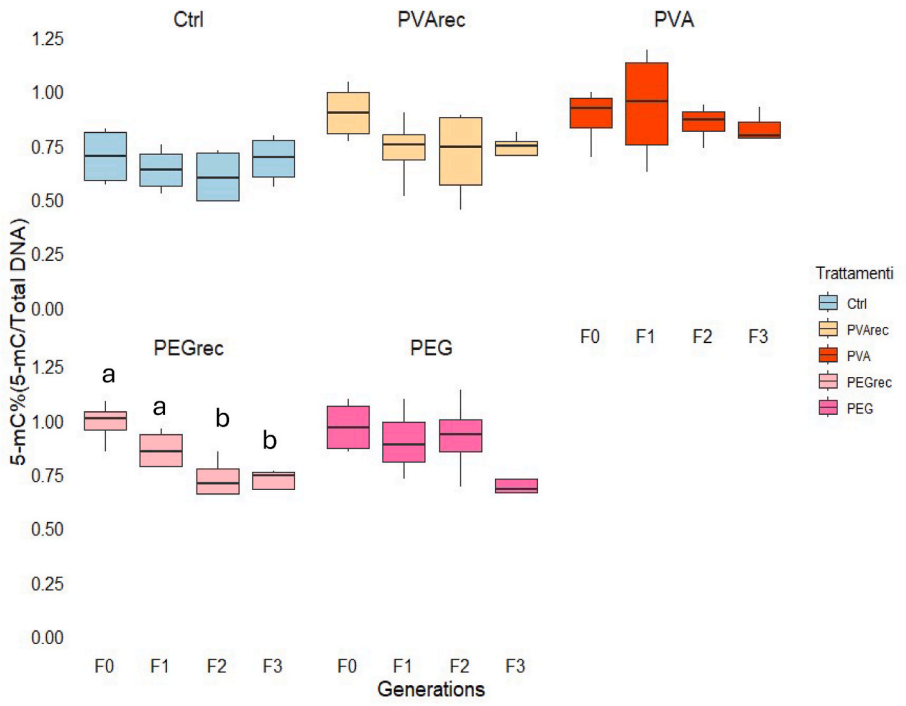

Fig. 4. Total 5-methylcytosine (5-mC) DNA methylation levels in the F_0, F_1, F_2, F_3 generations of *D. magna* exposed to 1 μg/L of polyvinyl alcohol (PVA) and polyethylene glycol (PEG) over all the generations (PEG and PVA treatments), exposed to PVA and PEG only in the parental generations (F_0) (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.

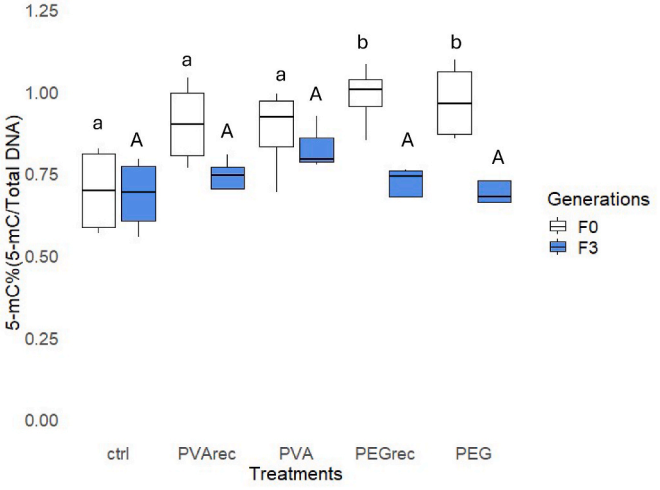

Fig. 5. Total 5-methylcytosine (5-mC) DNA methylation levels in F_0 and F_3 generations of *D. magna* exposed to 1 μg of polyvinyl alcohol (PVA) and polyethylene glycol (PEG) in both generations F_0 and F_3 (PEG and PVA treatments), the treatment exposed to PVA and PEG only in the parental generations (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. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

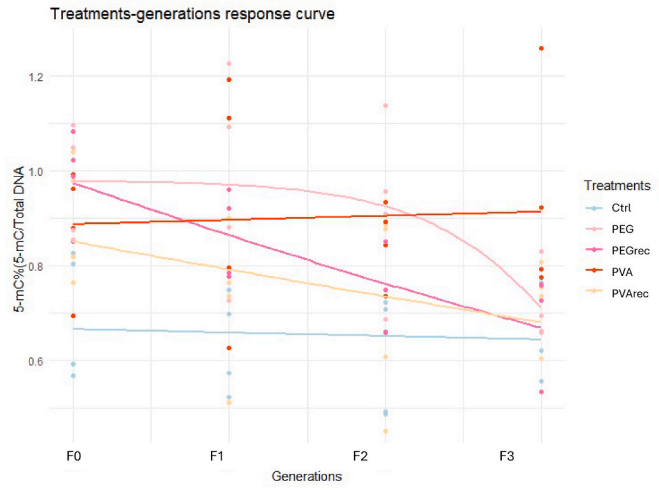

Fig. 6. Trend of the total 5-methylcytosine (5-mC) DNA methylation levels in the F_0, F_1,F_2, F_3 generations of *D. magna* exposed to 1 μg/L of polyvinyl alcohol (PVA) and polyethylene glycol (PEG) over all the generations (PEG and PVA treatments), and exposed to PVA and PEG only in the parental generations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

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 capability of *D. magna* (Table 1). However, the lack of significant

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 were analyzed through R version 4.3.

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 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 PVA and 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.

	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

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, Figs. 2 and 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 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 concentrations of some 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 drug 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, followed by 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 (Figs. 5 and 6) suggested an initial epigenetic response to PEG exposure, which diminished over time (Figs. 4 and 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 hypothesized 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.

CRediT authorship contribution statement

Lara Nigro: Writing – original draft, Methodology, Formal analysis, Conceptualization. **Andrea Binelli:** Writing – review & editing, Validation, Supervision, Funding acquisition, Conceptualization. **Iene Herman:** Writing – review & editing, Methodology, Investigation, Data curation. **Stefano Gazzotti:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Marco Aldo Ortenzi:** Writing – review & editing, Supervision, Methodology. **Jana Asselman:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Andrea Binelli reports financial support was provided by Cariplo Foundation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2025.121436.

Data availability

Data will be made available on request.

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