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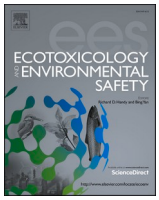

Genotoxic effects of particulate matter on larvae of a common and widespread butterfly along an urbanization gradient

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ABSTRACT

Biodiversity is currently declining worldwide. Several threats have been identified such as habitat loss and climate change. It is unknown if and how air pollution can work in addition or in synergy to these threats, contributing to the decline of current species and/or local extinction. Few studies have investigated the effects of particulate matter (PM), the main component of air pollution, on insects, and no studies have investigated its genotoxic effects through Micronucleus assay. Butterflies play an important role in the environment, as herbivores during larval stages, and as pollinators as adults. The aim of this study was to evaluate the genotoxic effects of PM_{10} from different sites along a gradient of population urbanization, on a common cabbage butterfly species (*Pieris brassicae*). PM_{10} was collected from April to September in an urban (Turin, Italy), a suburban (Druento, Italy) and a mountain site (Ceresole Reale, Italy) with different urbanization levels. *P. brassicae* larvae (n = 218) were reared in the laboratory under controlled conditions (26 °C, L:D 15:9) on cabbage plants (average 9.2 days), and they were exposed to PM_{10} organic extracts (20 and 40 m^3/mL) or dimethyl sulfoxide (controls) through vaporization. After exposure, larvae were dissected and cells were used for the Micronucleus (MN) assay. Results showed that all PM extracts induced significant DNA damage in exposed larvae compared to controls, and that increasing the PM dose (from 20 to 40 m^3/mL) increased genotoxic effects. However, we did not detect any significant differences between sites with different urbanization levels. In conclusion, PM at different concentrations induced genotoxic effects on larvae of a common butterfly species. More alarmingly, PM could work in addition to and/or in synergy with other compounds (e.g. pesticides) and, especially on species already threatened by other factors (e.g. fragmentation), thus affecting the vitality of populations, leading to local extinctions.

1. Introduction

Species are currently suffering rapid and alarming anthropogenic extinctions, known as the "sixth extinction wave" (e.g. Ceballos et al., 2010). Several causes have been indicated as possible threats to biodiversity such as habitat loss, alien species, climate change, overexploitation and high-altitude pastoral abandonment (Tilman et al., 2017). We do not know if and how air pollution can contribute to the current decline or local extinction of species. There are some cases in which local extinctions are poorly explained by environment and climate changes (Bonelli et al., 2011); in these cases, air pollution could have contributed to extinctions. Indeed, particulate matter (PM), a main component of air pollution, can include toxic compounds that are dangerous to animal health, such as polycyclic aromatic hydrocarbons (PAH) and heavy metals (HM) (Voutsa and Samara, 2002). PM can work in addition to or synergy with other threats and, thus, could contribute to the decline and local extinctions of particular species (in accordance with Santovito et al., 2020).

Air concentration of PM generally correlates with increasing urbanization. The highest $PM_{2.5}$ concentration per capita in Europe is within cities with populations between 0.75 and 1 million (Han et al., 2016). Indeed, higher urbanization entails a higher density of population, increasing vehicular traffic, and scarce dispersion of atmospheric pollutants (Han et al., 2021), also depending on city structures.

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Consequently, in urban areas, higher concentrations of PM with inorganic and/or organic hazardous components have been registered (Rockens et al., 2000). In Europe, urban transportation contributes substantially to the total emissions of PM (Pant and Harrison, 2013). However, the chemical composition of PM can change according to the source of emissions, seasons, characteristics of the sampling site and photochemical-meteorological conditions (Topinka et al., 2015).

Air pollution is known to induce several diseases in humans (Aoki, 2017), but its effects have been rarely investigated in wild animals. It has been previously shown that air pollution affects reproduction in mosquitos (Phanitchat et al., 2021), causes mortality in the final larval instars of larvae (Vanderstock et al., 2019), and can cause sublethal effects in *Drosophila melanogaster* (Meigen 1830; de Santana et al., 2018). PM can be absorbed by *Apis mellifera* (Linneo, 1758; Negri et al., 2015), but its genetic and behavioral effects on the species are still unknown. Of all invertebrates, butterflies are very sensitive to landscape changes, and ecosystems depend on their community stability (Da Rocha et al., 2010). For these reasons, understanding how these insect communities vary depending on the environmental quality is crucial to landscape/territorial management policies. In urban environments, butterfly richness, abundance and diversity indexes were found to be negatively correlated with increased levels of several pollutants such as NO_x, NO_2 and $PM_{2.5}$ (Meléndez-Jaramillo et al., 2021). However, the effect of PM on butterfly behavior is unknown, along with its possible ecological consequences for ecosystem services such as pollination.

It has been demonstrated that PM or other types of pollutants or anthropic-origin chemicals can cause genotoxic damage to DNA molecules and genomic instability (Araldi et al., 2015). This type of damage can be evaluated by different techniques, such as the Micronucleus (MN) assay. MN assays have been widely used to evaluate, in vitro and/or in vivo, the genotoxic damage induced by environmental pollutants on mammals (humans included) and other vertebrates (Santovito et al., 2020; Santovito et al., 2022). In invertebrates, MN assays have been positively used to assess the genomic impacts of the herbicide glyphosate on the protected butterfly larvae *Lycaena dispar* (Haworth 1803; Santovito et al., 2020), but no data are present in the literature about the possible MN presence in relation to PM on invertebrates.

In this article, we evaluated the possible genotoxic effects induced by exposure to coarse PM (PM with particles of less than 10 μm in diameter) on a common and widespread butterfly, *Pieris brassicae* (Linnaeus, 1758). In particular, the level of genotoxicity was assessed, along an urbanization gradient, using the MN assay. MNi represent small extranuclear bodies that have not been included in the daughter nuclei at telophase. They may arise from chromosome breakage or if a whole chromosome lags behind at anaphase, and fails to be incorporated into the new nuclei (Fenech et al., 2011). Chromosomal instability was also measured by scoring nuclear buds, which represent the process of elimination of amplified DNA and/or excess chromosomes from aneuploid cells (Fenech et al., 2011).

The starting hypothesis is that PM_{10} can increase baseline frequencies of genomic damage markers, with a consequent reduction in reproductive fitness and, in the final analysis, an increase in extinction risk, particularly for species classified as vulnerable.

2. Materials and methods

2.1. PM_{10} collection and extractions

PM_{10} was collected from 3 monitoring stations of the Regional Agency for Environmental Protection of Piedmont (ARPA Piemonte), Turin (urban traffic site, location 45°04'33.0"N, 7°40'41.3"E, altitude 243 m); Druento (suburban site, 45°10'32.8"N, 7°33'36.9"E, altitude 335 m) and Ceresole Reale (mountain site, 45°25'48.7"N, 7°14'43.5"E; altitude 1620 m). Sites are located within the Padana Plain (Italy) and are characterized by different urbanization levels; indeed, the resident population is equal to 848,885 people in the urban site, 8953 people in the suburban site and 159 people in the mountain site (resident population on 1st January 2022) (ISTAT, 2021). For each site, PM_{10} was collected daily on quartz-fiber filters (Ø = 47 mm) using low volume samplers (flow = 2.3 m^3/h) from 1st April 2019–30 th September 2019. This sampling period was selected because it corresponds to the larval season of *Pieris brassicae*. Moreover, the PM was sampled in six months in order to be representative of the PM organic extract of spring/summer in the studied area (i.e. Northern Italy). PM collected from the monitoring stations is naturally subject to abiotic conditions, such as rain, temperature, humidity and wind which might reduce PM concentration. Therefore, it represents the air PM concentrations of the monitored period. Daily mean PM_{10} concentrations from 1st April 2019–30 th September 2019 (PM sampling period) in mountain, suburban and urban sites were 11.8 μg/m^3, 18.0 μg/m^3, 17.5 μg/m^3, respectively (ARPA Piemonte DATA, 2019). These PM concentrations are below the European air quality standards - annual limit value = 40 μg/m^3 – and, for the mountain site, even below the WHO guidelines - annual guideline level = 15 μg/m^3 (European Commission Directive 2008/50/EC; Italian Legislative Decree 155/2010; WHO 2021). To perform the organic extraction of PM, daily filters were pooled to obtain one sample for each site (183 filter quarters for each site). Filter quarters of each pool were cut in small pieces, placed in a glass beaker and washed three times with acetone/cyclohexane (1:1) using an ultrasonic water bath. Then, filters and solvent (250 mL) were transferred in tubes, vortexed for 1 min and centrifuged at 4100 rpm for 10 min in order to remove filter debris. The supernatant was then evaporated using a rotary evaporator and re-suspended in dimethyl sulfoxide (DMSO) at a final concentration of 2000 m^3/mL (details in Schilirò et al., 2016). The extracts were stored at − 20 °C until analysis.

2.2. Larval rearing

To test if PM_{10} has genotoxic effects on butterfly larvae, we selected the most common butterfly species, cabbage butterflies belonging to the *Pieris* genus. To reduce genetic differences between treatments, we selected the species that lays eggs in large batches, *P. brassicae* (large cabbage butterfly). Le Masurier (1994) highlighted that different group sizes (5, 40 and 100) of *Pieris brassicae* larvae for the first three instars did not affect larval survival. The species is not protected neither threatened (Bonelli et al., 2018). Larvae, rather than adults, were used because they are more sedentary and thus it is easier to correlate genotoxic effects to PM exposure.

Eggs were collected from urban allotments as they were considered pests in that context and, thus, removed and sacrificed. We collected more than 500 eggs from the "Orti generali" urban allotment in Turin (45°00'43.5"N 7°37'37.4"E).

Eggs were raised in the laboratory and, before hatching, they were placed in Petri dishes. The day after hatching, larvae were equally divided between four plants of *Brassica oleracea var. Kapral*; each plant was kept in a net cage in a climate cell at 26 °C L:D 15:9 (as reported by Santovito et al., 2020 and Piccini et al., 2021) and corresponds to a different treatments: urban, suburban and mountain site extracts. Plant/larvae treatments were sprayed with PM_{10} dissolved in water and 1% or 2% DMSO (final PM_{10} doses = 20 m^3/mL and 40 m^3/mL, respectively); while for controls plant/larvae were treated with water and 1% or 2% DMSO in order to expose the control larvae to the same conditions as the treated larvae except for the PM organic compounds.

2.3. Experimental design

Two experiments were performed; the first experiment was carried out from 24th June to 9th July (15 days), exposing plants and larvae to 20 m^3/mL of PM_{10} or to 1% of DMSO (controls); the second one (2nd to 16th August and from 5th to 22nd September 2021, 31 days), exposing plants and larvae to a double dose of 40 m^3/mL of PM_{10} or to 2% of DMSO (controls 2x). A total of 22 individuals placed on controls treated

with double dose of DMSO (controls 2x) were found dead in the first 3 days in August, and only five individuals survived until the end of the experiment. This high mortality is likely related to some unexpected aspect of the plant. Thus, we run a second round of the experiment with double dose with other eggs and another plants in September 2021 (including double dose treatments and control). A total of 529 larvae were used: 246 for the first experiment and 283 for the second one (Table A2 in Appendix).

For both experiments, each leaf for all the plants was measured before the experiment started. Specifically, the length and the width of all the leaves were measured. The area of each leaf was estimate with the formula: leaf area = length * width /2 (assuming that the form of leaves is similar to a rhombus) and the total leaf area of the plant was then calculated as the sum of all the areas of the individual leaves.

Each treatment with PM_{10} dilutions was performed as follows (for details see Protocol in Appendix A1): the PM_{10} extracts (2000 m^3/mL) from each site were defrosted at room temperature at least 30 min before treatment and diluted in commercial mineral water (water analysis in Table A1 in Appendix A1) at a final volume of 5 mL (final PM_{10} doses = 20 m^3/mL and 40 m^3/mL). To avoid contamination between treatments, the solution was prepared in a different spray bottle for each treatment. Spray bottles were mixed and sprayed on different plants corresponding to the different treatments. The dilution was sprayed near the leaves all around the plant to avoid diffusion of the dilution into the environment, and to ensure that the entire plant received the PM_{10} dilution and control treatments. To avoid cross-contamination between treatments, each plant was individually treated outside the climatic chamber. Standard hygiene rules were observed at all times. During the experiment, plants were watered every 2–3 days and replaced every 5 days because they were completely eaten by larvae (Figure A1 in Appendix).

The treatment was repeated three times (15 mL of PM_{10} or DMSO dilutions for each type of extract), simulating rainy days during the summer period (≈8 rainy days/month) until the achievement of the final larval stage. Indeed, in the first experiment (20 m^3/mL), larvae were exposed to 236 μg/mL, 360 μg/mL and 350 μg/mL of PM_{10} organic extracts, respectively. In the second experiment (40 m^3/mL), plants were exposed to 472 μg/mL, 720 μg/mL and 700 μg/mL, respectively. These exposure doses were selected because they corresponded to the mean estimate of PM leaf deposition for herbs during summertime (Cai et al., 2017; see Appendix). Moreover, these doses are similar to that generally tested *in vitro* on cell lines (Schilirò et al., 2016 – 200 μg/m^3; Schilirò et al., 2015 – 200 and 500 μg/mL).

At the end of the experiment, larvae were weighted with analytical balance (0.0001 mg) and euthanatized (that was performed cutting the head of the larvae) at the last instars (8–13 days). Indeed, according to Springolo et al. (2021) 10.4 days is the average time to reach the fourth/fifth larval instar for *P. brassicae* at a rearing temperature of 26 °C.

2.4. Preparation of slides

Larval bodies were inserted into a Falcon tube containing a fixative solution of 3:1 methanol/acetic acid and stored at 4 °C prior to analysis. Successively, larvae were fragmented with a pestle or tip. Cells were collected by centrifugation; supernatants were discarded and the pellets were dissolved in a minimal volume of fixative. These were then seeded onto slides to detect MNi by conventional staining with 5% Giemsa (pH 6.8) prepared in Sörensen buffer (Santovito et al., 2020).

2.5. Genotoxic analysis

Microscopic analysis was performed at x400 magnification on a light microscope, whereas investigating and images of micro-nucleated cells were performed at x1000 magnification. MNi and buds (hereafter together "total abnormalities") were scored in 1000 cells per subject, following the established criteria for MN evaluation (Thomas et al., 2009).

To understand if the extracts induced non-lethal (hereafter sublethal) genotoxic effects, we analyzed MNi, buds and total abnormalities in six different models. For all models, we excluded individuals for which we counted less than 1000 cells (31 individuals; Table A2). Considering the high mortality of the control 2x in August, likely related to some aspect of the plant, we excluded five survived individuals from the analysis. To evaluate if treatments (sites and doses) affect genotoxic abnormalities, we modelled total abnormalities (MNi + buds), micronuclei and buds in generalized linear models (GLM) with sites as a categorical explanatory variable. Weight (g) was considered as an offset. The reference category was the control. Considering that response variables were counting data, we used Poisson family, and we checked that models were not over- or under-dispersed. For these two over- or under-dispersed models (see details in Appendix), we used negative binomial family. Then, to each model, we applied a Tukey post hoc analysis with Bonferroni correction.

To investigate if dose affected aberration presence, controls were excluded and total abnormalities, micronuclei and buds were modelled in GLM with dose as categorical explanatory variable.

Each model was fitted using the 'lme4' (Bates et al., 2015) package in R. To evaluate the dispersion of models with Poisson distribution, we used the 'Dharma' (Hartig, 2019) package in R software (R Development Core Team 2014).

3. Results

We raised 529 larvae of which 218 survived for at least 8 days (Table A2 and Figure A2). A total of 53 larvae (28 for single dose and 25 for double dose of PM_{10}) were treated only with DMSO, while 57 (29 for single dose and 28 for double dose of PM_{10}) were treated with mountain site extract, 59 (26 for 20 m^3/mL dose and 33 for 40 m^3/mL dose of PM_{10}) were treated with suburban site extract and 56 (25 for 20 m^3/mL dose and 31 for 40 m^3/mL dose of PM_{10}) with urban site extract. Descriptive analysis of data is present in Appendix (Table A3 and Fig. A2).

Mean larval weight and mean sum of plant leaf sizes were similar between treatments (see Fig. A3 in Appendix). Mortality was high (similarly to Piccini et al., 2021) but was not affected by PM treatment (Table A2 and Fig. A4 in Appendix).

3.1. Genotoxic analysis

Overall total abnormalities and micronuclei were significantly higher in treatments, both with 20 m^3/mL and 40 m^3/mL doses, compared to controls, while buds were significantly higher than controls for the double dose (Fig. 1). Conversely, there were no significant differences between sites from post-hoc analysis (Fig. 1; Tables A5, A7, A9, A11, A13 and A15 in Appendix).

Mountain, suburban and urban sites presented significantly higher number of total abnormalities compared to controls with single dose (Mountain site: z value= 0.98, $p < 0.001$; Suburban site: z value= 0.77, $p < 0.001$; Urban site: z value= 0.92, $p < 0.001$; Tables A4 and A5 in Appendix A1) and with double dose (Mountain site: z value= 1.47, $p < 0.001$; Suburban site: z value= 1.28, $p < 0.001$; Urban site: z value= 1.41, $p < 0.001$; Tables A6 and A7 in Appendix A1).

Similarly, all treatments presented a significantly higher number of **MNi** for single dose (Mountain site: z value= 1.18, $p < 0.001$; Suburban site: z value= 1.05, $p < 0.001$; Urban site: z value= 0.98, $p < 0.001$; Tables A8 and A9 in Appendix A1) and double dose (Mountain site: z value= 1.74, $p < 0.001$; Suburban site: z value= 1.51, $p < 0.001$; Urban site: z value= 1.66, $p < 0.001$; Tables A10 and A11 in Appendix A1).

Buds were numerically significantly lower in control 2x than in treatments (mountain, suburban and urban site extracts; Fig. 1f and Table A14 and A15 in Appendix). Conversely, buds were significantly similar between control and single dose treatments (mountain, suburban and urban site extracts; Fig. 1e and Table A12 and A13 in Appendix).

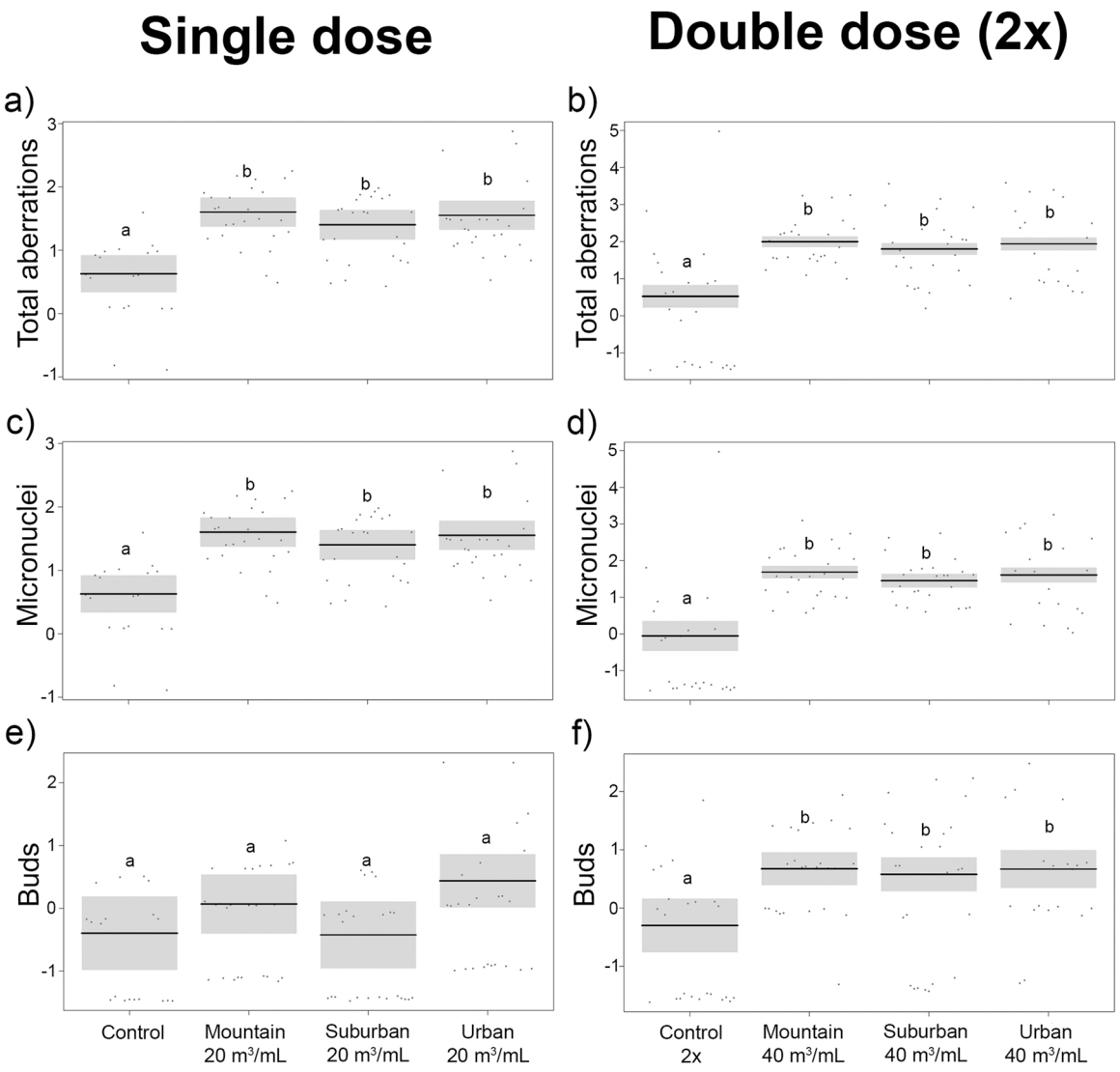

Fig. 1. Results for GLMs with (a, b) total abnormalities, (c, d) micronuclei (MNi) and (e, f) buds, for single dose (control and 20 m^3/mL) and double dose (control 2x and 40 m^3/mL). Dots are partial residuals and lines represent best-fit models. Letters above boxes identify significance as revealed by Tukey post hoc analyses with Bonferroni correction (for significance see Tables A4-A15 in Appendix).

3.2. Effect dose

To test the dose effect, GLM results showed that 40 m^3/mL have a higher effect on total abnormalities (z value= 5.499, $p < 0.001$; Fig. 2a), micronuclei (z value= 4.086, $p < 0.001$; Fig. 2b) and buds (z value= 3.871, $p < 0.001$; Fig. 2c).

4. Discussion

Butterfly decline began at the end of the XVIII century, and habitat loss, degradation and chemical pollution have been identified as the main causes of the decline (IUCN). Little information is known on lethal and sublethal effects of air pollutants. However, metal pollution has been identified as a possible cause of local extinctions of *Parnassius apollo* (Nieminen et al., 2001) and the decrease of smelter air pollution leads to Lepidoptera community recovery (Kozlov et al., 2022). Moreover, it has been shown that coal dust ingested by larvae affects mortality of final instars (Vanderstock et al., 2019).

Many studies have reported that coarse and fine PM have lethal and sublethal effects on humans, mice, and rats (Aoki, 2017), as well as on invertebrates like mosquitos (Phanitchat et al., 2021), and nematodes (Zhao et al., 2014). Considering that PM deposits on different types of leaves and different traits can influence this deposition (Chiam et al., 2019), herbivorous animals may absorb PM through leaf ingestion. Here, for the first time, we have demonstrated that *P. brassicae* larvae, after having eaten leaves with sprayed PM extracts, have significant DNA damage compared to controls.

4.1. PM_{10} genotoxic effects on larvae

Few studies have been conducted analyzing the effect of PM on insects. It has been previously shown that PM affects behavior and egg production in mosquitos (Phanitchat et al., 2021), as well as being absorbed by *Apis mellifera* (Negri et al., 2015) and, consequently, it can even be found in bee pollen (Papa et al., 2021). It could thus affect the behavior and physiology of this insect species (see Thimmegowda et al., 2020) and, consequently, it may also decrease pollination (see Ryalls et al., 2022). On the other hand, in only one previous study, the MN assay was used in order to evaluate the genomic damage on a phytophagous invertebrate at the larval stage (Santovito et al., 2020).

Here the genotoxic sublethal effects of PM_{10} on a common and widespread butterfly have been quantified. We found that PM_{10} of different sites along an urbanization gradient (urban, suburban and mountain sites) induce significant DNA damage, in terms of total

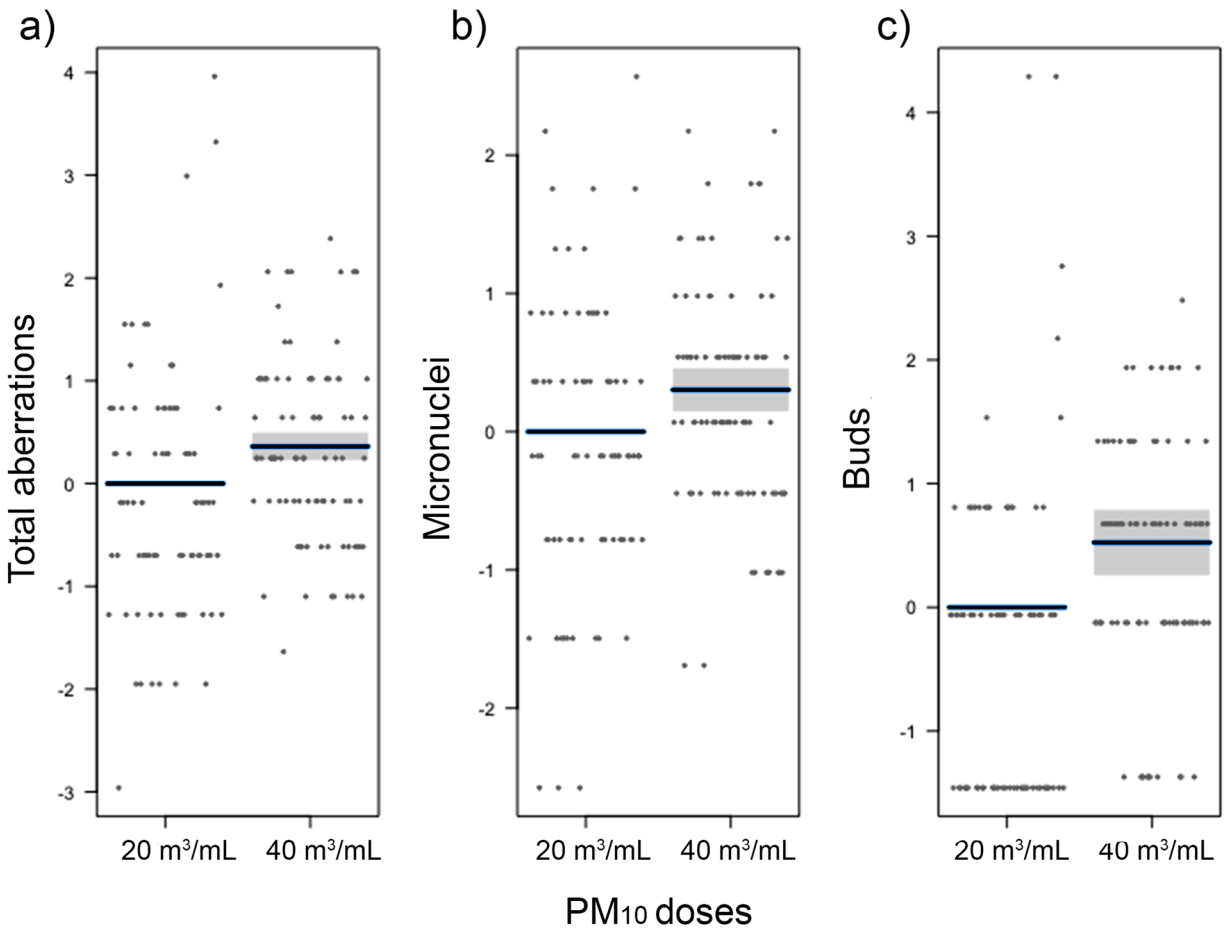

Fig. 2. Results for dose effects on a) total abnormalities, b) micronuclei and c) buds. Dots are partial residuals and lines represent best-fit models.

abnormalities, MNi and buds. Specifically, total abnormalities and MN were higher in all treatments with both doses (20 and 40 m^3/mL) compared to controls (control and control 2x). However, buds were significantly higher in treatments compared to controls only for the double dose (controls 2x and extract 40 m^3/mL). Indeed, the PM sampled in sites that are characterized by low PM levels (below the European air quality standards - annual limit value = 40 μg/m^3 – and, for the mountain site, even below the WHO guidelines - annual guideline level = 15 μg/m^3) are able to induce a significant genotoxic effect on butterfly larvae (European Commission Directive 2008/50/EC; Italian Legislative Decree 155/2010; WHO 2021)".

We observed a linear relationship between PM doses (from 20 to 40 m^3/mL) and genomic damage. Thus, higher doses of PM_{10} increased the genotoxic sublethal effects on larvae.

These results reflect a condition of genomic instability that could result in a reduction of vitality (Zhao et al., 2014). PM likely also affects other insect species, some of these might be threatened by other factors. For these species, the additive effect of genomic damage and other environmental factors (such as climate change, habitat loss, etc.) could increase the local extinction risk.

Although the MN assay requires dissection of the insect, this technique allows evaluation, in a short time, of the biological effects on larvae due to exposure to different PM concentrations. This study has provided important insights into the sublethal effects possibly occurring in insects due to PM exposure, even with concentrations below the current EU air quality standards (European Commission Directive 2008/50/EC) and WHO guidelines.

4.2. *Urbanization and PM_{10} genotoxic effects*

PM is a heterogeneous mixture of particles of different sizes derived from natural and/or anthropogenic sources (Pope and Dockery, 2006). Its chemical composition changes over time and space. During the summer, the elevated solar radiation, which can photo-decompose PM components, tends to modify the PM_{10} chemical constituents. In winter, the low temperatures facilitate the absorption of volatile compounds on particle surfaces (Perrone et al., 2010). PM composition and concentration change even in rural and urban areas. Mineral dust concentrations are higher in urban than in rural sites (Rodrìguez et al., 2004); indeed, at urban sites, vehicle exhaust products are the main contributor to PM_{10} (Ghio et al., 2012). The major sources of PM include vehicular and industrial emissions, power plants, crustal release, and refuse incineration. We only collected PM during the larval activity period (spring/summer) when sites have lower PAH concentrations, and thus lower toxicities. However, all sites present different PM concentrations (Urban: 17.6 μg/m^3; Suburban: 18.1 μg/m^3; Mountain 11.9 μg/m^3). Indeed, even the suburban site (Druento) presented a similar PM_{10} concentration to the urban site (Turin). This could be related to the intense vehicle transportation from suburban areas to Turin and/or to the weak pollutant dispersion rate due to the conformation of the territory (Cadum et al., 2009). Even the mountain site (Ceresole Reale) presented a similar concentration of PM_{10} (11.9 μg/m^3) to urban and suburban sites, this could be linked to the high presence of tourists in the area during spring and summer time.

Differences between sites did not reflect significant differences in the genotoxic damage on *P. brassicae* larvae. Indeed, we did not record significant differences between treatments (Fig. 1). For humans, Renzi et al. (2021) have found a comparable mortality risk between suburban

and urban settings regarding PM. Moreover, it has been shown that large urban areas could have an influence on PM concentrations up to 200 km, as was the case in the Beijing-Tianjin-Hebei urban agglomeration (Du et al., 2020).

Genomic damage of PM was significantly similar between sites (urban, suburban and mountain areas). Even the PM derived from the mountain site (Ceresole Reale) was found to cause DNA damage, thus several local butterfly species protected at the European level (such as *Parnassius apollo* and *P. mnemosyne*, listed in Habitats Directive 92/43/CEE) and/or threatened (Bonelli et al., 2011), could show genomic damage.

5. Conclusions

These are the first results of the non-lethal effects of PM_{10} on butterfly larvae. The sublethal effects of PM may be found even in other organisms, especially herbivorous insects that directly ingest PM deposited on plants. Even PM sampled in sites with low concentrations, below the current EU air quality standards and WHO guidelines, can cause genomic damage in herbivorous animals, and these effects can decrease average fitness and consequently increase local extinction risk. However, no studies have been conducted to evaluate this aspect.

In conclusion, butterfly larvae seem to be sensitive organisms to investigate air quality and PM genotoxicity (see Macrì et al., 2023). PM could work in addition to and/or in synergy with other compounds (such as pesticides, fungicides and heavy metals), which may cause genomic damage to insects (e.g. Santovito et al., 2020). Moreover, some invertebrates are threatened by several other factors such as climate change and habitat loss (Bonelli et al., 2011). The combination of all these factors may strongly affect the vitality of populations and lead to local extinctions. Among threatened species, there are also species efficient in providing ecosystem services (such as pollination), thereby aggravating the consequences of local biodiversity loss, and threatening even ecosystem functioning (Piccini et al., 2018).

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CRediT authorship contribution statement

Conceptualization: I. P., M.M, M.G. T.S., Sa.B., A.S. and Si.B; Formal analysis: I.P. and L.D.; Investigation: I.P., M.M., M.G., and L.D.; Supervision: T.S., Sa.B., A.S., and Si.B.; Visualization: I.P.; Roles/Writing – original draft: I.P., M.M., M.G. and L.D.; Writing – review & editing: I.P., M.M., M.G., L.D., T.S., Sa.B., A.S. and Si.B.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2023.114638.

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