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RESEARCH ARTICLE

Cabbage butterfly as bioindicator species to investigate the genotoxic effects of PM_{10}

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Abstract

Atmospheric pollution poses a serious threat to environment and human health, and particulate matter (PM) is one of the major contributors. Biological effects induced by PM are investigated through in vitro assays using cells and by in vivo tests with laboratory model animals. However, also the estimation of adverse effects of pollutants, including airborne ones, on wild animals, such as insects, is an essential component of environmental risk assessment. Among insects, butterflies are sensitive to environmental changes and are important wild pollinators, so they might be suitable as environmental bioindicator species. The aim of this study was to evaluate the suitability of a wild cabbage butterfly species (*Pieris brassicae*) as a bioindicator organism to assess the genotoxic effects of PM_{10} collected in different sites. PM_{10} was collected from April to September in urban, suburban, and rural sites. *P. brassicae* larvae were reared in laboratory under controlled conditions on cabbage plants and exposed to PM_{10} organic extracts or dimethyl sulfoxide (controls) through vaporization. After exposure, larvae were dissected, and cells were used for comet assay. All PM extracts induced significant DNA damage in exposed larvae compared to controls and the extract collected in the most polluted site caused the highest genotoxic effect. In conclusion, the study suggested that butterflies, such as *P. brassicae*, could be applied as sensitive and promising bioindicators to investigate air quality and PM genotoxicity. Indeed, the use of these organisms allows the detection of genotoxic effects induced by PM sampled also in low-polluted areas.

Keywords Air pollution · Bioindicator species · Cabbage butterfly · Caterpillars · Comet assay · Particulate matter

Introduction

Particulate matter (PM) is a mixture of solid and liquid particles with different shapes and origin that has an aerodynamic diameter of 0.001–100 µm (Mukherjee and Agrawal 2018). The PM composition is complex, mainly including

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inorganic ions, organic pollutants, metals, and other harmful compounds that can be toxic for organisms, such as polycyclic aromatic hydrocarbons (PAHs). Atmospheric inhalable PM (PM_{10}), which includes particles with aerodynamic diameters ≤ 10 μm, is considered one of the most important air pollution indicators (WHO 2021).

Epidemiological studies highlighted that the long-term exposure to PM_{10} increases risk of chronic bronchitis, coronary events, chronic kidney disease, type 2 diabetes, and cancer mortality, while the short-term exposure to PM_{10} was associated with cardiovascular and respiratory mortality (Cesaroni et al. 2014; Kim et al. 2018; Liu et al. 2019; Rojas-Rueda et al. 2021). The International Agency for Research on Cancer (IARC) designated PM as a Group I carcinogen (IARC 2016).

In order to protect human health, current European air quality Directive and World Health Organisation (WHO) guidelines establish limit/guideline values for PM concentrations (PM_{10} or $PM_{2.5}$) and for concentration of other air pollutants that can be adsorbed on PM (e.g., benzo(a)pyrene — BaP, one of the most toxic PAHs) (European Commission Directive 2004/107/EC; European Commission Directive 2008/50/EC; WHO 2021). Although the environmental and health effects induced by PM are related to its concentration and to its chemical composition, the PM effect cannot be easily deduced considering only its mass and the concentrations of some chemicals adsorbed on it. Indeed, PM is a complex chemical mixture, which changes according to emission sources, season, sampling site characteristics, and photochemical-meteorological conditions (Topinka et al. 2015; Pongpiachan et al. 2017), so it is not possible to quantify all chemicals on it. Moreover, the effects of all pollutants and of their metabolites are not always known and, in addition, synergistic/antagonistic interactions could occur among them, causing altogether an unpredictable biological effect.

The approach applied to evaluate the effect induced by the complex mixture of PM was generally based on the use of different in vitro bioassays on prokaryotic/eukaryotic cells. Results obtained highlighted that (according to different aerodynamic diameter, origin and composition) PM was able to induce different modification and alteration at cellular level (Møller et al. 2015; Heßelbach et al. 2017; Peixoto et al. 2017; Thompson 2018; Bonetta et al. 2019). The PM biological effects were also investigated in vivo using laboratory model animals (rats and mice) showing that PM can induce oxidative stress, cardiovascular and immune responses, brain and liver toxic effects, and mutagenicity and genotoxicity (Aoki 2017; Chen et al. 2022). However, also the estimation of adverse effects of pollutants, including airborne ones, on wild animals is an essential component of environmental risk assessment. Therefore, there is a need to develop new monitoring schemes and indicators to assess the air pollution impacts on different animal species. In particular, more studies on animals reared in areas characterized by high pollution levels may be helpful to establish the importance of sentinel organisms on risk assessment and to formulate regulatory procedures, as well as the evaluation of pathological manifestation occurrence (Losacco and Perillo 2018).

Several insect taxa, such as butterflies and moths, are successfully used in ecotoxicological research as a bioindicator of environmental pollution, due to their significance in ecosystems and for humans (Jha 2008; Augustyniak et al. 2016; Ghazanfar et al. 2016; Potts et al. 2016; Schowalter et al. 2018; Al-Alam et al. 2019). Indeed, wild pollinators, such as butterflies, are essential for food production (Ghazanfar et al. 2016; Potts et al. 2016; Rhodes 2018). Since their decline could affect human life and well-being (Potts et al. 2016), there is a need to assess the pollution impacts on both managed and wild pollinators (European Commission workshop Report 2022). Moreover, due to their sensitivity to environmental changes, these insects could be applied as sentinel organisms also for the assessment of air pollution effects.

In particular, butterflies could represent a valuable bioindicator to study environmental risks of PM. Indeed, butterfly larvae are phytophagous so they can be exposed to PM through direct contact but also through ingestion of PM settled on leaves. Moreover, some butterfly species are easy to grow, easy to manipulate, and are ubiquitous, so they could be reared in laboratory and experimentally exposed to PM, but they could also be sampled in the wild after their natural exposure to environmental PM.

PM could affect biological systems in a variety of possible ways (Chen et al. 2022). Since one of the recognized effects of air pollution and PM is the ability to induce a DNA damage (Bonetta et al. 2019), the genotoxicity can be an interesting sub-lethal effect that could be evaluated on sentinel organisms, giving important information on the ability of air pollution to affect species and functionality of ecosystems (Augustyniak et al. 2016).

One of the most applied bioassays to assess the pollutant genotoxicity is the comet assay, which detects single and double-strand breaks and alkali labile sites (Dhawan et al. 2009; Azqueta and Collins 2013; Araldi et al. 2015). Although, in recent years, the comet assay was applied on different insect species used as bioindicators (e.g., *Drosophila melanogaster*, *Spodoptera exigua*, *Ceraeochrysa claveri*, *Bombus atratus*) to evaluate the effect induced by environmental contaminants (e.g., cadmium, mercury, agrochemicals) (Augustyniak et al. 2016; de Santana et al. 2018; Gajski et al. 2019; Gastelbondo-Pastrana et al. 2019; Ceschi-Bertoli et al. 2020), the possible use of insect species as bioindicators of genotoxicity induced by PM with different origin and characteristics has been poorly explored (de Santana et al. 2018). In particular, to the best of our knowledge, the possible application of butterflies as a bioindicator to assess PM environmental risks has never been studied.

The aim of this study was to evaluate the usefulness of a common and widespread wild butterfly species, *Pieris*

brassicae, as bioindicator organism for investigating the genotoxic effects induced by PM_{10} samples. In particular, this species has a wide distribution from North Africa across Europe and Asia and is able to live in different habitats also located at different altitudes (Feltwell 1982). Larvae of *P. brassicae* (hatched from field collected eggs) were exposed in laboratory to organic extracts of PM_{10} sampled in different sites (with different pollution levels) in order to test the butterfly sensitivity at increasing levels of pollution. After exposure, the larvae were sacrificed, and the genomic damage was evaluated using the comet assay.

Materials and methods

PM_{10} collection and extraction

PM_{10} was collected from three monitoring stations of the Regional Agency for Environmental Protection of Piedmont (ARPA Piemonte) located within the Padana Plain in Northern Italy: Torino (urban traffic site, location 45°04′33.0″N, 7°40′41.3″E), Druento (suburban site, 45°10′32.8″N, 7°33′36.9″E) and Ceresole Reale (rural site, 45°25′48.7″N, 7°14′43.5″E) (Fig. 1). The stations are part of a monitoring network, which was designed by the Italian government in order to monitor the air quality as required by the European legislation (European Commission Directive 2008/50/EC; Italian Legislative Decree 155/2010). For each site, PM_{10} was daily collected on quartz-fiber filters (Ø = 47 mm) using low volume samplers (flow = 2.3 m^3/h) from 1 April 2019 to 30 September 2019. This sampling period was selected because it corresponds to the period of *P. brassicae*'s larval stage. Daily filters were pooled to obtain one sample for each site (183 filter quarters for each site), and each pool was chemically extracted in order to collect organic-extractable compounds following the method of Schilirò et al. (2016). Briefly, 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 (CP102, CEIA International, Roissy-en-France, France). Then, filters and solvent (250 mL) were transferred in tubes, vortexed for 1 min, and centrifuged at 4100 rpm for 10 min (Megafuge 16R, Thermo Fisher Scientific, Osterode am Harz, Germany) in order to remove filter debris. The supernatant was then evaporated using a rotary evaporator (Rotavapor R200, Buchi, Flawil, Switzerland) and re-suspended in dimethyl sulfoxide (DMSO) at a final concentration of 2000 m^3/mL. The extracts were stored at − 20 °C until analysis.

Air pollution data

Air pollution data were analyzed in order to establish the air pollution levels in the different sites.

Pollution data of each sampling site were collected from the ARPA Piemonte website (ARPA 2022). The mean concentrations of PM_{10} and four PAHs [BaP, benzo(a)anthracene (BA), benzo(b + j + k)fluoranthene (BF) and indeno(1,2,3-cd)pyrene (IP)] were calculated from 1 April 2019 to 30 September 2019, according to the larval season of *P. brassicae*.

PAHs data were used to obtain the toxic equivalency factor (TEF), which expresses the toxicity of PAH

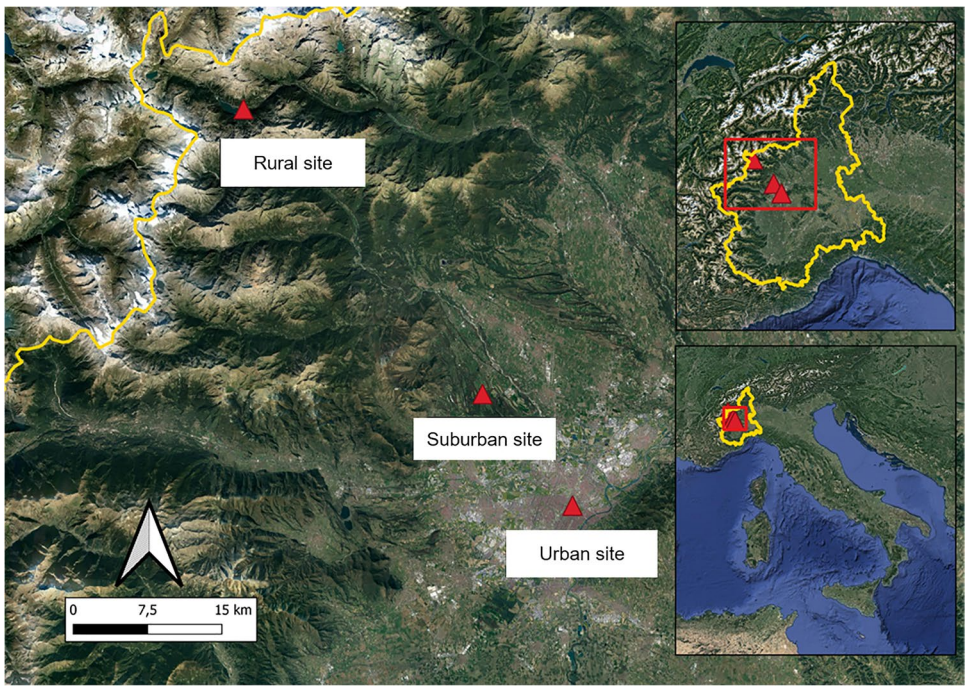

Fig. 1 Red triangles identify the three PM_{10} sampling sites: Torino (urban site), Druento (suburban site), Ceresole Reale (rural site). The sites are located in the Piedmont Region (marked in yellow), Northern Italy

mixtures as BaP equivalents. The PAH values below the limit of quantification (LOQ = 0.07 ng/m^3) were considered equal to half the LOQ (0.04 ng/m^3). Considering the carcinogenic potencies of PAHs in comparison to BaP (i.e., the reference PAH) (Nisbet and La Goy 1992; Samburova et al. 2017), TEF was calculated as:

$$TEF = BaP \times 1 + BA \times 0.1 + BF \times 0.1 + IP \times 0.1$$

Larval rearing and experimental design

Butterfly eggs were collected in the wild (urban garden *Orti generali* of Torino, Northern Italy) and placed in Petri dishes in the laboratory. The day after hatching, the larvae ($n = 283$) were equally divided into four plants of *Brassica oleracea var. Kapral* located in four separated net cages in a climate cell at 26 °C L:D 15:9 (as reported by Santovito et al. 2020 and Piccini et al. 2021). The four plants corresponded to four different treatments: three different PM_{10} extracts (rural, suburban, urban extracts) and one control.

The PM_{10} extracts (2000 m^3/mL in DMSO) of each site were diluted in commercial water at a final volume of 5 mL (final PM_{10} doses = 40 m^3/mL). For the control, a solution of water and DMSO was used. The diluted extracts and the control were sprayed near the leaves all around the plants assuring that the entire plants received the whole dilutions. To avoid cross-contamination among the treatments, each plant was singularly treated outside the climatic chamber. Plants and larvae were treated every 3 days (40 m^3/mL for each treatment), simulating rainy days during the summer period (≈ 8 rainy days/month) until the achievement of the last larval stage (8–13 days). The plants were watered every 2–3 days and replaced every 5 days because they were completely eaten by larvae (two plants were used for each extract). The exposure dose (40 m^3/mL) was selected because it is similar to the mean estimate of PM leaf deposition for herbs during summertime (Cai et al. 2017; as described in details in Supplementary Materials).

At the end of the experiment, the surviving larvae ($n = 117$) were sacrificed, and their cuticle was cut using a micro-scissor. Head and caudal parts were used for the comet assay. The experiments comply with the ARRIVE guidelines (Percie du Sert et al. 2020) and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council 2010).

Comet assay

The comet assay was performed according to Tice et al. (2000) with slight modifications (Bonetta et al. 2019). This assay is based on the ability of DNA fragments to migrate toward the anode in agarose gel under electrophoresis field, forming structures called comets.

After exposure, the head and caudal parts of each larva were gently mixed in 100 µL of low melting point agarose (LMP 0.7%). LMP agarose containing the disaggregated cells of the larvae (20 µL) was placed twice on microscope slides coated with 1% of normal melting agarose, with additional LMP agarose added as the top layer (the cells collected from each larva were placed on a single slide containing two replicates; each larva/slide was coded with a unique code). Slides were incubated for 2 h at 4 °C in lysis solution (8 mM Tris–HCl, 2.5 M NaCl, 100 mM EDTA disodium salt dihydrate, 1% TRITON X-100 and 10% DMSO, pH 10), immersed in an alkaline electrophoresis buffer (10 mM EDTA tetrasodium salt dihydrate, 300 mM NaOH, 10% DMSO, pH > 13) for 20 min and subjected to electrophoresis in the same buffer (20 min, 1 V/cm and 300 mA). Then, slides were neutralized for 3 min using a neutralization buffer (0.4 M Tris–HCl, pH 7.5, 4 °C), fixed using ethanol 70% (− 20 °C), and dried. For the analysis of DNA damage, the DNA of the cells was stained with ethidium bromide (20 µg/mL) and the percentage of DNA in the tail (%TI) of 100 cells for each larva was estimated using a fluorescence microscope (Axioskop HBO 50, Zeiss, Oberkochen, Germany) equipped with the Comet Assay IV analysis system (Perceptive Instruments, Instem, Staffordshire, UK). The fluorescence intensity obtained from the comet tail was used as an indicator of the amount of DNA damage. The results were reported according to the latest MIRCA guideline (Møller et al. 2020).

Statistical analysis

To understand if the exposure to PM_{10} extracts induced a significant genotoxic effect, the %TI (as mean of 100 cells) was modeled in a generalized linear mixed model (GLMM) with the site as categorical explanatory variable and egg batch as numerical explanatory variable as a random factor. Moreover, to understand the effects of TEF, we excluded controls, and %TI (as mean of 100 cells) was modeled in a generalized linear model (GLM) with the TEF as numerical explanatory variable. In both models, the reference category was the control, and individuals with count less than 100 cells (5 individuals) were excluded from the analysis. Considering that residuals were not normally distributed, Gamma distribution family was used in models (Zuur et al. 2009). Then, a post hoc analysis with Bonferroni correction was applied (Zuur et al. 2009). The model was fitted with the "lme4" R package in R software (R Development Core Team 2020).

Results

Air pollution data

Air pollution data in the three sites are reported in Table 1. The mean PM_{10} concentrations measured in the urban and in

the suburban sites were similar, while the lowest mean PM_{10} concentration was measured in the rural site. The PM_{10} concentrations of the three sites were below the Italian/European limit value (PM_{10} annual limit value = 40 μg/m^3) (European Commission Directive 2008/50/EC; Italian Legislative Decree 155/2010). However, only the PM_{10} concentration measured in the rural site was below the annual guideline level set by the WHO (PM_{10} annual guideline level = 15 μg/m^3) (WHO 2021).

Regarding PAH concentrations, BaP and BA concentrations were equivalent in the three sites (< LOQ), complying with Italian/European target value (BaP annual target value = 1 ng/m^3) (European Commission Directive 2004/107/EC; Italian Legislative Decree 155/2010). On the contrary, analyzing the other PAHs and TEFs, a concentration trend in agreement with the site type (rural, suburban, urban) was found in the three sites; indeed, the highest BF, IP, and TEF concentrations were measured in the urban site while the lowest concentrations were found in the rural site.

Genotoxic effects of PM_{10} extracts on larvae assessed by comet assay

The number of larvae involved in the experiment and finally used for the comet assay is reported in Table 2, together with the larval weight. Mean larval weight was not affected by PM treatment.

The results of the comet assay are reported in Fig. 2, while in Fig. S1 in Supplementary Materials, some examples of comets are shown. The statistical analysis showed that the DNA damage, expressed as %TI, was higher for larvae exposed to all treatments with respect to control (control: mean %TI = 6.30% ± 2.62%; rural extract: mean %TI = 9.44% ± 6.00%, t value = −3.245, p = 0.0012; suburban extract: mean %TI = 9.83% ± 4.80%, t value = −3.045, p = 0.0023; urban extract: mean %TI = 14.75% ± 8.27%, t value = −4.549, p < 0.001; Table S1 Supplementary Materials). This result was confirmed by the post hoc analysis with Bonferroni correction; indeed, all treatments (rural, suburban, and urban extracts) induced significantly higher %TI than those induced by control (rural extract: z value = −3.245, p = 0.0070; suburban extract: z value = −3.045, p = 0.0140; urban extract: z value = −4.549, p < 0.001; Table S2 in Supplementary Materials). Finally, the post hoc analysis with Bonferroni correction highlighted that the urban extract induced on larvae a higher DNA damage with respect to the rural extract (z value = −2.978, p = 0.0174) and the suburban extract (z value = −3.014, p = 0.0155; Table S2 in Supplementary Materials).

In addition, the GLM analysis highlighted that the mean %TI increased with an increase of TEF (t value = −3.468, p < 0.001; Fig. 3 and Table S3 in Supplementary Materials).

Discussion

Air pollution data

Overall, in the three sites, pollutant concentrations (PM and PAHs) were low (generally below the reference limits), and

Table 1 Concentrations of air pollutants in the three sites from 1 April 2019 to 30 September 2019 (larval season of *P. brassicae*). Data are reported as mean ± standard deviations. PM: particulate matter, BaP: benzo(a)pyrene, BA: benzo(a)anthracene, BF: benzo(b + j + k)fluoranthene, IP: indeno(1,2,3-cd)pyrene, TEF: toxic equivalency factor

Site type	PM_{10} (μg/m^3)	BaP (ng/m^3)	BA (ng/m^3)	BF (ng/m^3)	IP (ng/m^3)	TEF (mean) (ng/m^3)
Rural site	11.8 ± 2.5	0.040*	0.040*	0.040*	0.040*	0.052
Suburban site	18.0 ± 7.5	0.040*	0.040*	0.065 ± 0.043	0.045 ± 0.008	0.055
Urban site	17.5 ± 5.0	0.040*	0.040*	0.093 ± 0.078	0.063 ± 0.036	0.060

*0.040 corresponds to half of the quantification limit (LOQ)

Table 2 Number and weight of larvae used in the present study

Treatment	Dissected larvae for comet assay	Larval weight (mean ± SD, g)	Larvae considered for comet assay results[a]
Control (DMSO)	25	0.26 ± 0.11	25
Rural extract (40 m^3/mL)	28	0.23 ± 0.04	26
Suburban extract (40 m^3/mL)	33	0.25 ± 0.10	31
Urban extract (40 m^3/mL)	31	0.21 ± 0.05	30

SD: standard deviation

[a]Larvae with less than 100 cells suitable to score the %TI were excluded; the cells of each larva were placed on a single slide

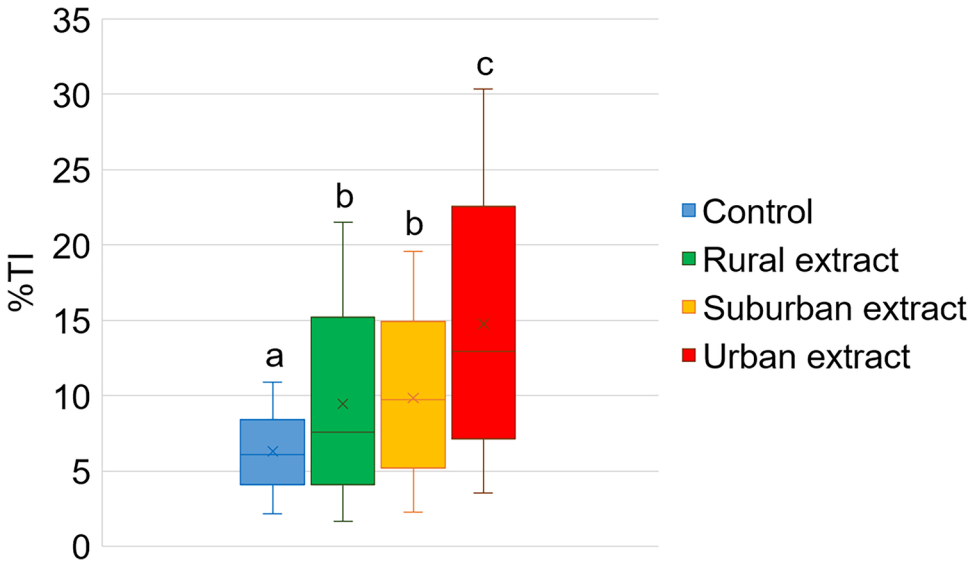

Fig. 2 %TI of larvae treated with organic PM_{10} extracts collected in different sites (tested dose = 40 m^3/mL). Data of larvae treated with DMSO are reported as control. a, b, c = boxplots identified by the same letter do not statistically differ (post hoc analysis with Bonferroni correction)

no marked difference was found between pollutant concentrations of the different sites. This result can be explained by considering that, as reported in previous studies (Gea et al. 2021; Marangon et al. 2021), in urban/suburban sites of the investigated area (the Padana Plain), the concentrations of pollutants have a seasonal trend with higher values in the cold period (October to March), while lower levels are observed in the warm period (April to September). Indeed, during summer, the elevated solar radiation can photodecompose PM components through exposure to ultraviolet light modifying the PM_{10} chemical constituents. On the contrary, in winter, the low temperatures and a lower pollutant dispersion facilitate the absorption of volatile compounds on particle surfaces (Perrone et al. 2010). This leads to a higher concentration of PAHs and nitro-PAHs during wintertime in Torino area (Schilirò et al. 2015; Bonetta et al. 2019). Moreover, this trend is also due to a difference in pollutant emission sources. Indeed, the release of air pollutants is generally lower in the summer months as in these months, there is a lack of domestic heating, a reduction of traffic, and the closure of many industrial and commercial activities, which are among the main sources of PM and PAHs (Kim et al. 2015; Patel et al. 2020). Conversely, in rural sites, pollutant concentrations generally do not show a marked seasonal trend. In fact, at these sites, pollution sources are generally lower and, due to the high altitude, the pollutant dispersion is generally greater than in urban and suburban sites. Moreover, unlike urban and suburban sites, in rural

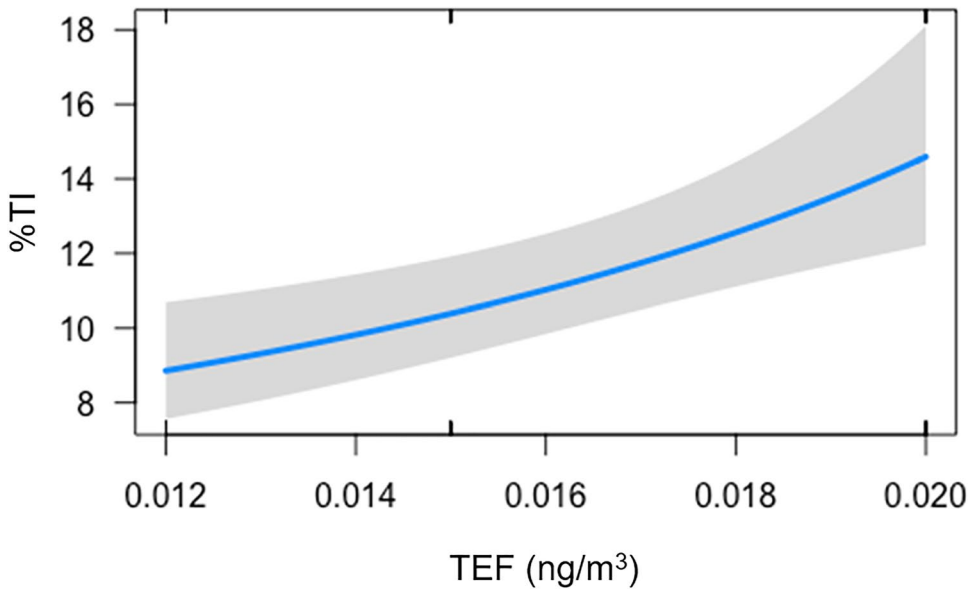

Fig. 3 Graphical results of GLM applied on comet assay data (expressed as %TI) in relation to TEF (as numerical explanatory variable)

sites, the release of pollutants may be greater in the summer months due to the greater influx of tourists (highest rate of tourists in mountain sites in 2019; Piedmont Region 2020).

Despite low pollutant concentrations and little difference between pollutant levels at different sites, PM extracts sampled between April and September were tested in this study on *P. brassicae* butterflies. PM samples were collected only during the larval period (spring/summer) when the investigated sites are characterized by low air pollution (Bonetta et al. 2019). Therefore, in the present study, it was assessed whether this organism was sensitive enough to detect the potential genotoxic effects of PM collected in low polluted periods and in low polluted sites. Moreover, it was studied whether this organism is suitable to detect a different effect between samples containing similar amounts of PM but different chemical composition.

Cabbage butterfly larvae as bioindicator of PM genotoxicity

The genotoxic effect of PM_{10} on *P. brassicae* larvae has been investigated with comet assay in order to assess the suitability of this species as a bioindicator. Butterflies could be good bioindicator organisms; indeed, as pollinators, they provide ecosystem services that are fundamental for ecosystem functioning and indirectly affect human life and well-being (Ghazanfar et al. 2016; Piccini et al. 2018). Among the different butterfly species, *P. brassicae* seems to be advantageous since it is a common and wide distributed butterfly that goes through at least three generations in 1 year accordingly to latitude; hence, it can be easily collected and identified on field. In addition, it is characterized by a fast life cycle and can be reared successfully on many cultivar and hybrids of cabbage (which are easily available); therefore, it can be used for laboratory studies throughout the year and independently of the seasonality of supplies from the wild (Feltwell 1982). Finally, this species lays eggs in large batches (up to 140 eggs/batch) (Higginson et al. 2011), allowing the reduction of genetic differences among individuals, and larvae of *P. brassicae* reach the last larval instar in few days providing large material on which to experiment (Feltwell 1982; Springolo et al. 2021).

The results of the present study support the suitability of *P. brassicae* as bioindicator organism, as this species showed a proper sensitivity to airborne PM (i.e., larvae were not too susceptible to PM exposure but were sensitive enough to show a genotoxic effect directly proportional to PM quality). Indeed, the PM_{10} collected in all the different sites (rural, suburban, and urban sites) induced a significant and increasing DNA damage, in terms of %TI with respect to control.

Although PM concentrations were similar among the three sites, the results of the comet assay showed that %TI was significantly higher after the exposure to the urban traffic extract (i.e., the highest %TI was found in the urban traffic site), suggesting that this butterfly could be considered a sensitive bioindicator to evaluate the genotoxic effect of PM characterized by different chemical composition. Indeed, the different genotoxic effect induced by the three extracts could be due to a different PM composition among sites, as demonstrated by differences in terms of BF, IP, and consequently TEF, which are higher in the urban site with respect to the suburban and rural sites. This aspect was also confirmed by the statistical analysis that showed an increase of %TI with the increase of TEF value.

These results are in accordance with the study of de Santana et al. (2018) that used *D. melanogaster* as model organism to study genotoxicity associated with air pollution exposure, which showed a higher genotoxic effect in animals exposed to the urban area than in ones exposed to the rural area. Moreover, the result is also in accordance with the study of Delgado-Rodríguez et al. (1999), in which a genotoxic activity of PM on insects was demonstrated using the somatic mutation and recombination test in wings of *D. melanogaster*.

Moreover, in the present study, it was demonstrated that the PM collected in months that are characterized by low PM levels (i.e., below the current European air quality standards) and the PM collected in a rural site (i.e., Ceresole Reale, where PM concentrations are even below the WHO guidelines) were able to induce a significant genotoxic effect on a possible bioindicator organism. Similarly, the exposure to low PM doses can induce an effect also in humans. Indeed, as reported by WHO (2021), PM adverse health effects were shown also by studies performed in countries with relatively clean air. In this study, only organic extracts of PM (i.e., organic pollutants) were tested; therefore, the genotoxic effect might be higher due to other inorganic pollutants adsorbed on it (e.g., metals).

Taken together, the sensitivity of *P. brassicae* to air pollutants and all its aforementioned characteristics make this butterfly also suitable for field studies that could be performed on larvae exposed in the wild in different areas. Larvae should be preferred with respect to adults, because they are more sedentary, and thus, it is easier to correlate the detected biological effects to PM exposure. Since larvae are considered pests, in the wild, they could be exposed not only to PM but also to pesticides, thus increasing DNA damage; this could represent a limitation to the use of larvae as air quality bioindicators. This limitation could be partially overcome with the use of butterflies in combination with other model organisms such as plants (Hasanovic et al. 2022).

Finally, the results of the present study highlight that comet assay, although requires the dissection of the insect, was proven to be a suitable assay for the evaluation, in a short time, of the biological effects on larvae due to acute exposure to different PM extracts. The usefulness of this

assay compared to the other genotoxicity tests (e.g., chromosomal aberrations, sister chromatid exchanges, and micronucleus assay) is confirmed by literature. Indeed, its advantages include sensitivity for detection of low levels of DNA damage, reduced number of cells per sample required, and flexibility in using proliferating as well as non-proliferating cells, low cost, easy application, and short time needed to complete a study (Dhawan et al. 2009).

Conclusions

The impact of air pollution on human health is well studied, while air pollution impact on wild insects, including those providing ecosystem services essential for humans, is largely unknown. The use of insects, such as butterflies, for ecotoxicological studies is desirable because insect rearing is inexpensive, and experiments can be performed on large scale in small space and time (Augustyniak et al. 2016). Despite the need to identify new bioindicators, to the best of our knowledge, the use of butterflies as a bioindicator of PM_{10} genotoxic effect has never been investigated before. This study demonstrated that PM collected in different sites is able to induce a different genotoxic effect on butterfly larvae, suggesting that butterfly larvae could be a sensitive and promising bioindicator to investigate the air quality and the genotoxicity of PM collected in sites with different pollution sources. Indeed, they were able:

1. To show a genomic damage induced by PM collected in months that are characterized by low PM levels
2. To detect a genomic damage induced also by PM collected in a rural area characterized by low air pollution
3. To identify a different level of DNA damage depending on the chemical characteristics of PM extract (i.e., PAH concentrations and TEF).

Therefore, butterfly larvae have been proven to be a helpful tool to assess the environmental risks related to PM exposure. Moreover, besides laboratory studies, future research could be performed on field in order to monitor the combined effects of air pollutants and other stressors on wild pollinators. These studies could be important for environmental monitoring considering that wild pollinators are essential for food production, so their decline could indirectly affect human life and well-being. Finally, it is important to underline that environmental monitoring provides crucial data that are used to design policies aimed at improving air quality.

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Data availability Data is contained within the article or Supplementary Material.

Declarations

Ethical approval The experiments comply with the ARRIVE guidelines (Percie du Sert et al. 2020) and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council 2010).

Consent to participate Not applicable.

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