Cabbage butterfly as bioindicator species to investigate the genotoxic effects of PM10

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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 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 PM10 collected in different sites. PM10 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 PM10 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 effect induced by PM sampled also in low-polluted areas.
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Dear Editor,

We are submitting the manuscript *"Cabbage butterfly as bioindicator species to investigate genotoxic effects of PM_{10}"* by Manuela Macrì, Marta Gea, Irene Piccini, Luca Dessì, Alfredo Santovito, Simona Bonelli, Tiziana Schilirò, Sara Bonetta on *ENVIRONMENTAL SCIENCE AND POLLUTION RESEARCH.*

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 generally investigated through *in vitro* assay using prokaryotic/eukaryotic cells and by *in vivo* test with laboratory model animals (rats and mice). 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 common 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 in an urban, a suburban and a rural sites. *P. brassicae* larvae were reared in the laboratory under controlled conditions on cabbage plants and they were exposed to PM_{10} organic extracts or dimethyl sulfoxide (controls) through vaporization. After exposure, larvae were dissected and cells were used for Comet assay to detect DNA damage.

The results of this study demonstrated that PM collected in different sites is able to induce a different genotoxic effects on butterfly larvae, suggesting that butterfly larvae could be a sensitive and promising bioindicator to investigate the air quality and the PM genotoxicity.

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We believe that the paper fits the aims and scope of the Journal, specifically, fits the following subjects:

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We have not submitted our manuscript to a preprint server before submitting it to *ENVIRONMENTAL SCIENCE AND POLLUTION RESEARCH*.

Hoping that the manuscript may fulfil the scientific standards of *ENVIRONMENTAL SCIENCE AND POLLUTION RESEARCH*, our best regards.

Sara Bonetta and Co-authors

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Cabbage butterfly as bioindicator species to investigate 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 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 effect induced by PM sampled also in low-polluted areas.

Keywords: Air pollution; Bioindicator species; Cabbage butterfly; Caterpillars; Comet assay; Particulate matter.

1. Introduction

Particulate matter (PM) is a mixture of solid and liquid particles with different shapes and origin that has an aerodynamic diameter in the range of 0.001–100 μm (Mukherjee and Agrawal 2017). The PM composition is complex, mainly including 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}), that includes particles with aerodynamic diameters ≤ 10 μm, is considered one of the most important air pollution indicators.

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 (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 concentrations of PM (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 using this approach. 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 effects, 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 aimed at assessing 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 (Augustyniak et al. 2016). Indeed, wild pollinators, such as butterfly, are essential for food production. 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 challenge biological systems in a variety of possible ways. Since one of the recognized effects of air pollution and PM is the ability to induce a DNA damage, 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.

One of the most applied bioassays to assess the pollutant genotoxicity is the Comet assay. This method can detect single and double strand breaks and alkali labile sites; it is based on ability of DNA fragments to migrate toward the anode in agarose gel under electrophoresis field, forming the comets. The fluorescence intensity obtained from the comet tail is used as an indicator of the amount of DNA damage (Araldi et al. 2015). Relative to other genotoxicity tests, such as chromosomal aberrations, sister chromatid exchanges, and micronucleus assay, the advantages of the Comet assay include its demonstrated sensitivity for detecting low levels of DNA damage, requirement for small number of cells per sample, flexibility to use proliferating as well as non-proliferating cells, low cost, ease of application, and the short time needed to complete a study (Dhawan et al. 2009).

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. cadium, mercury, agrochemicals etc.) (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.

2. Materials and methods

2.1 PM_{10} collection and extractions

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 1st April 2019 to 30th September 2019. This sampling period was selected because it corresponds to the larval season of *P. brassicae*. 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 (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. 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. The extracts were stored at -20°C until analysis.

2.2 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 1st April 2019 to 30th 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 mixtures as BaP equivalents. 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:

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

2.3 Larval rearing and experimental design

The larvae of *P. brassicae* were used as a bioindicator organism to evaluate the genotoxic effects of PM_{10} extracts. Butterfly eggs were collected in the wild, taken to the laboratory and placed in Petri dishes. The sampling site was the urban garden *Orti generali* of Torino (45°00'43.5"N 7°37'37.4"E). It was selected because here, butterfly eggs/larvae are considered pests and thus they are killed. The day after hatching, the larvae (n = 283) were equally divided into four plants of *Brassica oleracea var. Kapral* corresponding to four different treatments: exposure to 40 m^3/mL of three PM_{10} extracts (rural extract, suburban extract, urban extract) and exposure to DMSO (control treatment). Considering that the daily mean PM_{10} concentrations from 1st April 2019 to 30th September 2019 (PM sampling period), were 11.8 µg/m^3 in the rural site, 18.0 µg/m^3 in the suburban site, and 17.5 µg/m^3 in the urban site, larvae were exposed to 472 µg/mL, 720 µg/mL and 700 µg/mL of PM_{10} organic extracts, respectively. These exposure doses were selected because they are similar to the mean estimate of PM leaf deposition for herbs during summertime (Cai et al. 2017; see Supplementary Materials); moreover, these doses are similar to that generally tested *in vitro* on cell lines (Schilirò et al. 2015 – 200 and 500 µg/mL; Schilirò et al. 2016 – 200 µg/mL). Finally, the three PM_{10} extracts were diluted and tested at doses based on an equivalent volume of sampled air (m^3) instead of equivalent PM mass (µg), in order to simulate the real exposure. Indeed, the three investigated sites are characterized by different levels of PM_{10} (i.e. different PM mass/m^3 of air), so when plants, animals and humans are located in these sites they are exposed to different PM_{10} mass. However, they are exposed to the same amount of air volume.

Plants and larvae were kept 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) and they were treated with dilutions of each PM_{10} extract or DMSO every three days, simulating rainy days during the summer period (≈8 rainy days/month) until the achievement of the last larval stage (8–13 days), thus larvae were exposed to a total of three treatments. 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).

Each treatment with PM_{10} dilutions was performed as follows. The PM_{10} extracts (2000 m^3/mL) from each site were defrosted at room temperature and diluted in commercial water at a final volume of 5 mL (final PM_{10} doses = 40 m^3/mL). The dilution was sprayed near the leaves all around the plant to avoid the diffusion of the dilution in the environment and to assure that the entire plant received the PM_{10} dilution. To avoid cross-contamination among the treatments, each plant was singularly treated outside the climatic chamber.

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).

2.4 Comet assay

The Comet assay was performed according to Tice et al. (2000) with slight modifications (Bonetta et al. 2019). 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. 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) equipped with the Comet Assay IV analysis system (Perceptive Instruments, Instem).

2.5 Statistical analysis

To understand if the exposure to PM_{10} extracts induced a significant genotoxic effect, the %TI (as mean of 100 cells) was modelled 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 modelled in a 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 2014).

3. Results

3.1 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: BA concentration was 0.04 ng/m^3 in all sites and BaP was not detected during the whole period 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, 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.

3.2 Genotoxic effect 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 are 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. 3** 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; **Table S3 and Fig. S1 in Supplementary Materials**).

4. Discussion

4.1 Air pollution data

Overall in the three sites pollutant concentrations (PM and PAHs) were low (generally below the reference limits) and 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 months (October to March), while lower levels are observed in the warm months (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 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.

4.2 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 generation in one 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 in laboratory experimentation 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. These results highlight that Comet assay, although requires the dissection of the insect, allows for evaluation, in a short time, of the biological effects on larvae due to acute exposure to different PM extracts. 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 *Drosophila melanogaster* as model organism to study genotoxicity associated with air pollution exposure, that 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-Rodriguez et al. (1999), in which a genotoxic activity of PM on insects was demonstrated using the somatic mutation and recombination test in wings of *Drosophila 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.

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.

5. 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 effects 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:

i) to show a genomic damage induced by PM collected in months that are characterized by low PM levels;

ii) to detect a genomic damage induced also by PM collected in a rural area characterized by low air pollution;

iii) to identify a different 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.

TITLE PAGE

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

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STATEMENTS AND DECLARATIONS

Ethical Approval

Not applicable

Compliance with Ethical Standards

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

Consent to Publish

Not applicable

Authors Contributions

Conceptualization: Manuela Macrì, Marta Gea, Irene Piccini, Alfredo Santovito, Simona Bonelli, Tiziana Schilirò, Sara Bonetta; Methodology: Manuela Macrì, Marta Gea, Irene Piccini; Formal analysis: Irene Piccini, Luca Dessì; Investigation: Manuela Macrì, Marta Gea, Irene Piccini, Alfredo Santovito, Luca Dessì; Resources: Alfredo Santovito, Simona Bonelli, Tiziana Schilirò, Sara Bonetta; Supervision: Alfredo Santovito, Simona Bonelli, Tiziana Schilirò, Sara Bonetta; Visualization: Manuela Macrì, Marta Gea, Irene Piccini; Writing - original draft: Manuela Macrì, Marta Gea, Irene Piccini; Writing - review & editing: Alfredo Santovito, Simona Bonelli, Tiziana Schilirò, Sara Bonetta.

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Availability of data and materials

Data is contained within the article or Supplementary Material

TABLE CAPTIONS

Table 1 Concentrations of air pollutants in the three sites from 1st April 2019 to 30th September 2019 (larval season of *P. brassicae*). Data are reported as mean ± standard deviations

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

FIGURE CAPTIONS

Fig. 1 Localization of the PM_{10} sampling sites

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)

Fig. 3 Examples of cells with a different DNA damage (comets) detected after exposing butterfly larvae to PM extracts (photos taken during the present study)

Graphical Abstract

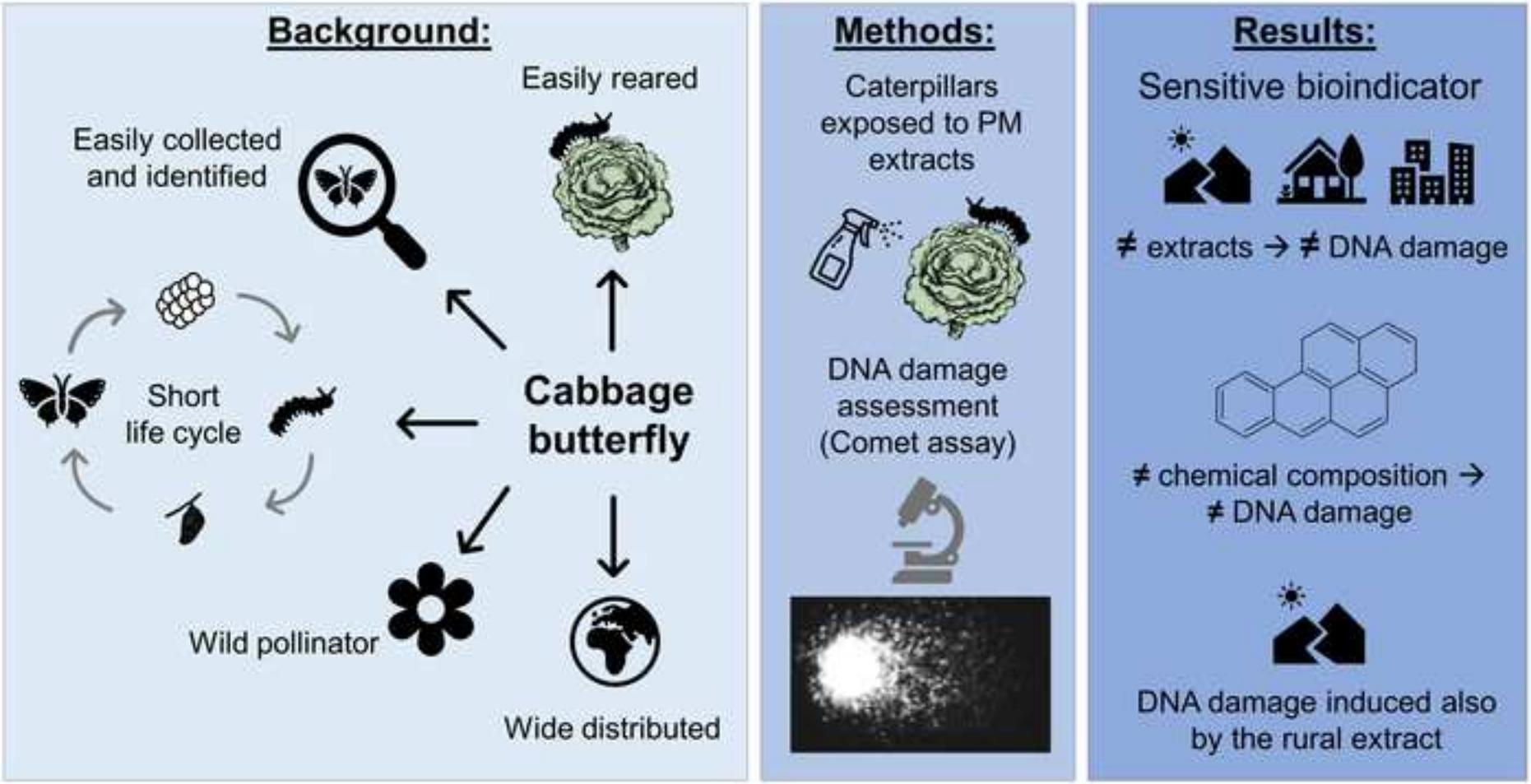

Figure 1

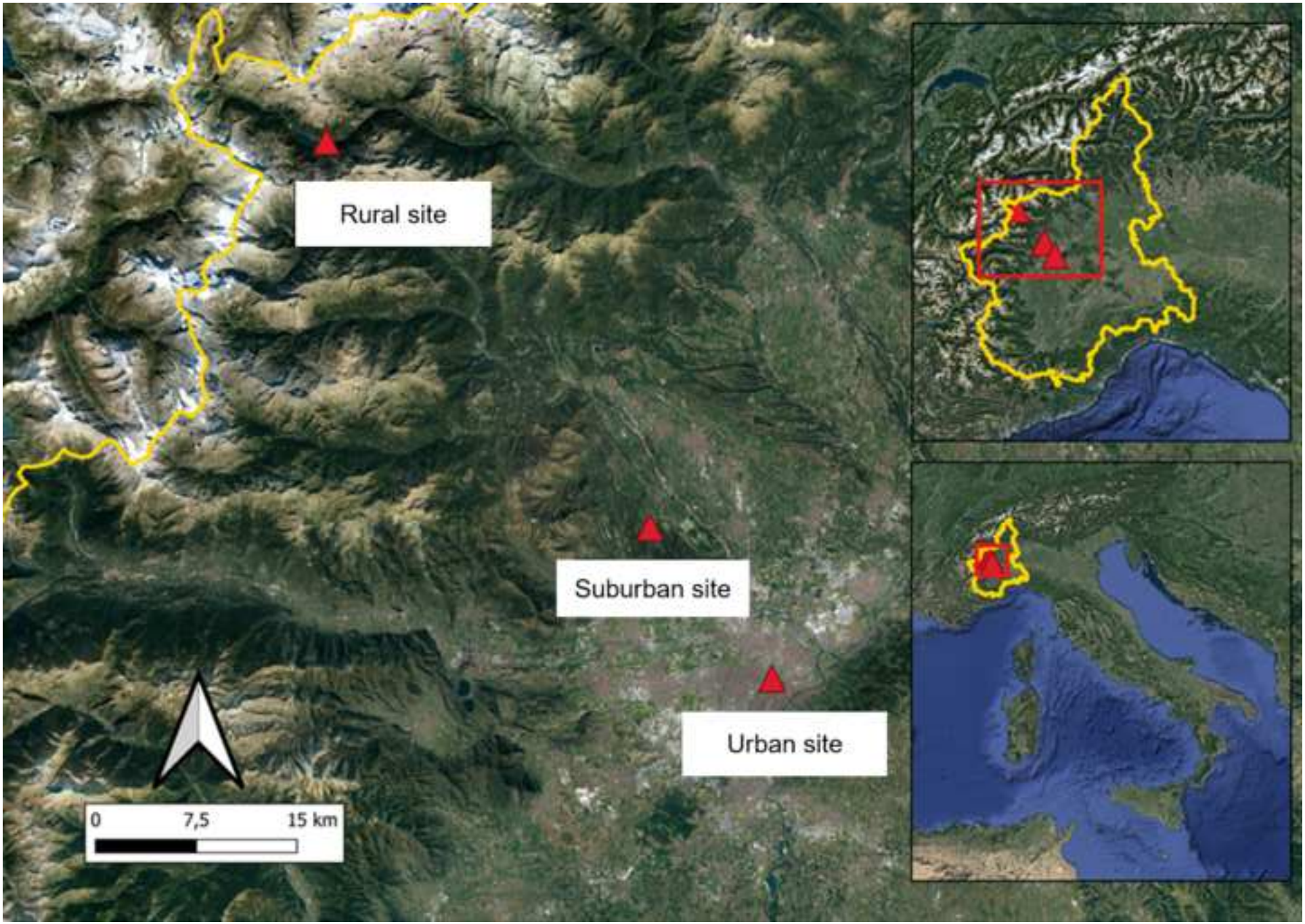

Figure 2

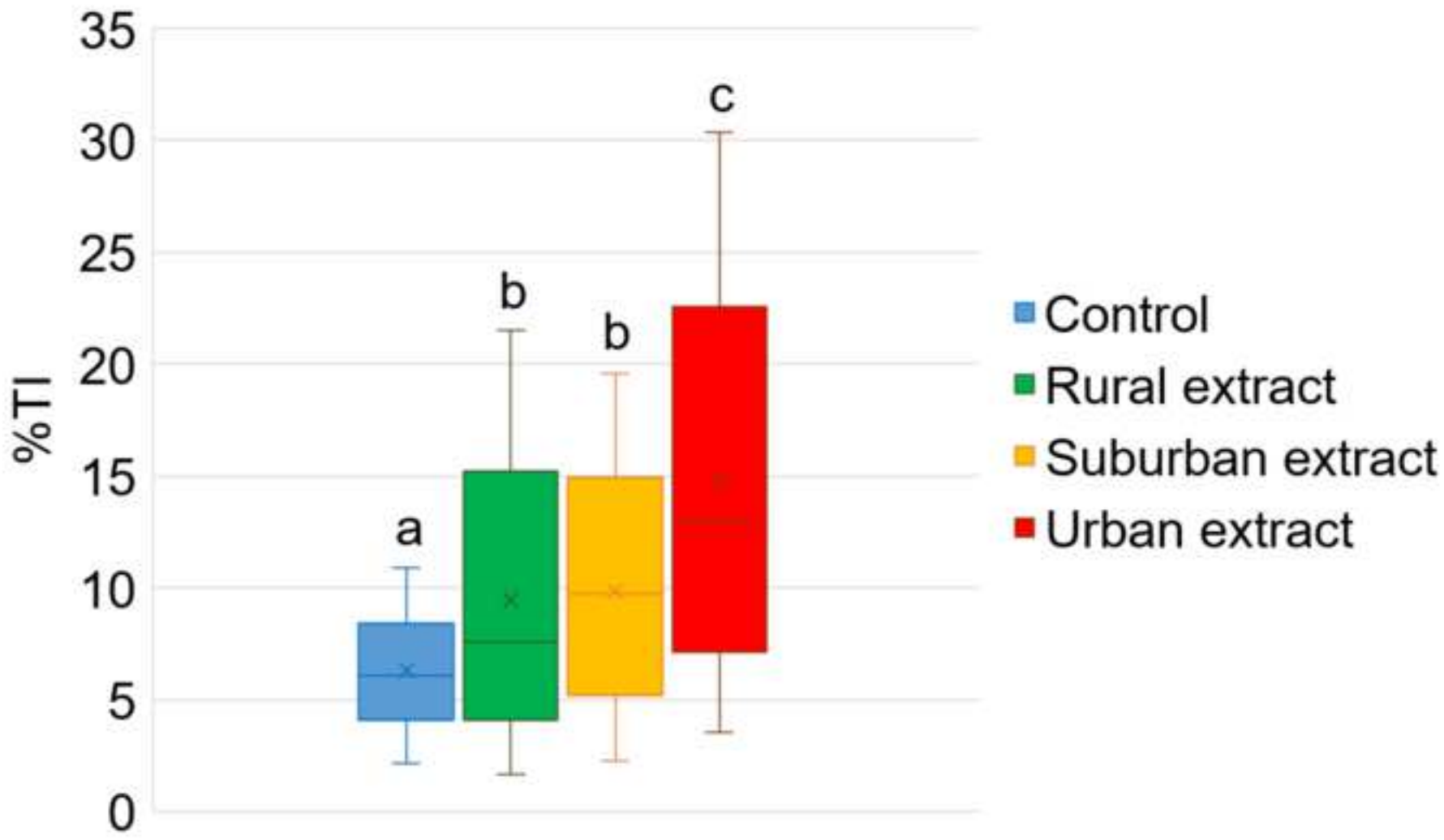

Figure 3

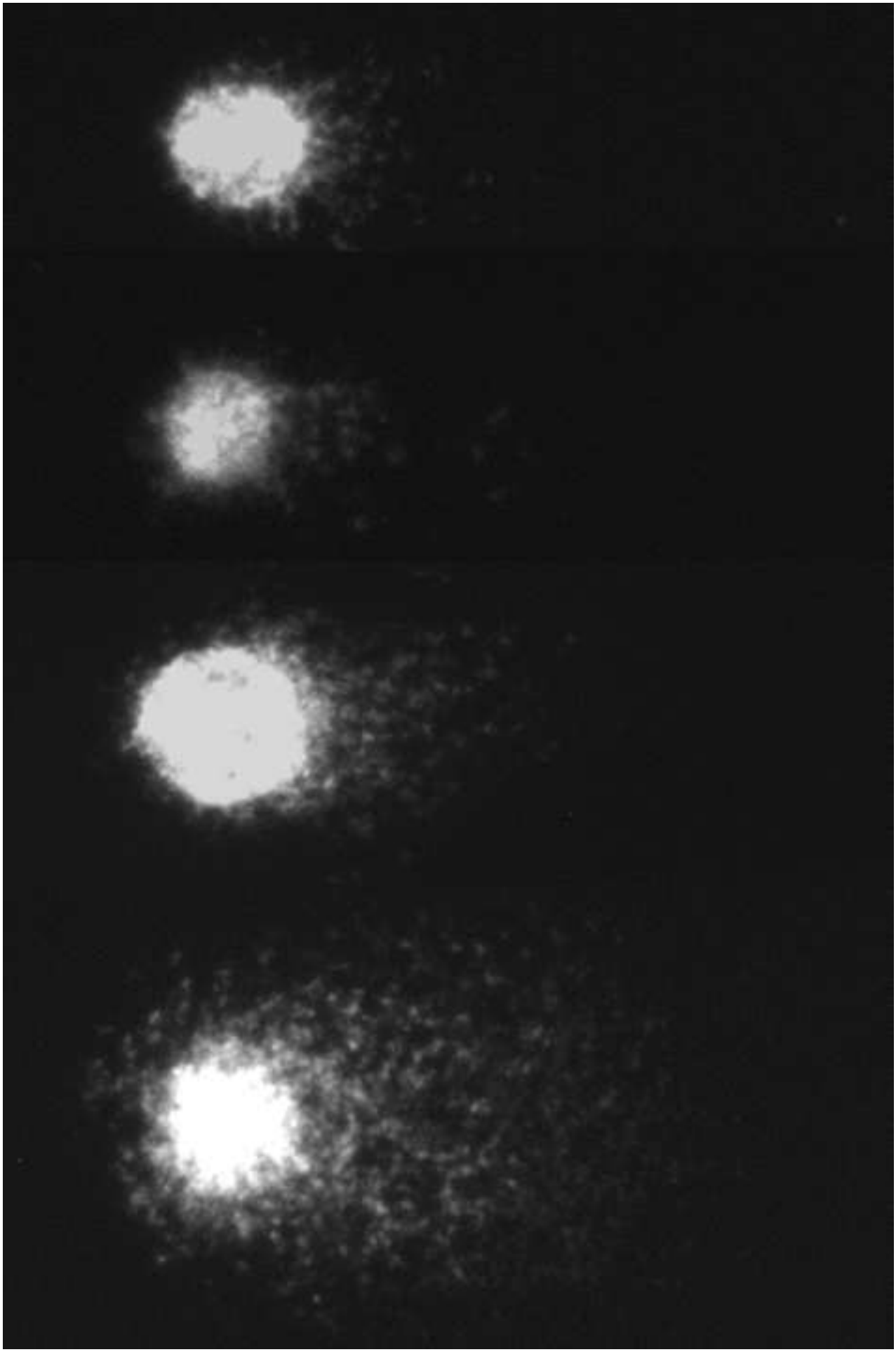

Table 1

Table 1 Concentrations of air pollutants in the three sites from 1st April 2019 to 30th September 2019 (larval season of *P. brassicae*). Data are reported as mean ± standard deviations

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	< lod	0.040 ± 0.001	0.040 ± 0.001	0.040 ± 0.001	0.012
Suburban site	18.0 ± 7.5	< lod	0.040 ± 0.001	0.065 ± 0.043	0.045 ± 0.008	0.015
Urban site	17.5 ± 5.0	< lod	0.040 ± 0.001	0.093 ± 0.078	0.063 ± 0.036	0.020

lod = limit of detection

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

[a] = larvae with less than 100 cells suitable to score the %TI were excluded; SD = standard deviation