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# Environmental Science and Pollution Research

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

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<b>Corresponding Author:</b>	Sara Bonetta University of Torino Torino, ITALY
<b>Corresponding Author Secondary Information:</b>	
<b>Corresponding Author's Institution:</b>	University of Torino
<b>Corresponding Author's Secondary Institution:</b>	
<b>First Author:</b>	Manuela Macri
<b>First Author Secondary Information:</b>	
<b>Order of Authors:</b>	Manuela Macri Marta Gea Irene Piccini Luca Dessi Alfredo Santovito Simona Bonelli Tiziana Schilirò Sara Bonetta
<b>Order of Authors Secondary Information:</b>	
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<b>Abstract:</b>	<p>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 (<i>Pieris brassicae</i>) 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. <i>P. brassicae</i> 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 <i>P. brassicae</i>, 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.</p>
<b>Suggested Reviewers:</b>	Marko Geric

	<p>Institute for Medical Research and Occupational Health, Zagreb, Croatia  mgeric@imi.hr  Expert in genetic and environmental toxicology. Comet assay expert</p>
	<p>Annamaria Buschini  University of Parma: Universita degli Studi di Parma  annamaria.buschini@unipr.it  Expert in genetic toxicology, research field in quantitative/qualitative evaluation of genetic hazard with Comet assay.</p>
	<p>Carina Ladeira  Instituto Politecnico de Lisboa Escola Superior de Tecnologia da Saude de Lisboa  carina.ladeira@estesl.ipl.pt  Expert in the field of genetic toxicology.</p>
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UNIVERSITÀ DEGLI STUDI DI TORINO  
DIPARTIMENTO DI SCIENZE DELLA SANITA' PUBBLICA E PEDIATRICHE  
DEPARTMENT OF PUBLIC HEALTH AND PEDIATRICS

DIRETTORE: PROF. SSA CARLA MARIA ZOTTI  
Piazza Polonia, 94 – 10126 Torino (Italia)  
Codice Fiscale 80088230018 – P.IVA IT02099550010

16<sup>th</sup> November 2022

Dear Editor,

We are submitting the manuscript "*Cabbage butterfly as bioindicator species to investigate genotoxic effects of PM<sub>10</sub>*" 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<sub>10</sub> collected in different sites.

PM<sub>10</sub> 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<sub>10</sub> 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.

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

- Environmental Analyses and Monitoring
- Impact of Chemicals/Pollutants on Human and Animal Health

All of the authors have read and approved the paper and it has not been published previously nor is it being considered by any other peer-reviewed journal. All authors are aware of and accept responsibility for the manuscript. All figures and tables were produced by the authors. Lastly, all authors declare no conflicting interests.

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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1 **Cabbage butterfly as bioindicator species to investigate genotoxic effects of PM<sub>10</sub>**

2 Manuela Macrì<sup>1a</sup>, Marta Gea<sup>2a</sup>, Irene Piccini<sup>1</sup>, Luca Dessì<sup>1</sup>, Alfredo Santovito<sup>1</sup>, Simona Bonelli<sup>1</sup>, Tiziana  
3 Schilirò<sup>2b</sup>, Sara Bonetta<sup>2b\*</sup>

4

5 <sup>1</sup>Department of Life Sciences and Systems Biology, University of Torino, via Accademia Albertina 13, 10123  
6 Torino, Italy

7 <sup>2</sup>Department of Public Health and Pediatrics, University of Torino, via Santena 5 bis, 10126 Torino, Italy

8

9 <sup>a</sup>These authors equally contributed to the research

10 <sup>b</sup>These authors equally contributed to the research

11 \* Corresponding author:

12 Sara Bonetta

13 sara.bonetta@unito.it

14 Department of Public Health and Pediatrics,

15 University of Torino,

16 Via Santena 5 bis, 10126 Torino, Italy.

17 Phone: +39 0116708192

18

19 **Abstract**

20 Atmospheric pollution poses a serious threat to environment and human health and particulate matter (PM) is  
21 one of the major contributors. Biological effects induced by PM are investigated through *in vitro* assays using  
22 cells and by *in vivo* tests with laboratory model animals. However, also the estimation of adverse effects of  
23 pollutants, including airborne ones, on wild animals, such as insects, is an essential component of  
24 environmental risk assessment. Among insects, butterflies are sensitive to environmental changes and are  
25 important wild pollinators, so might be suitable as environmental bioindicator species. The aim of this study  
26 was to evaluate the suitability of a wild cabbage butterfly species (*Pieris brassicae*) as a bioindicator organism  
27 to assess the genotoxic effects of PM<sub>10</sub> collected in different sites. PM<sub>10</sub> was collected from April to September  
28 in urban, suburban and rural sites. *P. brassicae* larvae were reared in laboratory under controlled conditions

29 on cabbage plants and exposed to PM<sub>10</sub> organic extracts or dimethyl sulfoxide (controls) through vaporization.  
30 After exposure, larvae were dissected and cells were used for Comet assay. All PM extracts induced significant  
31 DNA damage in exposed larvae compared to controls and the extract collected in the most polluted site caused  
32 the highest genotoxic effect. In conclusion, the study suggested that butterflies, such as *P. brassicae*, could be  
33 applied as sensitive and promising bioindicators to investigate air quality and PM genotoxicity. Indeed, the use  
34 of these organisms allows the detection of genotoxic effect induced by PM sampled also in low-polluted areas.

35

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

38

## 39 1. Introduction

40 Particulate matter (PM) is a mixture of solid and liquid particles with different shapes and origin that has an  
41 aerodynamic diameter in the range of 0.001–100 µm (Mukherjee and Agrawal 2017). The PM composition is  
42 complex, mainly including inorganic ions, organic pollutants, metals, and other harmful compounds that can  
43 be toxic for organisms, such as polycyclic aromatic hydrocarbons (PAHs). Atmospheric inhalable PM (PM<sub>10</sub>),  
44 that includes particles with aerodynamic diameters ≤ 10 µm, is considered one of the most important air  
45 pollution indicators.

46 Epidemiological studies highlighted that the long-term exposure to PM<sub>10</sub> increases risk of chronic bronchitis,  
47 coronary events, chronic kidney disease, type 2 diabetes, and cancer mortality, while the short-term exposure  
48 to PM<sub>10</sub> was associated with cardiovascular and respiratory mortality (Rojas-Rueda et al. 2021). The  
49 International Agency for Research on Cancer (IARC) designated PM as a Group I carcinogen (IARC 2016).

50 In order to protect human health, current European air quality Directive and World Health Organisation  
51 (WHO) guidelines establish limit/guideline values for concentrations of PM (PM<sub>10</sub> or PM<sub>2.5</sub>) and for  
52 concentration of other air pollutants that can be adsorbed on PM (e.g. benzo(a)pyrene – BaP, one of the most  
53 toxic PAHs) (European Commission Directive 2004/107/EC; European Commission Directive 2008/50/EC;  
54 WHO 2021). Although the environmental and health effects induced by PM are related to its concentration  
55 and to its chemical composition, the PM effect cannot be easily deduced using this approach. Indeed, PM is a  
56 complex chemical mixture, which changes according to emission sources, season, sampling site characteristics

57 and photochemical-meteorological conditions (Topinka et al. 2015; Pongpiachan et al. 2017), so it is not  
58 possible to quantify all chemicals on it. Moreover, the effects of all pollutants and of their metabolites are not  
59 always known and, in addition, synergistic/antagonistic interactions could occur among them, causing  
60 altogether an unpredictable biological effect.

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

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

81 In particular, butterflies could represent a valuable bioindicator to study environmental risks of PM. Indeed,  
82 butterfly larvae are phytophagous so they can be exposed to PM through direct contact but also through  
83 ingestion of PM settled on leaves. Moreover, some butterfly species are easy to grow, easy to manipulate and



84 are ubiquitous, so they could be reared in laboratory and experimentally exposed to PM but they could also be  
85 sampled in the wild after their natural exposure to environmental PM.

86 PM could challenge biological systems in a variety of possible ways. Since one of the recognized effects of air  
87 pollution and PM is the ability to induce a DNA damage, the genotoxicity can be an interesting sub-lethal  
88 effect that could be evaluated on sentinel organisms, giving important information on the ability of air pollution  
89 to affect species and functionality of ecosystems.

90 One of the most applied bioassays to assess the pollutant genotoxicity is the Comet assay. This method can  
91 detect single and double strand breaks and alkali labile sites; it is based on ability of DNA fragments to migrate  
92 toward the anode in agarose gel under electrophoresis field, forming the comets. The fluorescence intensity  
93 obtained from the comet tail is used as an indicator of the amount of DNA damage (Araldi et al. 2015). Relative  
94 to other genotoxicity tests, such as chromosomal aberrations, sister chromatid exchanges, and micronucleus  
95 assay, the advantages of the Comet assay include its demonstrated sensitivity for detecting low levels of DNA  
96 damage, requirement for small number of cells per sample, flexibility to use proliferating as well as non-  
97 proliferating cells, low cost, ease of application, and the short time needed to complete a study (Dhawan et al.  
98 2009).

99 Although, in recent years, the Comet assay was applied on different insect species used as bioindicators (e.g.  
100 *Drosophila melanogaster*, *Spodoptera exigua*, *Ceraeochrysa claveri*, *Bombus atratus*) to evaluate the effect  
101 induced by environmental contaminants (e.g. cadmium, mercury, agrochemicals etc.) (Augustyniak et al. 2016;  
102 de Santana et al. 2018; Gajski et al. 2019; Gastelbondo-Pastrana et al. 2019; Ceschi-Bertoli et al. 2020), the  
103 possible use of insect species as bioindicators of genotoxicity induced by PM with different origin and  
104 characteristics has been poorly explored (de Santana et al. 2018). In particular, to the best of our knowledge,  
105 the possible application of butterflies as a bioindicator to assess PM environmental risks has never been  
106 studied.

107 The aim of this study was to evaluate the usefulness of a common and widespread wild butterfly species, *Pieris*  
108 *brassicae*, as bioindicator organism for investigating the genotoxic effects induced by PM<sub>10</sub> samples. In  
109 particular, this species has a wide distribution from North Africa across Europe and Asia and is able to live in  
110 different habitats also located at different altitudes (Feltwell 1982). Larvae of *P. brassicae* (hatched from field  
111 collected eggs) were exposed in laboratory to organic extracts of PM<sub>10</sub> sampled in different sites (with different

112 pollution levels) in order to test the butterfly sensitivity at increasing levels of pollution. After exposure, the  
113 larvae were sacrificed and the genomic damage was evaluated using the Comet assay.

114

## 115 **2. Materials and methods**

### 116 **2.1 PM<sub>10</sub> collection and extractions**

117 PM<sub>10</sub> was collected from three monitoring stations of the Regional Agency for Environmental Protection of  
118 Piedmont (ARPA Piemonte) located within the Padana Plain in Northern Italy: Torino (urban traffic site,  
119 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  
120 (rural site, 45°25'48.7"N, 7°14'43.5"E) (**Fig. 1**). The stations are part of a monitoring network, which was  
121 designed by the Italian government in order to monitor the air quality as required by the European legislation  
122 (European Commission Directive 2008/50/EC; Italian Legislative Decree 155/2010). For each site, PM<sub>10</sub> was  
123 daily collected on quartz-fiber filters (Ø = 47 mm) using low volume samplers (flow = 2.3 m<sup>3</sup>/h) from 1<sup>st</sup> April  
124 2019 to 30<sup>th</sup> September 2019. This sampling period was selected because it corresponds to the larval season of  
125 *P. brassicae*. Daily filters were pooled to obtain one sample for each site (183 filter quarters for each site) and  
126 each pool was chemically extracted in order to collect organic-extractable compounds (Schilirò et al. 2016).  
127 Briefly, filter quarters of each pool were cut in small pieces, placed in a glass beaker and washed three times  
128 with acetone/cyclohexane (1:1) using an ultrasonic water bath. Then, filters and solvent (250 mL) were  
129 transferred in tubes, vortexed for 1 min and centrifuged at 4100 rpm for 10 min in order to remove filter debris.  
130 The supernatant was then evaporated using a rotary evaporator and re-suspended in dimethyl sulfoxide  
131 (DMSO) at a final concentration of 2000 m<sup>3</sup>/mL. The extracts were stored at -20°C until analysis.

132

### 133 **2.2 Air pollution data**

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

135 Pollution data of each sampling site were collected from the ARPA Piemonte website (ARPA 2022). The mean  
136 concentrations of PM<sub>10</sub> and four PAHs [BaP, benzo(a)anthracene (BA), benzo(b+j+k)fluoranthene (BF) and  
137 indeno(1,2,3-cd)pyrene (IP)] were calculated from 1<sup>st</sup> April 2019 to 30<sup>th</sup> September 2019, according to the  
138 larval season of *P. brassicae*.

139 PAHs data were used to obtain the Toxic Equivalency Factor (TEF), which expresses the toxicity of PAH  
140 mixtures as BaP equivalents. Considering the carcinogenic potencies of PAHs in comparison to BaP (i.e. the  
141 reference PAH) (Nisbet and La Goy 1992; Samburova et al. 2017), TEF was calculated as:

$$142 \quad TEF = BaP \times 1 + BA \times 0.1 + BF \times 0.1 + IP \times 0.1$$

143

### 144 **2.3 Larval rearing and experimental design**

145 The larvae of *P. brassicae* were used as a bioindicator organism to evaluate the genotoxic effects of PM<sub>10</sub>  
146 extracts. Butterfly eggs were collected in the wild, taken to the laboratory and placed in Petri dishes. The  
147 sampling site was the urban garden *Orti generali* of Torino (45°00'43.5"N 7°37'37.4"E). It was selected  
148 because here, butterfly eggs/larvae are considered pests and thus they are killed. The day after hatching, the  
149 larvae ( $n = 283$ ) were equally divided into four plants of *Brassica oleracea* var. *Kapral* corresponding to four  
150 different treatments: exposure to 40 m<sup>3</sup>/mL of three PM<sub>10</sub> extracts (rural extract, suburban extract, urban  
151 extract) and exposure to DMSO (control treatment). Considering that the daily mean PM<sub>10</sub> concentrations from  
152 1<sup>st</sup> April 2019 to 30<sup>th</sup> September 2019 (PM sampling period), were 11.8 µg/m<sup>3</sup> in the rural site, 18.0 µg/m<sup>3</sup> in  
153 the suburban site, and 17.5 µg/m<sup>3</sup> in the urban site, larvae were exposed to 472 µg/mL, 720 µg/mL and 700  
154 µg/mL of PM<sub>10</sub> organic extracts, respectively. These exposure doses were selected because they are similar to  
155 the mean estimate of PM leaf deposition for herbs during summertime (Cai et al. 2017; see Supplementary  
156 Materials); moreover, these doses are similar to that generally tested *in vitro* on cell lines (Schilirò et al. 2015  
157 – 200 and 500 µg/mL; Schilirò et al. 2016 – 200 µg/mL). Finally, the three PM<sub>10</sub> extracts were diluted and  
158 tested at doses based on an equivalent volume of sampled air (m<sup>3</sup>) instead of equivalent PM mass (µg), in order  
159 to simulate the real exposure. Indeed, the three investigated sites are characterized by different levels of PM<sub>10</sub>  
160 (i.e. different PM mass/m<sup>3</sup> of air), so when plants, animals and humans are located in these sites they are  
161 exposed to different PM<sub>10</sub> mass. However, they are exposed to the same amount of air volume.

162 Plants and larvae were kept in four separated net cages in a climate cell at 26 °C L:D 15:9 (as reported by  
163 Santovito et al. 2020 and Piccini et al. 2021) and they were treated with dilutions of each PM<sub>10</sub> extract or  
164 DMSO every three days, simulating rainy days during the summer period (≈8 rainy days/month) until the  
165 achievement of the last larval stage (8–13 days), thus larvae were exposed to a total of three treatments. The

166 plants were watered every 2–3 days and replaced every 5 days because they were completely eaten by larvae  
167 (two plants were used for each extract).

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

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

177

#### 178 **2.4 Comet assay**

179 The Comet assay was performed according to Tice et al. (2000) with slight modifications (Bonetta et al. 2019).  
180 After exposure, the head and caudal parts of each larva were gently mixed in 100 µL of low melting point  
181 agarose (LMP 0.7%). LMP agarose containing the disaggregated cells of the larvae (20 µL) was placed twice  
182 on microscope slides coated with 1% of normal melting agarose, with additional LMP agarose added as the  
183 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  
184 disodium salt dihydrate, 1% TRITON X-100 and 10% DMSO, pH 10), immersed in an alkaline electrophoresis  
185 buffer (10 mM EDTA tetrasodium salt dihydrate, 300 mM NaOH, 10% DMSO, pH > 13) for 20 min and  
186 subjected to electrophoresis in the same buffer (20 min, 1 V/cm and 300 mA). Then, slides were neutralized  
187 for 3 min using a neutralization buffer (0.4 M Tris-HCl, pH 7.5, 4°C), fixed using ethanol 70% (–20°C), and  
188 dried. For the analysis of DNA damage, the DNA of the cells was stained with ethidium bromide (20 µg/mL)  
189 and the percentage of DNA in the tail (%TI) of 100 cells for each larva was estimated using a fluorescence  
190 microscope (Axioskop HBO 50, Zeiss) equipped with the Comet Assay IV analysis system (Perceptive  
191 Instruments, Instem).

192

193

194 **2.5 Statistical analysis**

195 To understand if the exposure to PM<sub>10</sub> extracts induced a significant genotoxic effect, the %TI (as mean of 100  
196 cells) was modelled in a generalized linear mixed model (GLMM) with the site as categorical explanatory  
197 variable and egg batch as numerical explanatory variable as a random factor. Moreover, to understand the  
198 effects of TEF, we excluded controls and %TI (as mean of 100 cells) was modelled in a GLM with the TEF as  
199 numerical explanatory variable. In both models, the reference category was the control and individuals with  
200 count less than 100 cells (5 individuals) were excluded from the analysis. Considering that residuals were not  
201 normally distributed, Gamma distribution family was used in models (Zuur et al. 2009). Then, a post hoc  
202 analysis with Bonferroni correction was applied (Zuur et al. 2009). The model was fitted with the ‘lme4’ R  
203 package in R software (R Development Core Team 2014).

204

205 **3. Results**

206 **3.1 Air pollution data**

207 Air pollution data in the three sites are reported in **Table 1**. The mean PM<sub>10</sub> concentrations measured in the  
208 urban and in the suburban sites were similar, while the lowest mean PM<sub>10</sub> concentration was measured in the  
209 rural site. The PM<sub>10</sub> concentrations of the three sites were below the Italian/European limit value (PM<sub>10</sub> annual  
210 limit value = 40 µg/m<sup>3</sup>) (European Commission Directive 2008/50/EC; Italian Legislative Decree 155/2010).  
211 However, only the PM<sub>10</sub> concentration measured in the rural site was below the annual guideline level set by  
212 the WHO (PM<sub>10</sub> annual guideline level = 15 µg/m<sup>3</sup>) (WHO 2021).

213 Regarding PAH concentrations, BaP and BA concentrations were equivalent in the three sites: BA  
214 concentration was 0.04 ng/m<sup>3</sup> in all sites and BaP was not detected during the whole period complying with  
215 Italian/European target value (BaP annual target value = 1 ng/m<sup>3</sup>) (European Commission Directive  
216 2004/107/EC; Italian Legislative Decree 155/2010). On the contrary, analyzing the other PAHs, a  
217 concentration trend in agreement with the site type (rural, suburban, urban) was found in the three sites; indeed,  
218 the highest BF, IP and TEF concentrations were measured in the urban site while the lowest concentrations  
219 were found in the rural site.

220

221

### 222 **3.2 Genotoxic effect of PM<sub>10</sub> extracts on larvae assessed by Comet assay**

223 The number of larvae involved in the experiment and finally used for the Comet assay are reported in **Table**  
224 **2**, together with the larval weight. Mean larval weight was not affected by PM treatment.

225 The results of the Comet assay are reported in **Fig. 2**, while in **Fig. 3** some examples of comets are shown. The  
226 statistical analysis showed that the DNA damage, expressed as %TI, was higher for larvae exposed to all  
227 treatments with respect to control (control: mean %TI = 6.30% ± 2.62%; rural extract: mean %TI = 9.44% ±  
228 6.00%, t value = -3.245, p = 0.0012; suburban extract: mean %TI = 9.83% ± 4.80%, t value = -3.045, p =  
229 0.0023; urban extract: mean %TI = 14.75% ± 8.27%, t value = -4.549, p<0.001; **Table S1 Supplementary**  
230 **Materials**). This result was confirmed by the post hoc analysis with Bonferroni correction; indeed, all  
231 treatments (rural, suburban and urban extracts) induced significantly higher %TI than those induced by control  
232 (rural extract: z value = -3.245, p = 0.0070; suburban extract: z value = -3.045, p = 0.0140; urban extract: z  
233 value = -4.549, p<0.001; **Table S2 in Supplementary Materials**). Finally, the post hoc analysis with  
234 Bonferroni correction highlighted that the urban extract induced on larvae a higher DNA damage with respect  
235 to the rural extract (z value = -2.978, p = 0.0174) and the suburban extract (z value = -3.014, p=0.0155; **Table**  
236 **S2 in Supplementary Materials**).

237 In addition, the GLM analysis highlighted that the mean %TI increased with an increase of TEF (t value = -  
238 3.468, p<0.001; **Table S3 and Fig. S1 in Supplementary Materials**).

239

## 240 **4. Discussion**

### 241 **4.1 Air pollution data**

242 Overall in the three sites pollutant concentrations (PM and PAHs) were low (generally below the reference  
243 limits) and no marked difference was found between pollutant concentrations of the different sites. This result  
244 can be explained by considering that, as reported in previous studies (Gea et al. 2021; Marangon et al. 2021),  
245 in urban/suburban sites of the investigated area (the Padana Plain) the concentrations of pollutants have a  
246 seasonal trend with higher values in the cold months (October to March), while lower levels are observed in  
247 the warm months (April to September). Indeed, during summer the elevated solar radiation can  
248 photodecompose PM components through exposure to ultraviolet light modifying the PM<sub>10</sub> chemical  
249 constituents. On the contrary, in winter, the low temperatures and a lower pollutant dispersion facilitate the

250 absorption of volatile compounds on particle surfaces (Perrone et al. 2010). This leads to a higher concentration  
251 of PAHs and nitro-PAHs during wintertime in Torino area (Schilirò et al. 2015; Bonetta et al. 2019). Moreover,  
252 this trend is also due to a difference in pollutant emission sources. Indeed, the release of air pollutants is  
253 generally lower in the summer months as in these months there is a lack of domestic heating, a reduction of  
254 traffic and the closure of many industrial and commercial activities, which are among the main sources of PM  
255 and PAHs (Kim et al. 2015; Patel et al. 2020). Conversely, in rural sites, pollutant concentrations generally do  
256 not show a marked seasonal trend. In fact, at these sites pollution sources are generally lower and, due to the  
257 high altitude, the pollutant dispersion is generally greater than in urban and suburban sites. Moreover, unlike  
258 urban and suburban sites, in rural sites the release of pollutants may be greater in the summer months due to  
259 the greater influx of tourists (highest rate of tourists in mountain sites in 2019; Piedmont Region 2020).

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

267

#### 268 **4.2 Cabbage butterfly larvae as bioindicator of PM genotoxicity**

269 The genotoxic effect of PM<sub>10</sub> on *P. brassicae* larvae has been investigated with Comet assay in order to assess  
270 the suitability of this species as a bioindicator. Butterflies could be good bioindicator organisms, indeed, as  
271 pollinators, they provide ecosystem services that are fundamental for ecosystem functioning and indirectly  
272 affect human life and well-being (Ghazanfar et al. 2016; Piccini et al. 2018). Among the different butterfly  
273 species, *P. brassicae* seems to be advantageous since it is a common and wide distributed butterfly that goes  
274 through at least three generation in one year accordingly to latitude, hence it can be easily collected and  
275 identified on field. In addition, it is characterized by a fast life cycle and can be reared successfully on many  
276 cultivar and hybrids of cabbage (which are easily available), therefore it can be used for in laboratory  
277 experimentation throughout the year and independently of the seasonality of supplies from the wild (Feltwell

278 1982). Finally, this species lays eggs in large batches (up to 140 eggs/batch) (Higginson et al. 2011), allowing  
279 the reduction of genetic differences among individuals and larvae of *P. brassicae* reach the last larval instar in  
280 few days providing large material on which to experiment (Feltwell 1982; Springolo et al. 2021).

281 The results of the present study support the suitability of *P. brassicae* as bioindicator organism, as this species  
282 showed a proper sensitivity to airborne PM (i.e. larvae were not too susceptible to PM exposure but were  
283 sensitive enough to show a genotoxic effect directly proportional to PM quality). Indeed, the PM<sub>10</sub> collected  
284 in all the different sites (rural, suburban and urban sites) induced a significant and increasing DNA damage, in  
285 terms of %TI with respect to control. These results highlight that Comet assay, although requires the dissection  
286 of the insect, allows for evaluation, in a short time, of the biological effects on larvae due to acute exposure to  
287 different PM extracts. Although PM concentrations were similar among the three sites, the results of the Comet  
288 assay showed that %TI was significantly higher after the exposure to the urban traffic extract (i.e. the highest  
289 %TI was found in the urban traffic site), suggesting that this butterfly could be considered a sensitive  
290 bioindicator to evaluate the genotoxic effect of PM characterized by different chemical composition. Indeed,  
291 the different genotoxic effect induced by the three extracts could be due to a different PM composition among  
292 sites, as demonstrated by differences in terms of BF, IP and consequently TEF, which are higher in the urban  
293 site with respect to the suburban and rural sites. This aspect was also confirmed by the statistical analysis that  
294 showed an increase of %TI with the increase of TEF value.

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

301 Moreover, in the present study, it was demonstrated that the PM collected in months that are characterized by  
302 low PM levels (i.e. below the current European air quality standards) and the PM collected in a rural site (i.e.  
303 Ceresole Reale, where PM concentrations are even below the WHO guidelines) were able to induce a  
304 significant genotoxic effect on a possible bioindicator organism. Similarly, the exposure to low PM doses can



305 induce an effect also in humans. Indeed, as reported by WHO (2021), PM adverse health effects were shown  
306 also by studies performed in countries with relatively clean air.

307 Taken together, the sensitivity of *P. brassicae* to air pollutants and all its aforementioned characteristics make  
308 this butterfly also suitable for field studies that could be performed on larvae exposed in the wild in different  
309 areas. Larvae should be preferred with respect to adults, because they are more sedentary and thus it is easier  
310 to correlate the detected biological effects to PM exposure.

311

## 312 **5. Conclusions**

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

- 322 i) to show a genomic damage induced by PM collected in months that are characterized by low PM levels;
- 323 ii) to detect a genomic damage induced also by PM collected in a rural area characterized by low air pollution;
- 324 iii) to identify a different DNA damage depending on the chemical characteristics of PM extract (i.e. PAH  
325 concentrations and TEF).

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

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**TITLE PAGE**

**Cabbage butterfly as bioindicator species to investigate genotoxic effects of PM<sub>10</sub>**

Manuela Macri<sup>1a</sup>, Marta Gea<sup>2a</sup>, Irene Piccini<sup>1</sup>, Luca Dessì<sup>1</sup>, Alfredo Santovito<sup>1</sup>, Simona Bonelli<sup>1</sup>,  
Tiziana Schilirò<sup>2b</sup>, Sara Bonetta<sup>2b\*</sup>

<sup>1</sup>Department of Life Sciences and Systems Biology, University of Torino, via Accademia Albertina  
13, 10123 Torino, Italy

<sup>2</sup>Department of Public Health and Pediatrics, University of Torino, via Santena 5 bis, 10126 Torino,  
Italy

<sup>a</sup>These authors equally contributed to the research

<sup>b</sup>These authors equally contributed to the research

\* Corresponding author:

Sara Bonetta

sara.bonetta@unito.it

Department of Public Health and Pediatrics,

University of Torino,

Via Santena 5 bis, 10126 Torino, Italy.

Phone: +39 0116708192

**Other e-mail addresses:**

manuela.macri@unito.it

marta.gea@unito.it

irene.piccini@unito.it

luca.dessi@edu.unito.it

alfredo.santovito@unito.it

simona.bonelli@unito.it

tiziana.schiliro@unito.it

sara.bonetta@unito.it

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366

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## 509 **STATEMENTS AND DECLARATIONS**

### 510 **Ethical Approval**

511 Not applicable

512

### 513 **Compliance with Ethical Standards**

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

516

### 517 **Consent to Participate**

518 Not applicable

519

### 520 **Consent to Publish**

521 Not applicable

522



523 **Authors Contributions**

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

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536 **Competing Interests**

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

538

539 **Availability of data and materials**

540 Data is contained within the article or Supplementary Material

541

542 **TABLE CAPTIONS**

543 **Table 1** Concentrations of air pollutants in the three sites from 1<sup>st</sup> April 2019 to 30<sup>th</sup> September 2019 (larval  
544 season of *P. brassicae*). Data are reported as mean  $\pm$  standard deviations

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

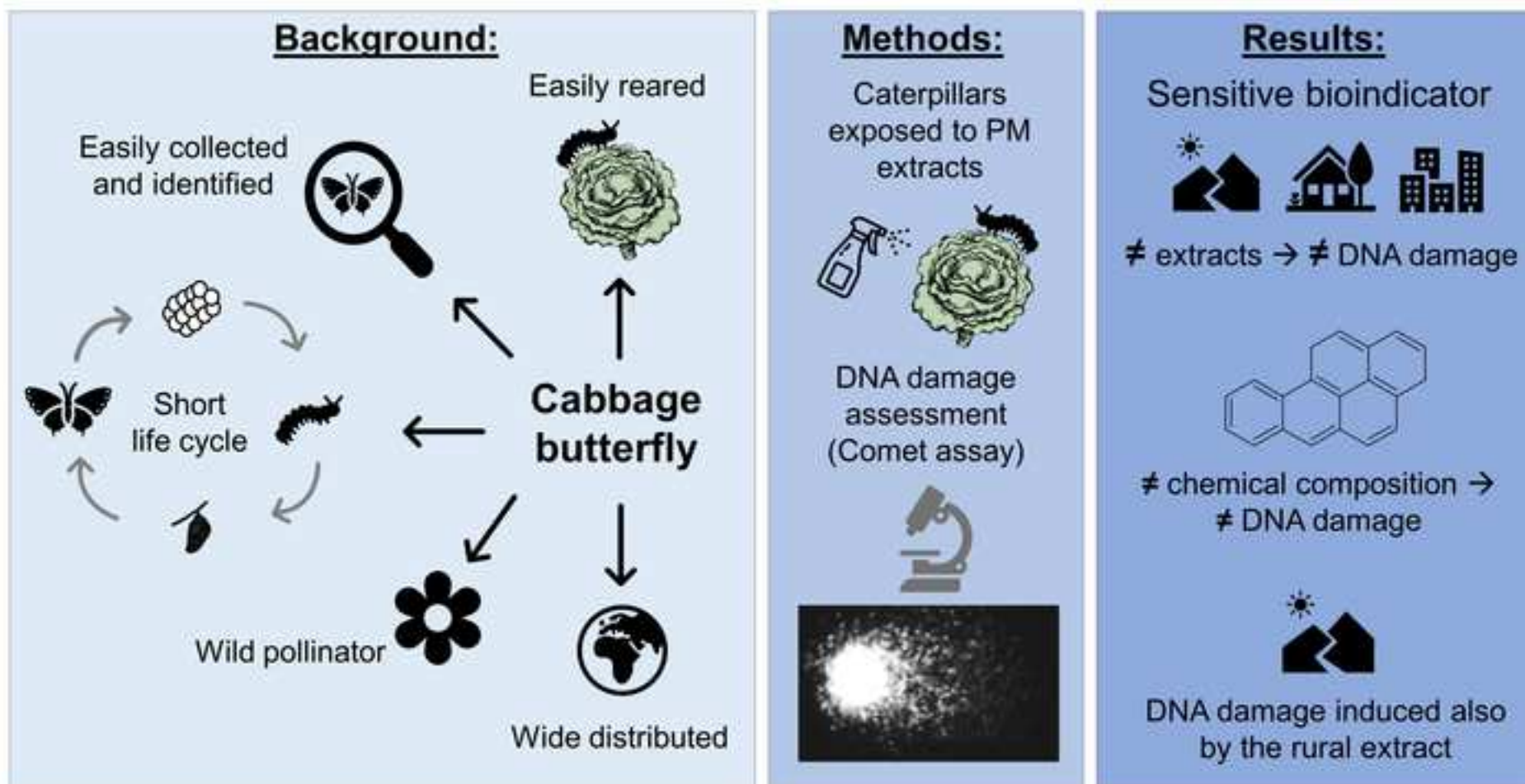
546

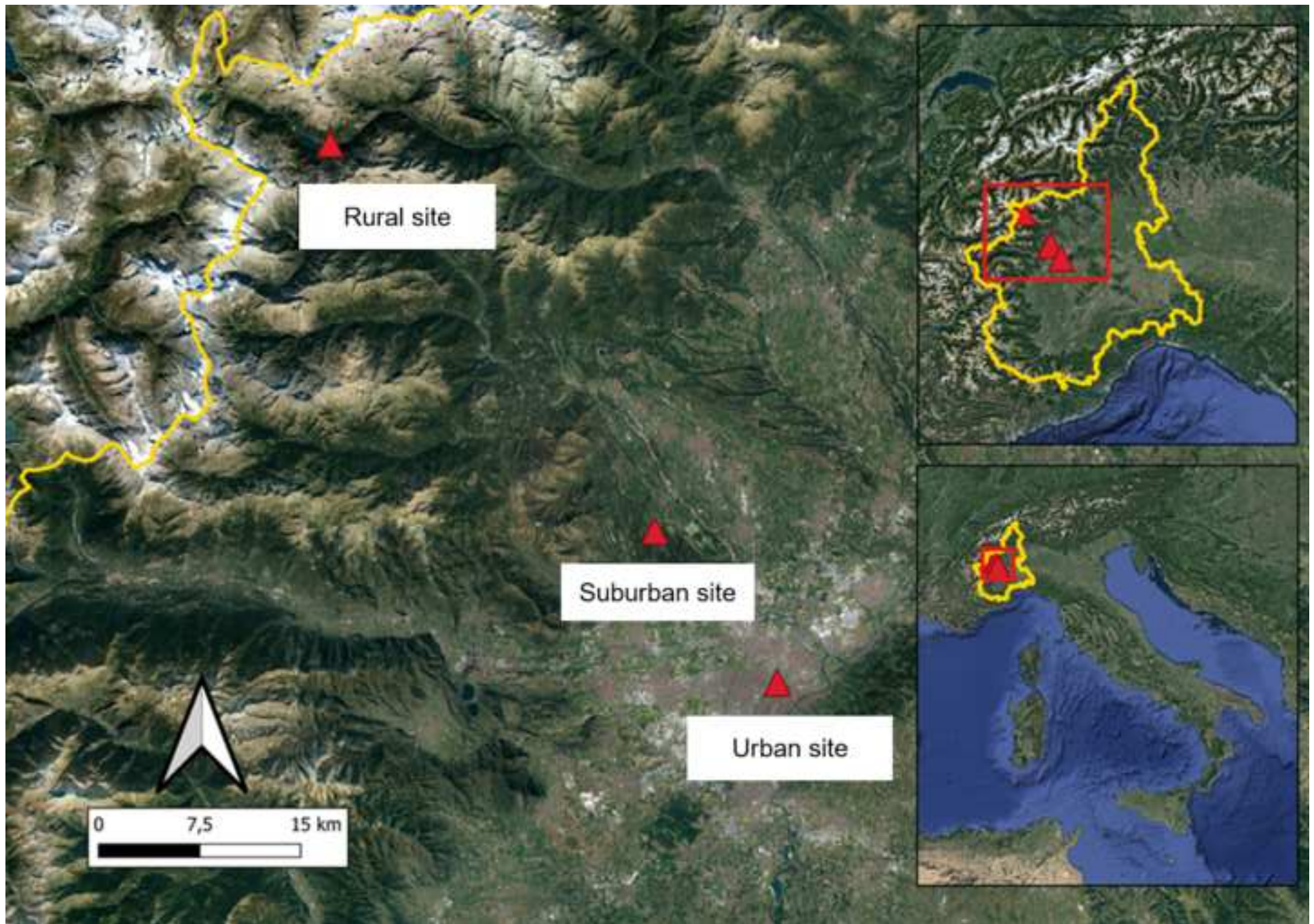
547 **FIGURE CAPTIONS**

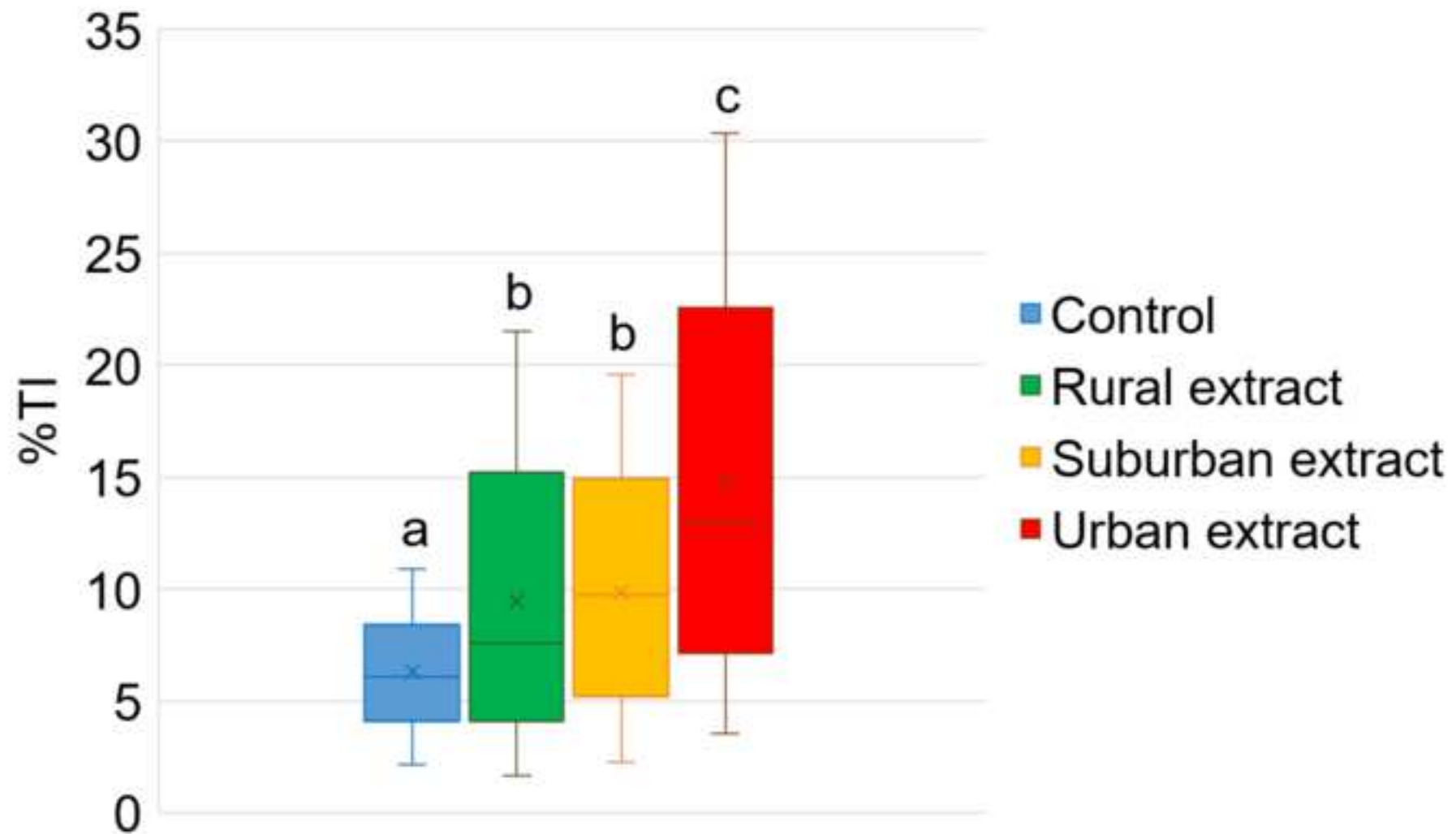
548 **Fig. 1** Localization of the PM<sub>10</sub> sampling sites

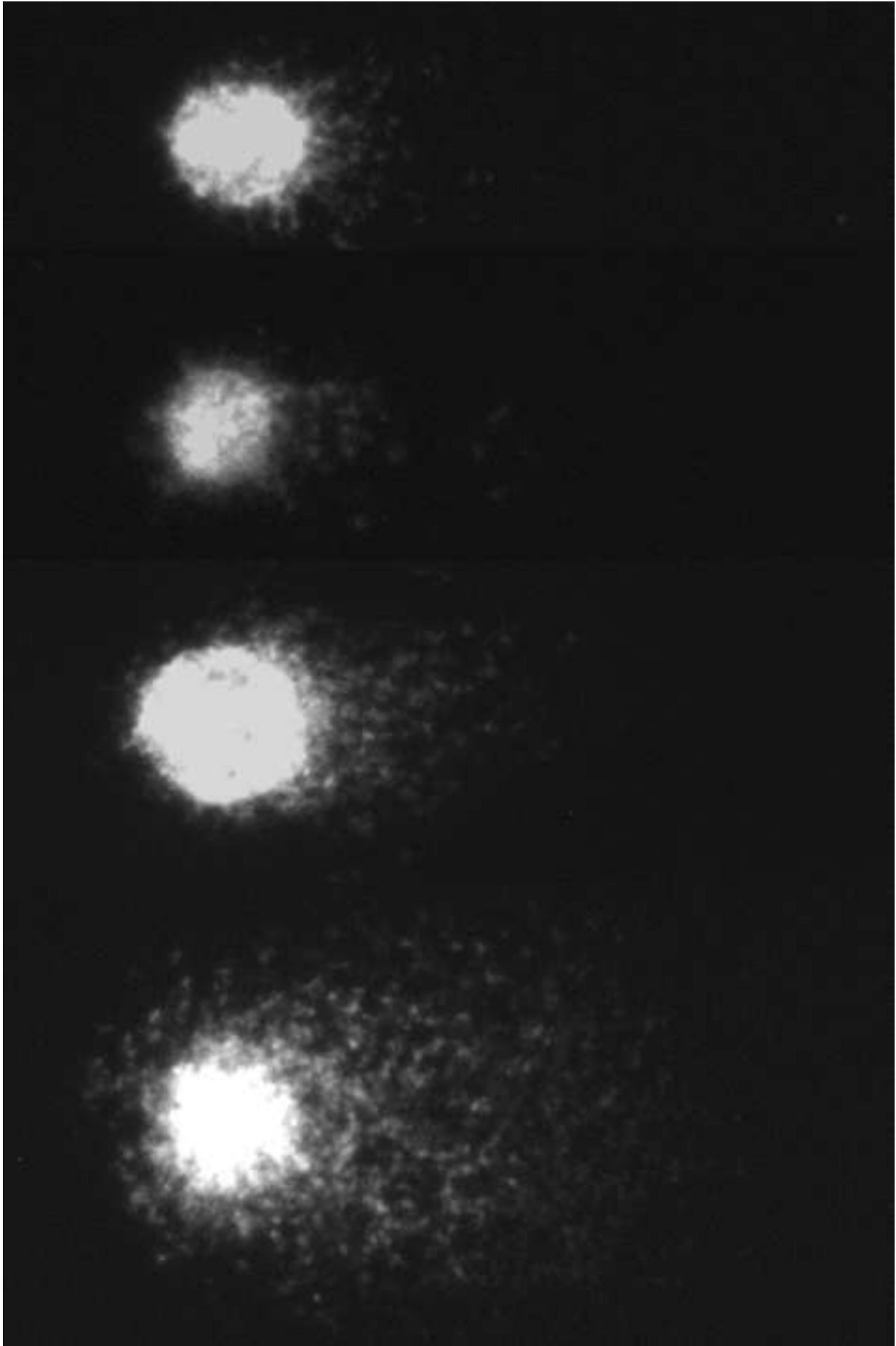
549 **Fig. 2** %TI of larvae treated with organic PM<sub>10</sub> extracts collected in different sites (tested dose = 40 m<sup>3</sup>/mL).  
550 Data of larvae treated with DMSO are reported as control. a, b, c = boxplots identified by the same letter do  
551 not statistically differ (post hoc analysis with Bonferroni correction)

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









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

Site type	PM <sub>10</sub> ( $\mu\text{g}/\text{m}^3$ )	BaP ( $\text{ng}/\text{m}^3$ )	BA ( $\text{ng}/\text{m}^3$ )	BF ( $\text{ng}/\text{m}^3$ )	IP ( $\text{ng}/\text{m}^3$ )	TEF (mean) ( $\text{ng}/\text{m}^3$ )
Rural site	11.8 $\pm$ 2.5	< lod	0.040 $\pm$ 0.001	0.040 $\pm$ 0.001	0.040 $\pm$ 0.001	0.012
Suburban site	18.0 $\pm$ 7.5	< lod	0.040 $\pm$ 0.001	0.065 $\pm$ 0.043	0.045 $\pm$ 0.008	0.015
Urban site	17.5 $\pm$ 5.0	< lod	0.040 $\pm$ 0.001	0.093 $\pm$ 0.078	0.063 $\pm$ 0.036	0.020

lod = limit of detection

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

<b>Treatment</b>	<b>Dissected larvae for Comet assay</b>	<b>Larval weight (mean <math>\pm</math> SD, g)</b>	<b>Larvae considered for Comet assay results<sup>a</sup></b>
<b>Control (DMSO)</b>	25	0.26 $\pm$ 0.11	25
<b>Rural extract (40 m<sup>3</sup>/mL)</b>	28	0.23 $\pm$ 0.04	26
<b>Suburban extract (40 m<sup>3</sup>/mL)</b>	33	0.25 $\pm$ 0.10	31
<b>Urban extract (40 m<sup>3</sup>/mL)</b>	31	0.21 $\pm$ 0.05	30

<sup>a</sup> = larvae with less than 100 cells suitable to score the %TI were excluded; SD = standard deviation



