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A Th2-type immune response and low-grade systemic inflammatory reaction as potential immunotoxic effects in intensive agriculture farmers exposed to pesticides

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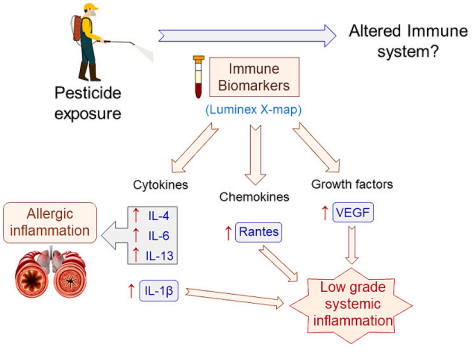
HIGHLIGHTS

- Serum levels of 27 immunoregulatory proteins were measured in greenhouse farmers and controls.
- These biomarkers were assessed at two time-points of a growing season (high and low pesticide use).
- Farmers had higher levels of IL-4, IL-6, IL-13, IL-1β, VEGF and RANTES than controls at the period of high pesticide exposure.
- Increased IL-4, IL-6 and IL-13 suggest a Th2-type immune response and allergic inflammation.
- Increased IL-1β, VEGF, RANTES suggest a low-grade systemic inflammation/ angiogenesis related to inflammatory-based diseases.

GRAPHICAL ABSTRACT



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ABSTRACT

Pesticides are chemicals widely used in agriculture to keep crops healthy and prevent them from being destroyed by pests, thus contributing to a sustainable food and feed production. However, long-term exposure to these compounds may be harmful to human health as they can affect the function of various organs systems, including the immune system. There is growing evidence that pesticides may increase the risk of developing immune-based diseases and inflammation. This study assessed whether greenhouse farmers occupationally exposed to pesticides presented alterations in immunoregulatory proteins, used as surrogate biomarkers of immune function. The study population consisted of 175 greenhouse workers occupationally exposed to pesticides and 91 non-exposed

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controls. Serum levels of 27 cytokines, chemokines and growth factors were measured using a magnetic bead-based immunoassay in a subpopulation of 111 greenhouse workers and 79 non-exposed controls. Since analytical determinations were performed in two periods of the same crop season with different use of pesticides (period of high and low pesticide exposure), linear mixed models for repeated measures were used to optimize statistical inference. The increase in IL-13, IL-4 and IL-6 observed in greenhouse workers compared to controls, and in the period of high exposure to pesticides relative to that of low exposure, suggest an altered Th1/Th2 balance towards the Th2 response. This finding points to a type-2 inflammation commonly presented as allergic inflammation, which has often been reported in farm-workers and in which pesticide exposure is considered a risk factor. Furthermore, the increase in IL-1β and VEGF, mediators of inflammation and angiogenesis, may suggest a low-grade systemic inflammation that might underlie chronic pathological conditions linked to pesticide exposure.

1. Introduction

Pesticides are widely used to prevent or control pests in agriculture, public health, and in the home. Almost all human populations are unavoidably exposed to low levels of pesticides due to the presence of these chemicals (or their metabolites or degradation products) in the environment or foodstuff. Pesticide exposure mostly occurs in occupational environments, such as open-air agriculture and intensive greenhouse farming. Greenhouse agriculture represents a high-yield intensive production system that resulted in a substantial change in crop production. Greenhouse farm workers are considered the group of population with the highest exposure to pesticides because of the enclosed architecture of the greenhouse farm design and the frequent use of pesticides required for pest control (Amoatey et al., 2020; Khan et al., 2013). Hence, these workers are exposed to a higher risk level than open-air farm workers. Intensive agriculture in plastic-covered greenhouses is a popular and profitable practice in Spain's Mediterranean coast, particularly in Almería province (Southeastern Spain), where the largest concentration of greenhouses in the world is located (Caparrós-Martínez et al., 2020). The favourable weather conditions and advance technology enhance crop productivity by creating optimal conditions year-round. However, the combination of high work and pesticide use intensity, multi-tasking and quick re-entry into pesticide-treated areas increase occupational pesticide exposure compared with open field farming (Tefera et al., 2019).

Although pesticides are highly regulated chemicals, their widespread use has raised health concerns. Apart from acute intoxications, which have clear and well-defined effects, chronic exposure to pesticides has been associated with target organ/system toxicity, including liver, kidney, immune and nervous system diseases, among others (Gangemi et al., 2016a; Strelitz et al., 2014). Occupational exposure to pesticides has been associated with a number of health outcomes, such as cancer, neurological diseases, mental disorders, respiratory diseases (including asthma), biomarkers of genotoxicity, and reproductive and developmental disorders (Ohlander et al., 2020). However, the clinical manifestation of these diseases is preceded by biochemical alterations in target organ/systems followed by functional and/or structural changes. The use of molecular biomarkers of early toxic effect provides mechanistic information on impaired toxicity pathways, thus increasing their predictive capacity and allowing early interventions aimed at preventing the development of diseases in a reversible stage (Gangemi et al., 2016b).

Experimental and epidemiological studies suggest that chronic pesticide exposure may affect the function of the immune system by impairing the cytokine balance (Corsini et al., 2008). The altered immune function may be an indicator of increased potential for the development of immunologically-based diseases (Fenga et al., 2014). These are the result of either a decreased immunocompetence (immunosuppression), which enhance the risk of infections and cancer, an inappropriate immunostimulation, leading to allergic reactions and autoimmune diseases, or inflammation (non-specific immunity) which contributes to tissue and organ damage (Corsini et al., 2013; Gangemi et al., 2016b).

Inflammation can be considered as a natural reaction aimed at repairing tissue damage triggered by harmful stimuli, including toxic substances (Medzhitov, 2008). It is therefore an adaptive response that comprises the release of mediators and effectors, followed by either resolution of the damaged tissue or persistence of the response, leading to fibrosis or subsequent organ failure (Serhan and Savill, 2005).

Evidence from animal models indicates that certain pesticides impair the immune function, yet the occurrence of immune disorders depends on the dose and duration of exposure (Gangemi et al., 2016a). In contrast, data in humans are scarce. Human studies should preferably be carried out by comparing pre- and post-exposure findings in the same group of subjects with a matched control group. The observed changes, even if slight, should be interpreted for prognostic purposes (Corsini et al., 2013). On this basis, the present study aimed to examine whether occupational exposure to pesticides in an intensive agriculture area was associated with alterations of immunoregulatory proteins such as cytokines, chemokines, and growth factors measured at two periods of a crop season with different pesticide exposure.

2. Material and methods

2.1. Study design and area of study

A longitudinal study was conducted on 280 individuals (aged 18–66 years) from Almería province (Southeastern Spain). Almería is well-known as the first horticultural production area of intensive agriculture under plastic greenhouse in Spain, representing almost half of the Spanish greenhouse surface (approx. 30,000 ha vs. 65,000 ha, respectively) (INE, 2022). The study was approved by the Research Ethics Committee of the University of Granada (reference 29/2009) and was in agreement with the Declaration of Helsinki for International Health Research.

2.2. Study population and exposure assessment

The study population was recruited during their scheduled annual occupational health surveillance. After receiving information on the objectives of the study, they agreed to participate and signed an informed consent form. Participants were also informed on their right to withdraw at any time from the research without consequences. Individuals with a clinical diagnosis of chronic diseases (e.g., diabetes, cancer, kidney, neurological, liver diseases or cancer at any site) were excluded from the study. Participants were assessed over two periods of the same crop season: May–June 2011 (low pesticide exposure period) and October–November 2011 (high exposure period).

The exposed group consisted of 189 greenhouse workers under an integrated production system, who were recruited on-site, in the greenhouses themselves, taking advantage of the periodic occupational health surveillance that they had scheduled before starting the workday, which included the collection of blood and urine samples. Fourteen workers were excluded for not providing blood samples in the two study periods assessed. Information on the tasks performed by greenhouse workers was obtained through structured face-to-face interviews and

verified by the foreman responsible for crop management.

Periods of high and low pesticide exposure were categorized depending on the development stage of the greenhouse crops grown in the study area (tomatoes, cucumbers, and zucchini). Each growth cycle spanned various development stages, each necessitating specific tasks, such as plant staking, pruning, weeding, thinning, and pesticide application. Since these tasks were performed at different stages of the crop growth cycle, all greenhouse workers carried out the same task during each stage. Sensitive periods in the crop's growth were flowering, fruit set and fruiting, in which crops were more vulnerable to pests and diseases. Therefore, the timely application of pesticides during these periods was crucial. These sensitive periods typically occurred in October and November, marking the peak of pesticide use. However, as the crop cycle approached its end in May/June of the following year, the focus shifted to harvesting the crops and preparing the soil for the next cycle. Therefore, pesticide application of pesticides was minimal or even unnecessary during this period. Greenhouse workers were responsible of mixing, loading and applying pesticides to the crops. The selection of pesticides for pest control was determined by agronomists supervising the study area, who provided guidance on the type of pesticides to use, application rates per hectare, and treatment duration. According to the information they provided, the pesticides most often used over the study period were insecticides (macrocyclic lactones, neonicotinoids, pyrethroids, *N*-methylcarbamates and *Bacillus thuringiensis*) and fungicides (triazoles, anilino-pyrimidines, and copper salts, among others) (Supplementary Table S1). The amount of pesticides used in the crops grown in the study area is shown in Supplementary Table S2.

The control group consisted of 91 healthy subjects, mainly officials (i.e., administrative staff and primary and secondary school teachers) living in municipalities within a radius of <2 km from the greenhouses selected for this study. They had no previous or current occupational or residential (e.g., garden, orchard …) pesticide exposure, which was an exclusion criterion. They were recruited at the Centre for Prevention of Occupational Hazards from Almería province, which monitors the health and safety conditions of employed workers of the province of Almería, as well as prevention and promotion activities. Recruitment took place in the same periods of the year as greenhouse workers, taking advantage their appointment for occupational health examination, which also included the collection of blood and urine samples. In order to avoid differences in background exposure to pesticides, control individuals were selected from the same geographical area as the greenhouse workers, such that non-occupational (i.e., environmental or dietary) exposure to pesticides was considered to be similar in both groups. The main difference between them thus lay in pesticide exposure as a consequence of performing agricultural activities in greenhouses.

2.3. Sample collection and preparation

In each of the two study periods, all study participants had venous blood drawn early in the morning after a fasting period of at least 10 h and processed under the same procedure. Blood was filled into serum separator tubes with clot activator and shipped in a portable fridge to the laboratory within 4 h. Serum was separated by centrifugation for 20 min at 400 ×*g* and 1 ml aliquots were stored frozen at −40 °C. After 2 or 2.5 years, depending on whether samples were collected in the period of high or low exposure, respectively, they were thawed at 4 °C and analysed for immunoregulatory proteins in the same batch without undergoing any freeze-thaw cycle.

2.4. Immunoregulatory proteins analysis

Twenty-seven immunological mediators including cytokines, chemokines, and growth factors (see the complete list in Table 1) were determined in serum by magnetic bead suspension array using the Bio-Plex Pro™ Human Cytokine 27-plex Assay kit (#M500KCAF0Y, Bio-Rad, Hercules, CA, USA), which allows the simultaneous detection of circulating analytes. The commercial kit was run according to the pre-optimized protocol based on the methodology provided by the manufacturer. Samples were measured in duplicate in every run and the concentration of immunological markers was calculated from standard curves and expressed as pg/ml. Optimization of standard curves across all of the assayed analytes was carried out to remove outliers. Data were acquired on a Luminex 200 system running xPONENT® 3.1 software.

Table 1
List of serum immunoregulatory proteins determined by multiplex immunoassays.

Type	Immunoregulatory molecule
Cytokines	IL-1β, IL1-Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, IFN-γ, TNF-α
Chemokines	MCP-1, MIP-1α, MIP-1β, RANTES, Eotaxin, IL-8, IP-10
Growth factors	VEGF, bFGF, PDGF-BB; G-CSF, GM-CSF

IL: interleukin; IL-1Ra: interleukin 1 receptor agonist; IFN-γ: interferon gamma; TNF-α: tumor necrosis factor.
MCP-1: monocyte chemoattractant protein-1 (CCL2); MIP-1α: macrophage inflammatory protein 1-alpha (CCL3); MIP-1β: macrophage inflammatory protein 1-beta (CCL4); RANTES: regulated on activation normal T cell expressed and secreted (CCL5); Eotaxin (CCL11); IL-8 (aka CXCL8); IP-10: Interferon gamma-induced protein 10 (CXCL10). CCL: C—C motif chemokine ligand.
VEGF: vascular endothelial growth factor; bFGF: basic fibroblast growth factor (aka fibroblast growth factor 2 and FGF-β); PDGF-BB: platelet-derived growth factor BB; G-CSF: granulocyte colony-stimulating factor: GM-CSF: granulocyte-macrophage colony-stimulating factor.

2.5. Statistical analysis

The descriptive analysis of immunoregulatory proteins was performed using median values and interquartile range (25th and 75th percentiles). Since the distribution of the immune biomarkers did not meet the normality criteria (assessed by the Kolmogorov-Smirnov test), the non-parametric Mann-Whitney *U* test was used to compare biomarker levels between greenhouse workers and controls. The non-parametric paired samples Wilcoxon test was used to compare changes in biomarkers levels between the two study periods (high and low pesticide exposure).

Subsequently, data for cytokines, chemokines, and growth factors were log-transformed to normalize their distribution, and analysed using linear mixed models because repeated measures of the immunoregulatory proteins were available for the same individual. Linear mixed models assumed that individuals represent random effects whereas exposure status (greenhouse workers vs. non-exposed controls), crop season period (high vs. low pesticide exposure), gender (male vs. female), and nationality (Spanish vs. Moroccan), age and body mass index (BMI) were added as fixed effects. Mixed models included the interaction term "greenhouse workers vs. controls" × "period of high vs. low pesticide exposure", such that only significant associations found for this interaction term were considered the most relevant, since the observed effects could be attributed to pesticide exposure. An unstructured variance model was chosen for these analyses since it offered a better fit according to the maximum likelihood test. For IL-2, IL-8, IL-15 and colony stimulating factors (GM-CSF and G-CSF), mixed model analysis was not performed because the percentage of non-detects exceeded 25 % of individuals. For the remaining immunoregulatory proteins, non-detect values were imputed with half the LOD.

A discriminant analysis was performed by Fisher's linear discriminant method to classify individuals as greenhouse workers or controls using all the immunoregulatory proteins studied. Statistical analysis was performed using the SPSS statistical software package (SPSS 21.0 for windows).

3. Results

The main characteristics of the study population are depicted in Table 2. The mean age of the participants was 41.9 years, with greenhouse workers being younger than controls. The greenhouse workers group had a greater proportion of men and Moroccans. Additionally, they consumed less tobacco and alcohol than the control group. No differences were observed by BMI.

The descriptive and bivariate analyses of the immunoregulatory proteins studied are shown in Table 3 (cytokines), Table 4 (growth factors) and Table 5 (chemokines). From the 27 immune biomarkers, IL-2, IL-15 (cytokines), GM-CSF, G-CSF (growth factors) and IL-8 (chemokine) were undetectable in >25 % of the study population. All cytokines studied, except IL-15, were raised in greenhouse workers relative to controls in the period of low pesticide exposure. In contrast, in the period of high exposure, a few cytokines (IL-2, IL-13, IL-1ra and IFN-γ) failed to show significant differences (Table 3). Regarding growth factors, greenhouse workers showed significantly higher levels of b-FGF than controls in the period of high pesticide exposure, and of b-FGF, PDGF-BB and VEGF in the period of low exposure (Table 4). As for chemokines, greenhouse workers had significantly greater levels of MIP-1α, MIP-1β and eotaxin, and lower levels of IP-10 than controls in the period of high exposure to pesticides. In the period of low exposure, levels of MIP-1α, MIP-1β, RANTES and eotaxin were significantly raised in greenhouse workers relative to controls (Table 5). On the other hand, the longitudinal analysis throughout the crop season showed significantly higher levels of almost all immunoregulatory proteins studied in the period of high exposure compared to the one of low exposure, except for the chemokines MCP-1 and RANTES, whose levels were significantly higher in the period of low exposure to pesticides.

Supplementary Tables S3 and S4 show the results of the mixed model analysis of the fixed factors studied: exposure status (greenhouse workers vs. controls), period of the crop season (high vs. low exposure period), sex (males vs. females), nationality (Spanish vs. Moroccan), age and BMI. All immunoregulatory proteins except VEGF, MCP-1 and eotaxin showed significantly greater levels in the period of high exposure to pesticides compared to that of low exposure. Likewise, greenhouse workers showed significantly higher values than controls for all the immunomodulatory proteins studied but the chemokine MCP1. Analysis by sex showed higher levels in males for all the cytokines studied and, in the case of growth factors and chemokines, only for PDGF-BB, IL-8, MIP-1α and eotaxin. Regarding nationality, significantly lower levels of immunoregulatory proteins were observed in Spaniards as compared to Moroccans except for MCP-1 (whose levels were significantly higher) and IL-13, IFN-γ and PDGF-BB, which failed to show significant differences. As for age, a statistically significant inverse association was observed for all cytokines studied but IL-2 and IL-15 in the period of low pesticide exposure, which can be considered closed to basal levels. For growth factors and chemokines, significant inverse associations were observed only for VEGF and b-FGF, and IL-8, MIP1α and RANTES, respectively, in the period of low pesticide exposure. Finally, BMI failed to be significantly associated with all the immunoregulatory proteins but PDGF-BB, which showed a significant and direct association.

The results of the mixed model analysis of each immunoregulatory protein studied adjusted for the fixed factors and co-variates indicated in Material and Methods (Statistical analysis) as well as the interaction term [exposure status (greenhouse worker vs. control) × period of the crop season (high vs. low period of pesticide exposure)] is shown Tables 6 and 7. Statistically significant differences were observed for the interaction term for IL-1β, IL-6, IL-13 and VEGF, and near-significant differences for IL-4 and RANTES, meaning that these biomarkers were increased in greenhouse workers compared to controls and in the period of high exposure to pesticides relative to that of low exposure (Fig. 1). The discriminant analysis showed a correct classification of 68.8 % of greenhouse workers in the period of low pesticide exposure and 66.7 % in that of high exposure. Better results were observed for controls, with 83.3 % being correctly classified in the period of low pesticide exposure and 87.3 % in the period of high exposure. By the canonical discriminant functions, the ordination of data shows that there are differences among the two study groups, despite the occurrence of overlaps (Supplementary Table S5).

Table 2
General sociodemographic characteristics and lifestyles of the study population.

	Total	Controls	Greenhouse workers	p
Age (years)	41.9 ± 10.4 (n = 223)	48.4 ± 8.4 (n = 82)	38.1 ± 9.6 (n = 141)	<0.001
Gender	280	91 (32.5 %)	189 (67.5 %)	
Male	131 (46.8 %)	25 (27.5 %)	106 (56.1 %)	<0.001
Female	149 (53.2 %)	66 (72.5 %)	83 (43.9 %)	
BMI (kg/m^2)	25.7 ± 4.7 (n = 241)	25.8 ± 4.5 (n = 90)	25.7 ± 4.8 (n = 151)	0.650
Tobacco	89	26 (29.2 %)	63 (70.8 %)	<0.001
Smoker	52 (58.4 %)	24 (92.3 %)	28 (44.4 %)	
Former smoker	19 (21.3 %)	0 (0 %)	19 (30.2 %)	
Non-smoker	18 (20.2 %)	2 (7.7 %)	16 (25.4 %)	
Alcohol	202	78 (38.6 %)	124 (61.4 %)	<0.001
Yes	85 (42.1 %)	47 (60.3 %)	38 (30.6 %)	
No	117 (57.9 %)	31 (39.7 %)	86 (69.5 %)	
Nationality	280	91 (32.5 %)	189 (67.5 %)	<0.001
Spanish	140 (50.0 %)	91 (100 %)	49 (25.9 %)	
Moroccan	140 (50.0 %)	0 (0 %)	140 (74.1 %)	

4. Discussion

This study assessed whether intensive agriculture farmers exposed to pesticides showed alterations in serum levels of immunoregulatory proteins, such as cytokines, chemokines and growth factors. Overall, increased levels of IL-4, IL-13, IL-1β, IL-6, VEGF and RANTES were found in greenhouse workers relative to controls and in the period of high exposure to pesticide relative to that of low exposure (Fig. 1). IL-4 is the regulator of lymphocyte functions (Th2 differentiation and B-cell switching to IgG1 and IgE class), while IL-13 is an effector cytokine that regulates smooth muscle contraction and mucus production in the airway epithelium, as occurs in allergic asthma, an inappropriately controlled inflammatory response (Junttila, 2018). IL-6 is a pleiotropic cytokine that plays an essential role in Th2-mediated allergic response. It can shift the Th1/Th2 balance towards the Th2 direction by promoting IL-4 production and Th2 differentiation, and by inhibiting IFN-γ production and Th1 differentiation (Diehl and Rincón, 2002). IL-6 is produced by different cells, including respiratory epithelial cells, in response to a variety of stimuli, such as allergens and inhaled environmental toxicants (Rincon and Irvin, 2012). The increase observed in our study in these three cytokines (IL-4, IL-13 and IL-6) suggest an altered Th1/Th2 balance towards the Th2 response, indicative of a type-2 inflammation commonly presented as allergic inflammation (Bao and Reinhardt, 2015).

Th2 dominance is often seen in immediate-type allergic reactions (i.e., asthma, atopic dermatitis) and is considered as a pathognomonic factor indicating progression of many chronic inflammatory diseases (Akdis et al., 2020) and various autoimmune diseases (Iwaszko et al., 2021). Type 2 inflammation usually manifests itself as asthma and allergy in developed countries, while in developing countries it is the result of repeated or chronic exposure to helminth infections (Bao and Reinhardt, 2015). Various allergic responses such as asthma, have been widely reported in workers at greenhouse, and open-field farms (Nordgren and Bailey, 2016). Though exposure to agricultural dusts containing a complex mixture of organic and inorganic materials (including pesticides) has been claimed as the most common cause of asthma among agricultural workers (https://www.hse.gov.uk/asthma/agriculture.htm), there is still some controversy as farm

Table 3
Descriptive analysis of the cytokines measured in the study population (stratified by exposure) in the period and high and low exposure to pesticides.

	High exposure period				Low exposure period				p value[a]
	n	% < LOD	MED	P25–P75	n	% < LOD	MED	P25–P75	
Controls									
IL-1β	63	0.0	0.58	0.20–1.25	79	1.3	0.24	0.13–0.45	<0.001
IL-2	63	54.0	NC	NC – 0.60	79	64.6	NC	NC – 0.60	–
IL-4	63	7.9	0.51	0.18–0.95	79	7.6	0.24	0.14–0.36	<0.001
IL-5	63	1.6	0.98	0.40–4.40	79	31.6	NC	NC – 0.65	<0.001
IL-6	63	0.0	1.56	0.62–2.95	79	10.1	0.65	0.30–0.68	<0.001
IL-7	63	20.6	1.35	0.51–2.52	79	8.9	1.01	0.51–1.96	0.023
IL-9	63	1.6	6.24	3.27–8.89	79	7.6	2.56	1.19–6.93	0.001
IL-10	63	1.6	3.44	1.85–7.77	79	13.9	1.18	0.74–3.70	<0.001
IL-12p70	63	3.2	7.43	4.78–13.30	79	6.3	3.58	1.30–8.94	<0.001
IL-13	63	0.0	2.83	1.65–4.16	79	2.5	1.20	0.70–1.70	<0.001
IL-15	63	68.2	NC	NC – 1.84	79	73.4	NC	NC – 0.75	–
Il-17	63	9.5	18.87	4.53–39.18	79	11.4	6.20	1.92–14.44	<0.001
IL-1ra	63	1.6	30.68	14.45–71.55	79	0.0	12.65	4.74–24.82	<0.001
IFN-γ	63	4.8	173.16	39.49–302.02	79	1.3	22.56	14.09–40.76	<0.001
TNF-α	63	1.6	11.45	4.07–15.99	79	1.3	5.43	3.94–10.32	<0.001
Greenhouse workers									
IL-1β	111	0.0	1.26**	0.72–1.70	93	3.2	0.73**	0.34–1.33	0.002
IL-2	111	33.3	0.60	NC – 2.17	93	40.9	0.60**	NC – 2.17	0.444
IL-4	111	1.8	0.91**	0.57–1.24	93	1.1	0.59**	0.39–1.01	0.006
IL-5	111	0.0	2.92**	1.54–4.49	93	17.2	0.65**	0.50–1.56	<0.001
IL-6	111	0.0	2.31**	1.42–3.00	93	5.4	1.56**	0.80–2.70	0.018
IL-7	111	0.9	2.31**	1.59–3.33	93	3.2	2.57**	1.51–4.05	0.512
IL-9	95	1.0	7.27*	4.89–10.17	93	2.1	5.18**	3.15–9.56	0.200
IL-10	111	0.0	6.67*	4.14–9.64	93	2.1	3.76**	1.95–8.59	0.002
IL-12p70	111	0.0	11.44**	6.95–16.13	93	2.1	7.14**	3.14–13.59	0.024
IL-13	111	0.0	2.57	1.96–3.68	93	0.0	2.12**	1.34–3.25	0.011
IL-15	111	87.4	NC	NC – NC	93	58.1	NC	NC – 1.52	–
Il-17	111	0.9	42.06**	24.62–68.95	93	3.2	13.88**	7.59–29.83	<0.001
IL-1ra	111	0.0	37.00	24.26–63.54	93	1.1	28.98**	12.72–52.59	0.102
IFN-γ	111	0.0	85.81	56.91–281.03	93	1.1	41.57**	25.43–69.05	<0.001
TNF-α	111	0.9	15.36**	9.69–20.93	93	2.1	13.54**	7.40–20.80	0.669

n: number of individuals; % < LOD: percentage values under limit of detection; MED: median; P25–P75: interquartile range (percentiles 25–75 %). NC: not calculated.
Units: pg/ml for all biomarkers.
[a] Wilcoxon test.
* $p < 0.05$.
** $p < 0.01$ for comparison between controls and greenhouse workers (Mann-Whitney test).

Table 4
Descriptive analysis of growth factors measured in the study population (stratified by exposure) in the period and high and low exposure to pesticides.

	High exposure period				Low exposure period				p value[a]
	n	% < LOD	MED	P25–P75	n	% < LOD	MED	P25–P75	
Controls									
VEGF	63	7.9	5.07	1.81–8.787	79	10.1	2.87	1.27–5.44	<0.001
b-FGF	63	0.0	19.23	10.24–28.27	79	1.3	10.03	7.57–17.06	<0.001
PDGF-BB	63	0.0	304.48	123.31–541.28	79	0.0	117.75	64.49–225.01	0.001
GM-CSF	63	74.6	NC	NC – 1.92	79	60.8	NC	NC – 0.40	–
G-CSF	63	41.3	1.60	NC – 1.60	79	91.1	NC	NC – NC	–
Greenhouse workers									
VEGF	111	7.2	5.95	2.51–11.80	93	1.1	8.47*	4.41–15.18	0.066
b-FGF	111	0.0	26.58*	17.12–36.12	93	1.1	17.55*	12.16–27.07	0.008
PDGF-BB	111	0.0	328.16	210.20–484.42	93	1.1	243.39*	121.47–441.51	0.412
GM-CSF	111	81.1	NC	NC – NC	93	62.4	NC	NC – 12.64	–
G-CSF	111	19.8	1.60	1.41–2.01	93	36.6	1.60	NC – 1.76	0.414

n: number of individuals; % < LOD: percentage values under limit of detection; AM: arithmetic mean; SD: standard deviation; MED: median; P25–P75: interquartile range (percentiles 25–75 %).
Units: pg/ml for all biomarkers.
[a] Wilcoxon test.
* $p < 0.01$ for comparison between controls and greenhouse workers (Mann-Whitney test).

environments have been associated with a lower risk of asthma development, particularly in subjects born before 1970 (Madsen et al., 2022). The present study provides some mechanistic support for the contribution of pesticide exposure to the development of asthma.

IL-4 and IL-13 antagonize Th1-driven proinflammatory immune response by downregulating synthesis of many proinflammatory cytokines (e.g., IL-1β, IL-8, TNFα), and also suppress other mediators of inflammation, such as granulocyte-macrophage colony-stimulating

Table 5
Descriptive analysis of chemokines measured in the study population (stratified by exposure) in the period and high and low exposure to pesticides.

	High exposure period				Low exposure period				p value[a]
	n	% < LOD	MED	P25–P75	n	% < LOD	MED	P25–P75	
Controls									
IL-8 (CXCL8)	63	30.2	NC	NC – 1.57	79	26.6	NC	NC – 1.39	0.097
IP-10 (CXCL10)	63	0.0	195.31	110.26–351.65	79	0.0	122.82	86.20–221.33	0.080
MCP-1 (CCL2)	63	14.3	5.72	0.83–7.25	79	8.9	2.84	1.22–5.51	0.020
MIP-1α (CCL3)	63	0.0	1.88	1.14–2.54	79	1.3	1.13	0.76–1.69	<0.001
MIP-1β (CCL4)	63	0.0	4.10	4.88–9.82	79	0.0	6.17	4.80–7.82	0.098
RANTES (CCL5)	63	0.0	1576.2	1263.1–1716.6	79	1.3	1646.3	1275.4–2316.3	0.002
Eotaxin (CCL11)	63	1.6	8.55	4.82–13.69	79	13.7	8.15	4.34–13.07	0.305
Greenhouse workers									
IL-8 (CXCL8)	111	9.0	2.61	1.52–3.99	93	7.5	2.58	1.71–3.88	0.320
IP-10 (CXCL10)	111	0.0	126.56**	85.10–308.60	93	0.0	115.51	85.50–148.26	0.373
MCP-1 (CCL2)	111	24.3	3.71	0.83–7.41	93	14.0	4.69	1.33–8.34	0.033
MIP-1α (CCL3)	111	0.0	2.49**	1.83–3.39	93	1.1	1.59**	1.16–2.59	0.007
MIP-1β (CCL4)	111	0.0	9.05**	6.55–13.00	93	0.0	8.55**	6.49–11.07	0.667
RANTES (CCL5)	111	0.9	1649.6	1325.7–2432.7	93	0.0	2822.0**	2049.3–3819.5	0.003
Eotaxin (CCL11)	111	0.9	14.61**	9.90–21.35	93	4.3	14.06*	6.68–19.79	0.238

n: number of individuals; % < LOD: percentage values under limit of detection; AM: arithmetic mean; SD: standard deviation; MED: median; P25–P75: interquartile range (percentiles 25–75 %).
Units: pg/ml for all biomarkers.
[a] Wilcoxon test.
* $p < 0.05$.
** $p < 0.01$ for comparison between controls and greenhouse workers (Mann-Whitney test).

Table 6
Mixed model analysis of serum levels (log-transformed) of cytokines in the study population (greenhouse workers and controls) over the two study periods of the grown season studied (high vs. low exposure to pesticides)[a].

	High exposure period		Low exposure period		B-coefficient (95 % CI)	p
	Greenhouse workers	Controls	Greenhouse workers	Controls		
IL-1β (n = 174)	−0.041 ± 0.055	−0.253 ± 0.068	−0.189 ± 0.051	−0.635 ± 0.062	−0.234 (−0.420; −0.049)	0.013
IL-4 (n = 174)	−0.149 ± 0.057	−0.386 ± 0.069	−0.270 ± 0.046	−0.657 ± 0.058	−0.150 (−0.323; 0.022)	0.088
IL-5 (n = 173)	0.281 ± 0.071	0.019 ± 0.085	−0.133 ± 0.056	−0.489 ± 0.069	−0.095 (−0.328; 0.139)	0.425
IL-6 (*n* = 174)	0.268 ± 0.050	0.120 ± 0.066	−0.131 ± 0.058	−0.304 ± 0.071	−0.286 (−0.467; −0.105)	0.002
IL-7 (*n* = 174)	0.351 ± 0.053	0.091 ± 0.065	0.348 ± 0.053	−0.050 ± 0.063	−0.137 (−0.318; 0.043)	0.134
IL-9 (*n* = 158)	0.819 ± 0.064	0.628 ± 0.072	0.639 ± 0.062	0.357 ± 0.075	−0.091 (−0.293; 0.111)	0.374
IL-10 (*n* = 169)	0.737 ± 0.075	0.476 ± 0.096	0.486 ± 0.077	0.125 ± 0.095	−0.099 (−0.348; 0.149)	0.429
IL-12p70 (*n* = 170)	1.052 ± 0.048	0.816 ± 0.064	0.796 ± 0.062	0.514 ± 0.072	−0.046 (−0.238; 0.146)	0.636
IL-13 (*n* = 172)	0.389 ± 0.034	0.437 ± 0.045	0.297 ± 0.043	0.106 ± 0.050	−0.240 (−0.364; −0.115)	< 0.001
IL-17 (*n* = 173)	1.558 ± 0.074	1.607 ± 0.091	1.047 ± 0.068	0.749 ± 0.082	0.192 (−0.058; 0.442)	0.132
IL-1rα (*n* = 173)	1.630 ± 0.062	1.355 ± 0.078	1.400 ± 0.058	1.016 ± 0.073	−0.110 (−0.304; 0.084)	0.265
IFL-γ (n = 174)	2.075 ± 0.072	1.885 ± 0.087	1.627 ± 0.058	1.434 ± 0.072	−0.004 (−0.219; 0.211)	0.972
TNF-α (*n* = 174)	1.148 ± 0.047	0.930 ± 0.059	1.079 ± 0.044	0.766 ± 0.054	−0.095 (−0.244; 0.053)	0.206

Units: pg/ml for all immune biomarkers (log-transformed).
Data correspond to mean ± standard error. Fixed-effect coefficient β represents the group difference (greenhouse workers – controls) from the high to the low pesticide exposure period. This means difference was calculated as: (mean of greenhouse workers – mean of controls at the high exposure period) – (mean of greenhouse workers – mean of controls at the low exposure period).
p: statistical significance for the β-coefficient of the interaction term [controls vs. greenhouse workers] × [high vs. low exposure period].
[a] Mixed model analysis with non-structured covariance adjusted for high vs. low exposure period, greenhouse workers vs. controls, sex, nationality, age, BMI, and the interaction term [high vs. low exposure period] × [controls vs. greenhouse workers].

factor (GM-CSF). IL-4 and IL-13 also suppress release of cytokines by Th17 cells resulting in downregulation of Th17-mediated inflammation (Iwaszko et al., 2021), which is involved in the development of many autoimmune diseases. However, in our study, GM-CSF was detected in a small proportion of greenhouse workers and controls, and the fully adjusted mixed models analysis found no significant differences in IL-17. On the other hand, IL-4 and IL-13 bind endothelial cells with high affinity and both cytokines exert similar, non-additive effects on endothelial cells, inducing vascular cell adhesion molecule (VCAM)-1 expression and subsequent eosinophils transmigration (Schnyder et al., 1996).

VEGF induces adhesion molecules (e.g., VCAM-1, ICAM-1 −intracellular cell adhesion molecule-1−, and *E*-selectin) on endothelial cells during inflammation, mainly through NF-κB activation (Kim et al., 2001). The Th2 cytokines IL-4 and IL-13 also enhance the production and release of VEGF by airway smooth muscle cells. Furthermore, IL-6 stimulates Th2 type cytokine secretion and upregulates VEGF. IL-1β, in contrast to other Th1 cytokines, is able to induce VEGF production (Wen et al., 2003). VEGF is involved in angiogenesis in the asthmatic airway and plays important roles in bronchial asthma disease progression (Shoda et al., 2016). Several reports have described an association between VEGF and allergic airway inflammation as VEGF may be released by different cells associated with allergic inflammation of the airways (Koczy-Baron et al., 2016).

RANTES (CCL5) is a potent chemoattractant for monocytes/macrophages and eosinophils recruitment, and it is involved in enhanced chronic inflammation (Ruster and Wolf, 2008). The increase in RANTES levels observed in our study may be an adaptive mechanism that

Table 7
Mixed model analysis of serum levels (log-transformed) of growth factors and chemokines in the study population (greenhouse workers and controls) over the two study periods (high vs. low exposure to pesticides)[a].

	High exposure period		Low exposure period		B-coefficient (95 % CI)	p
	Greenhouse workers	Controls	Greenhouse workers	Controls		
VEGF (n = 173)	0.725 ± 0.067	0.575 ± 0.082	0.856 ± 0.060	0.345 ± 0.073	−0.361 (−0.580; −0.142)	0.001
b-FGF (n = 173)	1.402 ± 0.037	1.160 ± 0.047	1.211 ± 0.041	0.962 ± 0.049	−0.008 (−0.139; 0.123)	0.904
PDGF-BB (n = 174)	2.497 ± 0.053	2.316 ± 0.069	2.280 ± 0.061	2.096 ± 0.073	−0.003 (−0.194; 0.188)	0.977
IL-8 (CXCL8) (n = 174)	0.372 ± 0.030	0.203 ± 0.039	0.366 ± 0.038	0.107 ± 0.044	−0.090 (−0.209; 0.030)	0.140
IP-10 (CXCL10) (n = 173)	2.272 ± 0.043	2.201 ± 0.052	2.132 ± 0.034	2.075 ± 0.044	0.013 (−0.112; 0.139)	0.832
MCP-1 (CCL2) (n = 173)	0.573 ± 0.068	0.282 ± 0.085	0.580 ± 0.071	0.209 ± 0.084	−0.081 (−0.336; 0.174)	0.533
MIP-1α (CCL3) (n = 173)	0.430 ± 0.036	0.218 ± 0.046	0.213 ± 0.041	−0.008 ± 0.048	−0.009 (−0.134; 0.117)	0.890
MIP-1β (CCL4) (n = 174)	1.001 ± 0.032	0.811 ± 0.039	0.946 ± 0.026	0.777 ± 0.032	0.002 (−0.004; 0.009)	0.686
RANTES (CCL5) (n = 174)	3.230 ± 0.026	3.228 ± 0.033	3.376 ± 0.035	3.273 ± 0.039	−0.101 (−0.213; 0.012)	0.079
Eotaxin (CCL11) (n = 173)	1.166 ± 0.056	0.806 ± 0.071	1.077 ± 0.054	0.809 ± 0.067	0.092 (−0.080; 0.264)	0.293

Units: pg/ml for all biomarkers (log-transformed).
Data correspond to mean ± standard error. Fixed-effect coefficient β represents the group difference (greenhouse workers – controls) from the high to the low pesticide exposure period. This means difference was calculated as: (mean of greenhouse workers – mean of controls at the high exposure period) – (mean of greenhouse workers – mean of controls at the low exposure period).
p: statistical significance for the β-coefficient of the interaction term [controls vs. greenhouse workers] × [high vs. low exposure period].
[a] Mixed model analysis with non-structured covariance adjusted for high vs. low exposure period, greenhouse workers vs. controls, sex, nationality, age, BMI, and the interaction term [high vs. low exposure period] × [controls vs. greenhouse workers].

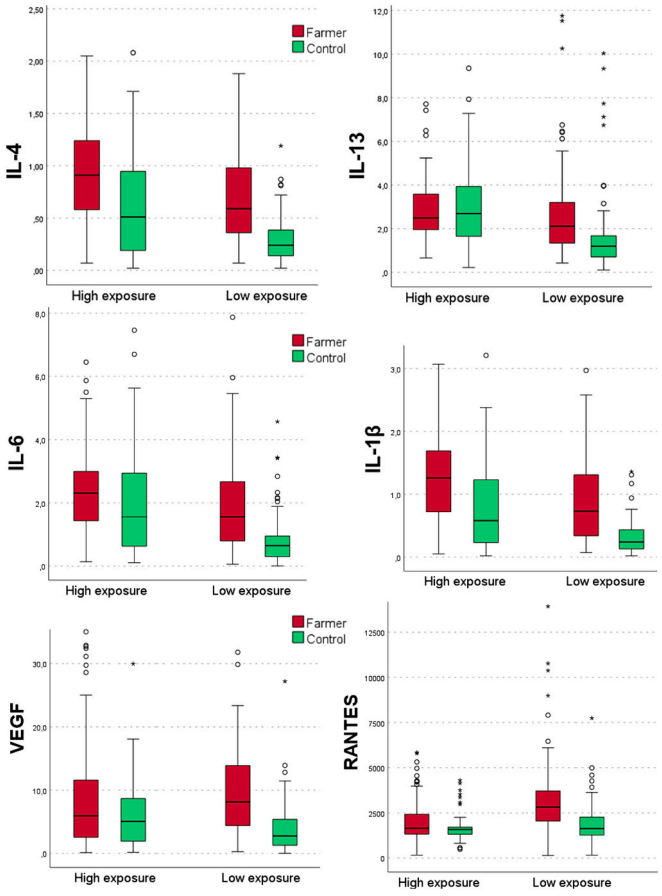

Fig. 1. Box plots of the immunoregulatory proteins for which relevant associations with occupational exposure to pesticides were observed (IL-4, IL-13, IL-1β, IL-6, VEGF and RANTES).

contributes to decreasing the dominant Th2-type cytokine response and also indicates an ongoing immune response or inflammation in the body. Increased circulating levels of this chemokine have been reported after ivermectin administration as anti-helminth treatment to decrease dominant Th2-type cytokine response in onchocerciasis patients (Cooper et al., 1996; Fendt et al., 2005). Although the information available on pesticides and RANTES is limited, a rise in plasma levels of RANTES has been observed in adult rats treated with permethrin, a pyrethroid insecticide, in early life (Vadhana et al., 2011).

The increased levels of IL-1β and VEGF found in greenhouse farmers at the period of high exposure to pesticides may suggest a systemic low-grade inflammatory state as both proteins are mediators of inflammation and angiogenesis, which are closely integrated processes in a number of physiological and pathological conditions including obesity, autoimmune diseases, and cancer (Shaik-Dasthagirisaheb et al., 2013). IL-1β is a pro-inflammatory agent associated with angiogenesis and increased vascular permeability though VEGF upregulation (Vinores et al., 2003). Besides, these two immunoregulatory proteins are considered the main factors responsible in establishing and maintaining tumor-mediated angiogenesis (Gelfo et al., 2020). Therefore, IL-1β and VEGF might play a role in the mechanisms underlying chronic diseases linked to pesticide exposure.

The discriminant analysis identified the immunoregulatory proteins that allow greenhouse workers to be distinguished from controls, with the latter being classified more correctly than greenhouse workers in the two study periods. Discriminant function analysis revealed that IL-4, IP-10, IL-8 and IL-17 have the highest power of discrimination in the period of low exposure to pesticides and IL-8, TNFα, MCP-1 and G-CSF in the period of high exposure. However, despite these findings, some overlaps exist among the studied biomarkers, suggesting that the distinction between workers and controls is not absolute. Other contributing factors not captured by the selected biomarkers may also play a role.

Different chemical classes of pesticides (organochlorines, carbamates, organophosphates, and several fungicides) can alter human inflammatory process. A literature review was performed in PubMed and Web of Science databases (Supplementary Table S6). The search string used, eligibility criteria and PRISMA flow diagram summarising the literature screening process are shown in Supplementary Fig. S1. Few studies have addressed the relationship between long-term occupational exposure to pesticides and changes in molecular biomarkers of immune function. Blood levels of several organochlorine pesticides (hexachlorobenzene, γ-hexachlorocyclohexane, dichlorodiphenyldichloroethylene (DDE), and pentachlorophenol (PCP) have been associated with suppression of Th1 cytokines (e.g., IL-2 and IFN-γ), and induction of Th2 cytokines (e.g., IL-4) (Daniel et al., 2002).

Some epidemiological studies have reported an increased risk of several cancers among farmers, specially non-Hodgkin's Lymphoma (Ohlander et al., 2020, 2022). Since this association may be related to the detrimental effect of pesticides on the immune system, changes in biomarkers of immunotoxicity deserve more attention as potential

predictive markers for cancer detection at early stages.

This study has several limitations. First, participants were not randomly selected, but consisted of farmworkers who volunteered to participate. However, as recruitment coincided with their periodic occupational health surveillance appointment, participation rate was nearly 100 %, which minimizes possible selection bias. The differences observed for socio-demographic characteristics between greenhouse workers and non-exposed controls could impact the effect estimates due to the potential existence of unmeasured or inadequately controlled confounding factors. To further evaluate the impact of potential confounders, we investigated the change in β coefficients before and after adjusting for a potential confounder. We focused on the six immunoregulatory proteins (IL-1β, IL-4, IL-6, IL-13, RANTES and VEGF) which were significantly, or near-significantly, associated with the interaction term "greenhouse workers *vs.* controls" × "period of high *vs.* low pesticide exposure" in Tables 6 and 7. In most cases, the change in β coefficients was <10 %, indicating little or no confounding effect. In a few cases, the change was >10 %, thus suggesting a confounding effect (see Supplementary Table S7). To minimize the impact of confounding, the mixed models analyses shown in Tables 6 and 7 were adjusted for covariates indicated therein as fixed effects.

Another limitation is that data on individual exposure to specific pesticides could not be collected through face-to-face interviews and we relied on detailed agronomists' reports on growing conditions, plant pests and diseases affecting the target crops in the study area. The classification between 'high' and 'low' exposure periods was largely based on operational definitions rather than direct measurements. This approach may lead to misclassification of exposure as it is susceptible to recall biases and inaccuracies. Generalization of exposure by the types of pesticides used during certain periods of the growing season may not accurately reflect the actual exposure scenario for each individual worker. Therefore, the findings observed, despite reflecting a real-life complex exposure scenario, cannot be attributed to any pesticide in isolation, but rather to the overall exposure to the pesticides used in the crop season studied. The absence of quantitative information on individual exposure prevented us from conducting more precise analyses on the dose-response relationship between exposure to specific pesticides and observed changes in immune function biomarkers. This limitation is in line with previous studies in which occupational exposure to pesticides is often poorly characterised (EFSA PPR Panel, 2017). The literature review shown in Table S6 revealed that many studies presented limited information on the specific pesticides used, reporting only crude exposure to pesticides or pesticide mixtures. It should be noted that, as greenhouse workers did not use organophosphate pesticides, the observed effects cannot be attributed to exposure to these compounds. On the other hand, cytokine levels may decrease over time, losing approximately 10–20 % each year after 2 years of storage as a result of storage effect, repetitive freeze-thawing cycles, or matrix interference (Keustermans et al., 2013). However, the approach used in this study ensured that any breakdown of immunoregulatory proteins would occur equally in both groups (greenhouse workers and controls) and in both study periods (high and low pesticide exposure), such that potential loss of stability due to storage time was minimized. Apart from these limitations, this study also has strengths. The main novelty is the use of a panel of molecular biomarkers to assess immune function in greenhouse workers, who are the subgroup of farmworkers with the highest occupational exposure to pesticides. In addition, this is a longitudinal study, wherein participants were repeatedly examined to assess changes in the immunoregulatory molecules that might occur over two periods of the same growing season with different exposure to pesticides, so that each individual served as a comparison with him/herself.

Further research is warranted to confirm and extend the results observed in this study, particularly by conducting a robust assessment of occupational pesticide exposure at individual level. This can be achieved by direct measurement of pesticide exposure, using e.g. patch dosimeter, surface wipe, and hand washing methods to measure external dermal exposure; or by biomonitoring of pesticides in operator urine or hair samples using multi-residue analytical methods. Both approaches will help validate self-reported results and provide more precise data.

5. Conclusion

This study found increased levels of IL-4, IL-6 and IL-13 in greenhouse workers exposed to pesticides, suggesting an impaired Th1/Th2 balance towards the Th2 response indicative of a type-2 inflammation usually presented as allergic inflammation. These finding are consistent with previous studies reporting that exposure to pesticides may cause allergic diseases, such as asthma, allergic rhinitis, and atopic dermatitis. On the other hand, the increased levels of VEGF IL-1β also observed in greenhouse workers may suggest a low-grade systemic inflammation and angiogenesis that may underlie chronic pathological conditions linked to pesticide exposure. However, these results should be interpreted with caution since they could be influenced by other factors not measured or inadequately controlled in our study. Further studies are warranted using direct quantitative measurements of individual pesticide exposure to better understand whether the effects of long-term pesticide exposure may be extended beyond innate immune dysfunction to an increased risk of chronic inflammatory-based diseases.

CRediT authorship contribution statement

David Lozano-Paniagua: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Tesifón Parrón:** Writing – review & editing, Resources, Formal analysis. **Raquel Alarcón:** Investigation, Data curation. **Mar Requena:** Investigation, Data curation. **Marina Lacasaña:** Writing – review & editing, Methodology, Conceptualization. **Antonio F. Hernández:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.173545.

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