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Physiological impact of air quality in
urban gulls and its implications for
their use as pollution sentinels in
coastal cities



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its implications for their use as pollution sentinels in
coastal cities**

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Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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(Catarina Fernandes de Oliveira)

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Abstract

Urban air pollutants pose significant threats to both wildlife and humans due to their persistence and potential for bioaccumulation. Sentinel species such as the yellow-legged gull (*Larus michahellis*) are valuable to assess environmental contamination. Due to their stationary nesting behaviour and continuous exposure to local air conditions, gull chicks are particularly valuable as early warning sentinels for urban air quality. In this study, we assessed the impact of airborne pollutants (PM_{2.5}, PM₁₀ and NO₂) on detoxification and oxidative stress biomarkers in 56 yellow-legged gull chicks in the overpopulated city of Barcelona, Spain. We analysed five biomarkers – cholinesterases (AChE, BChE), carboxylesterases (pNPA-CE, pNPB-CE), and glutathione S-transferase (GST) - in relation to the cumulative PM_{2.5}, PM₁₀, and NO₂ levels during the entire life of each gull chick. After accounting for individual variation in size, developmental stage, and trophic ecology (via stable isotope analysis), we found significant associations between air pollution and enzyme activity. Specifically, BChE and pNPB-CE activities were positively associated with PM₁₀ levels, while GST activity showed a positive correlation with NO₂ concentrations and a negative one with chick body weight. Our findings suggest that incorporating enzymatic biomarkers such as BChE, CE, and GST into physiological biomonitoring programs offers a practical and sensitive approach for evaluating the impact of urban air pollution, supporting integrated environmental and public health efforts within the One Health framework.

Keywords: B-esterases, GSTs, Pollution biomarkers, Air pollutants, Sentinel species

Resumo

Nas últimas décadas, tem-se observado uma maior influência da poluição urbana e dos poluentes antropogénicos na vida selvagem. Os contaminantes atmosféricos são uma ameaça ao meio ambiente, devido à sua persistência e potencial bioacumulação em organismos vivos. Neste estudo, investigamos o impacto de contaminantes, nomeadamente PM_{2.5}, PM₁₀ e NO₂, em crias de gaivotas-de-pata-amarela nascidas na cidade espanhola de Barcelona.

Frequentemente, a coleção de dados de poluição atmosférica envolve a concretização de medições precisas, através de estações de monitorização fixas ou de tecnologia de satélites avançada. Para além dos métodos tradicionais, os investigadores estão cada vez mais a usar espécies sentinelas (organismos que servem como bioindicadores de contaminação ambiental) para identificar os impactos da poluição atmosférica. Gaivotas urbanas como a gaivota-de-patas-amarelas (*Larus michahellis*) estão entre as espécies sentinelas consideradas mais fiáveis para monitorização da poluição urbana. As gaivotas-de-patas-amarelas são predadoras extremamente adaptadas a ambientes urbanos, construindo os ninhos em cidades costeiras e zonas industriais, áreas com elevada exposição a poluentes antropogénicos. Em relação à dieta, o seu comportamento oportunista e frequente contacto com água, solo e ar contaminados fazem delas excelentes bioindicadores de contaminação ambiental. As crias de gaivota-de-patas-amarelas são bioindicadores particularmente eficientes. Ao contrário das gaivotas adultas, as crias permanecem numa localização fixa (o ninho), inalando ar poluído continuamente. As crias de gaivota-de-patas-amarelas utilizadas neste estudo foram expostas à poluição atmosférica durante todo o seu tempo de vida (de três a quatro semanas).

Para confirmar o potencial das crias de gaivota-de-patas-amarelas como organismos sentinela, este estudo estabeleceu associações entre níveis de poluição atmosférica e alterações na atividade enzimática de biomarcadores específicos. Entre os biomarcadores mais utilizados atualmente para avaliar a poluição ambiental, selecionamos três enzimas pertencentes à família B-esterases e responsáveis por hidrolisar ligações éster: acetilcolinesterase (AChE), butirilcolinesterase (BChE) e carboxilesterases (CE). Para complementar, também selecionamos enzimas pertencentes à família das glutathione-S-transferases (GSTs), reconhecidas pelo seu papel crucial nas reações de conjugação e

processos de destoxificação.

Trabalho de campo e de laboratório foi realizado para investigar o impacto de contaminantes atmosféricos nas crias de gaivotas-de-patas-amarelas. Desde Março a Junho de 2023, 56 gaivotas foram capturadas em ninhos localizados na cidade de Barcelona. As atividades das enzimas hepáticas AChE e BChE foram determinadas de acordo com o protocolo de Ellman et al. (1961) e as atividades de carboxilesterases no fígado foram lidas seguindo o método descrito por Hosokawa e Satoh (2001). A atividade da enzima GST no mesmo órgão foi avaliada segundo o protocolo estabelecido por Habig et al. (1974). Os dados de poluição atmosférica dos poluentes $PM_{2.5}$, PM_{10} e NO_2 foram obtidos a partir de medições concretizadas por estações de monitorização fixas ao longo de todo o tempo de vida das gaivotas. A análise estatística foi realizada no programa RStudio (versão 4.2.2) e modelos lineares foram utilizados para avaliar a relação entre a exposição a poluentes atmosféricos e a atividade enzimática hepática.

A nossa hipótese inicial de que a atividade das enzimas no fígado das gaivotas poderia ser influenciada pela poluição atmosférica verificou-se correta. As enzimas hepáticas BChE e pNPB-CE revelaram associações positivas com as concentrações de PM_{10} . Por outro lado, os resultados mostraram que a GST tem uma relação positiva significativa com a concentração de NO_2 na atmosfera e tem uma correlação negativa com a massa corporal dos indivíduos. A relação inversa com a massa corporal sugere que crias mais jovens ou mais pequenas exibem atividades mais elevadas de GST, possivelmente devido a uma maior sensibilidade ao stress oxidativo durante o desenvolvimento inicial.

Um declínio na atividade da BChE era exetável com o aumento da exposição aos poluentes, principalmente devido à sensibilidade da enzima a produtos neurotóxicos, os quais a inibem ligando-se ao seu sítio ativo ou perturbando cofatores essenciais. No entanto, os nossos resultados mostraram uma associação positiva entre a atividade de BChE e os níveis de PM_{10} , o que sugere que os poluentes atmosféricos urbanos poderão não atuar através de neurotoxicidade, mas através da indução de inflamação sistémica e de stress oxidativo. O equilíbrio destes dois mecanismos poderá estimular a síntese enzimática no fígado. O aumento de atividade da BChE observado também poderá ser justificado por mecanismos de resposta de adaptação, nos quais a BChE atua como “bioscavanger” para neutralizar os contaminantes.

O aumento semelhante de atividade da carboxilesterase em relação à concentração de PM_{10} poderá também refletir uma resposta de destoxificação fisiológica. Este poluente contém vários compostos orgânicos lipofílicos, como hidrocarbonetos aromáticos policíclicos (PAHs) e flatatos, reconhecidos por ativarem recetores nucleares que regulam a expressão das CEs como parte da resposta adaptativa ao stress xenobiótico. O contaminante PM_{10} também induz stress oxidativo, contribuindo para a indução da CE através de vias de sinalização de ROS. Níveis moderados de ROS funcionam como mensageiros secundários, induzindo mecanismos de defesa antioxidante, incluindo a ativação de esterases hepáticas. O aumento paralelo das atividades enzimáticas da BChE e CE poderá representar uma resposta enzimática coordenada ao stress induzido por poluentes atmosféricos.

O presente estudo também demonstrou uma correlação positiva entre a atividade enzimática da GST e concentração atmosférica de NO_2 . Poluentes como o NO_2 contribuem significativamente para o aumento do stress oxidativo, aumentando os níveis de ROS e RNS nos tecidos. Este estudo evidencia que o aumento da atividade da GST no fígado é resultado de uma resposta adaptativa das crias de gaviotas-de-patas-amarelas à poluição urbana causada por radicais de NO_x , visto que as GSTs estão envolvidas na destoxificação destes compostos.

Este projeto contribui para o conceito de One Health (“Uma saúde”), que realça a interligação entre a saúde humana, a vida animal e o meio ambiente. De uma perspetiva de conservação e planeamento urbano, os nossos resultados enfatizam a necessidade de se implementarem estratégias de mitigação da poluição urbana em cidades com elevada densidade demográfica, como é o caso de Barcelona. Reduzir a emissão de partículas finas e óxidos de nitrogénio, através de um aperfeiçoamento dos meios de transporte público e da monitorização de emissões poluentes, poderá beneficiar tanto a população humana, como a vida selvagem. A análise espacial realizada identificou os bairros Eixample, Poblenou e Gràcia como as zonas urbanas mais poluídas. Estas zonas deverão ser alvos prioritários para a incorporação da biomonitorização fisiológica no planeamento urbano e em ações de conservação e de mitigação de poluição atmosférica.

Palavras-chave: B-esterases, GSTs, Biomarcadores de poluição, Poluentes Atmosféricos, Espécies sentinelas

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List of Abbreviations

AChE – Acetylcholinesterase

AIC – Akaike Information Criterion

AhR – Aryl hydrocarbon receptor

BChE – Butyrylcholinesterase

CAR – Constitutive androstane receptor

CBs – Carbamates

CDNB – 1-chloro-2,4-dinitrobenzene

CE – Carboxylesterase

CI – Confidence interval

DTNB – 5,5'-dithiobis(2-nitrobenzoic acid)

GIS – Geographic Information Systems

GPx – GSH peroxidase

GSH – Reduced glutathione

GSSG – Oxidized glutathione

GSTs – Glutathione-S-Transferases

OPs – Organophosphates (OPs)

PAH – Polycyclic aromatic hydrocarbon

PBDEs – Polybrominated Diphenyl Ethers

PCBs – Polychlorinated biphenyls

PM₁₀ – Particulate matter with a diameter of 10 μm or less ($\leq 10 \mu\text{m}$)

PM_{2.5} – Particulate matter with a diameter of 2.5 μm or less ($\leq 2.5 \mu\text{m}$)

POPs – Persistent organic pollutants

PXR – Pregnane X receptor

RNS – Reactive Nitrogen Species

ROS – Reactive oxygen species

pNPA – p-nitrophenyl acetate

pNPA-CE – Carboxylesterase with p-nitrophenyl acetate as substrate

pNPB – p-nitrophenyl butyrate

pNPB-CE – Carboxylesterase with p-nitrophenyl butyrate as substrate

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1. Introduction

1.1. Air pollution

Urbanization is a driver of environmental change, leading to biodiversity loss on a global scale (Worm *et al.*, 2006; Goddard, Dougill and Benton, 2010). Urban pollution has been growing throughout the last decades, introducing anthropogenic pollutants that affect both humans and wildlife (Pant and Singh, 2024; Jalil, 2025). Among the different urban pollutants, air pollution, primarily caused by vehicular emissions as well as industrial and construction activities, poses a significant threat to the environment (Haq, Waseem and Baqi, 2021; Agbontaen *et al.*, 2024). Some examples of air pollutants are particulate matter (PM), nitrogen oxides (NO_x), and sulphur dioxide (SO₂) (Haq, Waseem and Baqi, 2021; Agbontaen *et al.*, 2024). The presence of airborne contaminants in the atmosphere severely endangers the environment due to their persistence and potential bioaccumulation in living organisms (Haq, Waseem and Baqi, 2021; Agbontaen *et al.*, 2024).

Particulate Matter (PM) is classified according to its size: PM₁₀ (coarse particulate matter) includes particles with a diameter of 10 µm or less (≤ 10 µm), while PM_{2.5} (fine particulate matter) comprises particles with a diameter of 2.5 µm or less (≤ 2.5 µm) (Manisalidis *et al.*, 2020). The group of PM_{2.5} includes fine particles such as soot, tobacco smoke, smog, sulfates, nitrates, biological contaminants such as bacteria, viruses, allergens, metals (Burton, Suh and Koutrakis, 1996; Rajšić *et al.*, 2008; Manisalidis *et al.*, 2020). On the other hand, PM₁₀ encompasses larger particles such as fly ash, cement dust, fungi, mold spores, and settled dust, along with allergens like pollen and household dust (Burton, Suh and Koutrakis, 1996; Rajšić *et al.*, 2008; Manisalidis *et al.*, 2020). Both particle types can remain suspended in the atmosphere for extended periods, allowing them to travel long distances and infiltrate biological systems through inhalation or ingestion (Kumar *et al.*, 2025).

NO_x, primarily composed of nitrogen monoxide (NO) and nitrogen dioxide (NO₂), originates from both human activities (power generation, transportation, agriculture, and industrial processes) and natural sources (soil emissions and lightning) (Di Maio and Valachovic, 2024; Lee *et al.*, 2025). Nitrogen dioxide, a toxic gas, plays a role in the formation of tropospheric ozone (O₃) through photochemical reactions with volatile organic compounds, carbon monoxide (CO), and methane (CH₄) (Andino-Enríquez, Hidalgo-Bonilla and Ladino, 2018) and mainly results from the combustion of fossil fuels (Sangale *et al.*, 2020). It also contributes to the formation of PM_{2.5} (Zhao and Heinrich, 2024) and is involved in the formation of acid rain, which can damage ecosystems, degrade water quality, and harm organisms (Błaszczak, 2024). Additionally, NO_x can enhance nutrients pollution in coastal waters, favouring the formation of algal blooms (Lan *et al.*, 2024).

Prolonged exposure to air pollutants, particularly PMs, poses a major threat to human health. The World Health Organization estimated that air pollution is responsible for approximately seven million deaths annually (WHO, 2012). Previous studies have linked smog episodes to spikes in mortality (Correia *et al.*, 2013), as observed in Beijing's 2013 smog crisis (Cao, Liang and Niu, 2017; Zhang *et al.*, 2017). It has been reported that short-term exposure to PM₁₀ is correlated with a rise in mortality rates by approximately 0.5% for every 10 µg·m⁻³ increase in daily concentration (WHO, 2006). Previous research has also emphasized that polycyclic aromatic hydrocarbons (PAHs) present in PMs are recognized as carcinogenic, with a higher concentration of harmful compounds found in PM_{2.5} (Sosa *et al.*, 2017). In environments with high pollution levels, the lifetime cancer risk due to PAH exposure exceeds the strict safety limits set by the US Environmental Protection Agency (Sosa *et al.*, 2017). Research in industrial areas has shown that PMs can break down into nanoparticles smaller than 200 nm in aqueous environments, which can be absorbed by human cells, potentially contributing to respiratory diseases (Morozesk *et al.*, 2021). High concentrations of PM_{2.5} and PM₁₀ have also been associated with disruptions in the human pharyngeal microbiota, increasing susceptibility to infections and respiratory complications (Qin *et al.*, 2019). Long-term exposure to NO₂ impacts health condition, including hypertension, reduced lung function, chronic obstructive pulmonary disease (Lamichhane, Leem and Kim, 2018; Lyons *et al.*, 2020) and an increased susceptibility to viral infections (Jurado *et al.*, 2020). Research has shown that prolonged indoor exposure to NO₂ weakens

immune defenses, increasing vulnerability to illnesses such as influenza (Frampton *et al.*, 1989).

With the aim to mitigate air pollution and safeguard public health, the European Union countries must comply with the maximum pollution levels set by the Ambient Air Quality Directives and fulfill emission reduction commitments for five key airborne pollutants, including NO₂ and PM_{2.5} (EU Directive 2024/288). In Spain, various regulatory measures have been implemented to reduce air pollution. Notably, the Climate Change and Energy Transition Act (2021) establishes that all newly purchased cars need to be zero-emission by 2040, prohibits new hydrocarbon exploration, and requires cities with over 50,000 residents must establish Low Emission Zones (LEZs) (Salvador & Matías, n.d.). Additionally, the National Air Pollution Control Programme 2023-2030 sets ambitious emission reduction goals, aiming for an 88% decrease in sulfur dioxide, a 62% reduction in nitrogen oxides, and a 50% cut in PM_{2.5}, covering various sectors, including energy, transport, industry, and agriculture (Ministerio para la Transición Ecológica y el Reto Demográfico, 2024.).

1.2. Monitoring air pollutants by wildlife sentinels

Addressing air pollution requires the accurate measurement of pollutant levels and an assessment of their impacts. Usually, air pollution data is collected through fix air monitoring stations located in urban and industrial areas, as well as through advanced satellite technology (Gautam *et al.*, 2024). In addition to traditional methods, researchers are increasingly relying on biological sentinels, organisms that serve as indicators of environmental contamination, to assess the presence and impact of pollution (Dini *et al.*, 2024; Iacob *et al.*, 2024). These organisms, in special the ones in the top of food webs, can bioaccumulate pollutants, exhibit physiological or genetic stress responses, and provide early warning signals for environmental degradation (Ramírez *et al.*, 2020; Frixione *et al.*, 2022; Galimany *et al.*, 2023).

Synanthropic wildlife - *species that live in close association with people* - are particularly effective as sentinels due to their direct exposure to human-related pollutants and their ecological proximity with human populations (Dini *et al.*, 2024). These species play a crucial role in monitoring chemical, microbial, and genetic pollutants (Dini *et al.*, 2024). Research has shown significant bioaccumulation of heavy metals in urban birds (Manjula, Mohanraj

and Devi, 2015; Cooper *et al.*, 2017; Iacob *et al.*, 2024), and genotoxic effects in species inhabiting industrial and metropolitan areas (Baos *et al.*, 2006; Ceyca-Contreras *et al.*, 2020; Frixione *et al.*, 2022). Pollution monitoring using sentinel species allows researchers to assess not only environmental quality but also human health risks, given that many of these pollutants pose common threats across various species (Reif, 2011; Hassell *et al.*, 2019; García-Fernández *et al.*, 2020).

Among synanthropic wildlife, urban gulls such as the yellow-legged gull (*Larus michahellis*) (Figure 1), the model species used in the present study, are particularly reliable biological sentinels for monitoring urban pollution (*e.g.* Max *et al.*, 2025; Nos *et al.*, 2024). This predator species thrives in urbanized environments, often nesting in coastal cities and industrial zones - areas with high exposure to anthropogenic pollutants (Nos *et al.*, 2024; Max *et al.*, 2025). Their opportunistic foraging behaviour and frequent contact with contaminated water, soil, and air make them excellent integrative bioindicators of environmental contamination (Galimany *et al.*, 2023). Gull chicks, in particular, offer valuable insights into the health impacts of cumulative atmospheric pollution. Unlike adult gulls, which travel extensively, chicks stay in a fixed location (the nest) during their rearing period, continuously inhaling air pollutants from their immediate surroundings. The prolonged exposure and limited mobility makes them suitable for assessing the direct effects of air pollution on their health status. For example, previous studies have related pollutant exposure in birds to oxidative stress, altered immune responses, and developmental abnormalities, emphasizing the relevance of gull chicks as biological sentinels (Koivula and Eeva, 2010; Herrera-Dueñas *et al.*, 2014; Morrissey *et al.*, 2014; Bauerová *et al.*, 2017).



Figure 1. Picture showing one adult and two chicks of yellow-legged gull from the urban population of the city of Barcelona (Spain). Picture taken by Pablo Cermeño (BCNGulls).

1.3. Physiological markers to investigate the impact of pollutants on health

Modifications on enzymatic activities can serve as biomarkers for assessing how environmental pollutants can influence the health of organisms. For that, associations between pollution levels and biomarker activities have to be established. Among the biomarkers more currently used to assess environmental pollution, we selected three well-established esterase enzymes - acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and carboxylesterase (CE) - all belonging to the B-esterase family, a group of serine hydrolases involved in the breakdown of ester-containing compounds (Roy *et al.*, 2005) and glutathione-S-Transferases (GSTs), an enzyme involved in conjugation reactions and detoxification (Maurice *et al.*, 1991).

AChEs are present in all excitable nervous tissues and muscles, where they play a key role in synaptic transmission by breaking down the ester neurotransmitter acetylcholine (Van Dyk and Pletschke, 2011). Butyrylcholinesterase (BChE), also referred to as pseudocholinesterase or plasma cholinesterase, is a non-specific cholinesterase enzyme produced in the liver (Van Dyk and Pletschke, 2011). While its exact physiological function remains unclear, it is mainly recognized for its role in protecting AChE (Sridhar and Gumpeny, 2024). BChE is also involved in several cellular processes, including cell proliferation and morphogenic

movements (Robitzki *et al.*, 1997), and is recognized as a key regulator in lipid and lipoprotein metabolic pathways (Kutty, 1980). Both AChE and BChE have been used as biomarkers to identify the presence of chemical pollutants, mostly pesticides, in the environment, but they present differences in substrate selectivity and sensitivity to pollutant inhibition (Van Dyk and Pletschke, 2011). It is also advantageous that there are not known endogenous inhibitors of these enzymes, which supports the assumption of the correlation between enzyme activity and enzyme protein content (Khattab and Ali, 2007).

CEs are ubiquitous enzymes, mainly expressed in barrier tissues exposed to xenobiotics, including lung and gut epithelia, liver, kidney and skin (Williams, 2008; Hatfield *et al.*, 2011). They play a role in detoxification through hydrolysis of ester bonds found in both natural and synthetic molecules (Cashman *et al.*, 1996). CEs also protect AChE from inhibition by organophosphorus pesticides due to their high affinity for these compounds (Wheelock and Nakagawa, 2010; Nos *et al.*, 2020). CEs are suitable biomarkers for environmental pollution monitoring (Estévez, Vilanova and Sogorb, 2019). Unlike other short-lived and thermosensitive enzymes, they are highly stable and can withstand prolonged storage and freezing conditions, making them ideal for long-term analysis. Previous research has also shown that these esterases are inhibited by the presence of heavy metals (Laranjeiro *et al.*, 2020), which may be found in airborne particulate matter, highlighting their potential role as air pollution biomarkers.

GSTs are responsible for the conjugation between glutathione and hydrophobic and electrophilic compounds (Maurice *et al.*, 1991). They are involved in various metabolic pathways such as detoxification of xenobiotics, steroid metabolism, organic anion transport, carcinogenesis, and cell growth (Maurice *et al.*, 1991). GSTs are highly conserved across species, from plants to humans, which emphasizes their evolutionary significance (Sheehan *et al.*, 2001). One of the toxic compounds detoxified by GST is benzo[a]pyrene, a common air pollutant and polycyclic aromatic hydrocarbon (PAH), suggesting a possible application of GST as a biomarker for air pollution (Bocedi *et al.*, 2019).

1.4. Barcelona as a case study of an overpopulated polluted European city that hosts an urban population of yellow-legged gulls

Barcelona is one of the European cities with higher population density and traffic congestion and the NO₂ and PM_{2.5} levels frequently exceed the recommended safety limits (Schembari *et al.*, 2014). In recent years, additional regulatory measures have been implemented within the city including the extension of low-emissions zones to restrict high-emission vehicles and highlighted the contribution of its port to air pollution (Velásquez *et al.*, 2025). The Barcelona City Council has also invested in improving public transport infrastructure and developing cycling and pedestrian lanes to provide more sustainable mobility options. Another initiative is to provide incentives for low-emission ships, the use of liquefied natural gas as an alternative fuel, and electrification projects to reduce reliance on fossil fuels (Velásquez *et al.*, 2025).

Barcelona's air quality stations spread across different neighborhoods, continuously monitoring the atmosphere. Each station is equipped with sensors that measure key pollutants such as NO₂, PM₁₀, PM_{2.5}, SO₂, and CO. The collected data is centralized and made publicly available through the city's open data platform (Figure 2) (Open Data BCN).

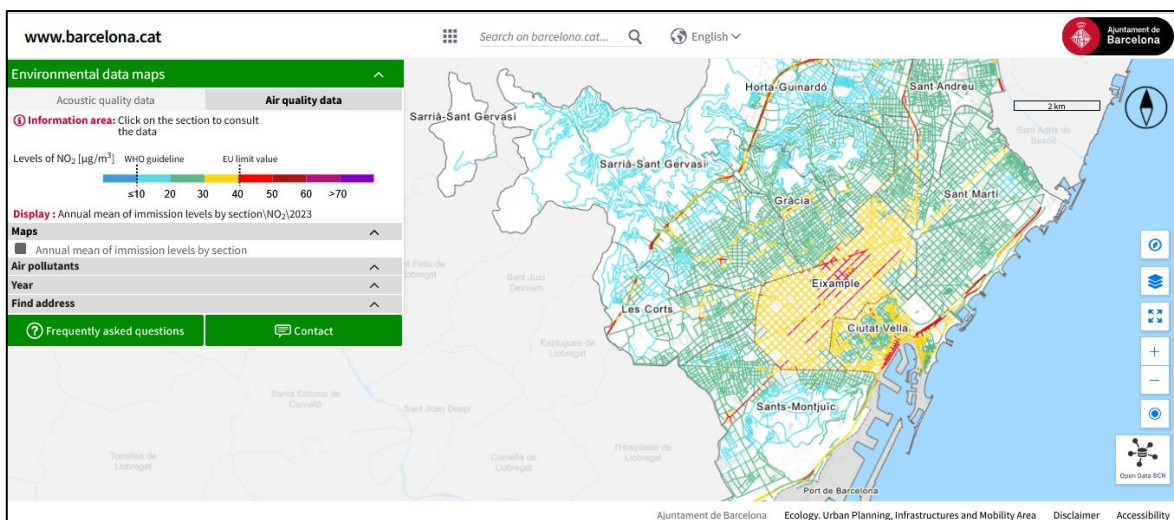


Figure 2. Screenshot from Barcelona's air quality data platform, illustrating the spatial distribution of annual NO₂ concentrations in 2023 (Open Data BCN, Servei de dades obertes de l'Ajuntament de Barcelona), one of the air pollutants used in the present study.

Recent estimates suggest that Barcelona hosts approximately 350 breeding pairs of yellow-legged gulls (Martín-Vélez and Navarro, 2025) which predominantly nest on building

rooftops (Martín-Vélez *et al.*, 2022). This species has been identified as sentinel species of urban pollution in Barcelona (Navarro *et al.*, 2019; Galimany *et al.*, 2023). Over the past few years, urban yellow-legged gulls have been used to monitor various environmental threats in the city, including plastic pollution (Max *et al.*, 2025), the presence of persistent organic pollutants such as PCBs and PBDEs (Nos *et al.*, 2024), and the circulation and dispersion of zoonotic pathogens (Martín-Vélez *et al.*, 2024; Navarro *et al.*, 2019). These studies have shown the species' ability to inform about the health status of urban environments, offering insights into the pressures associated with living in cities.

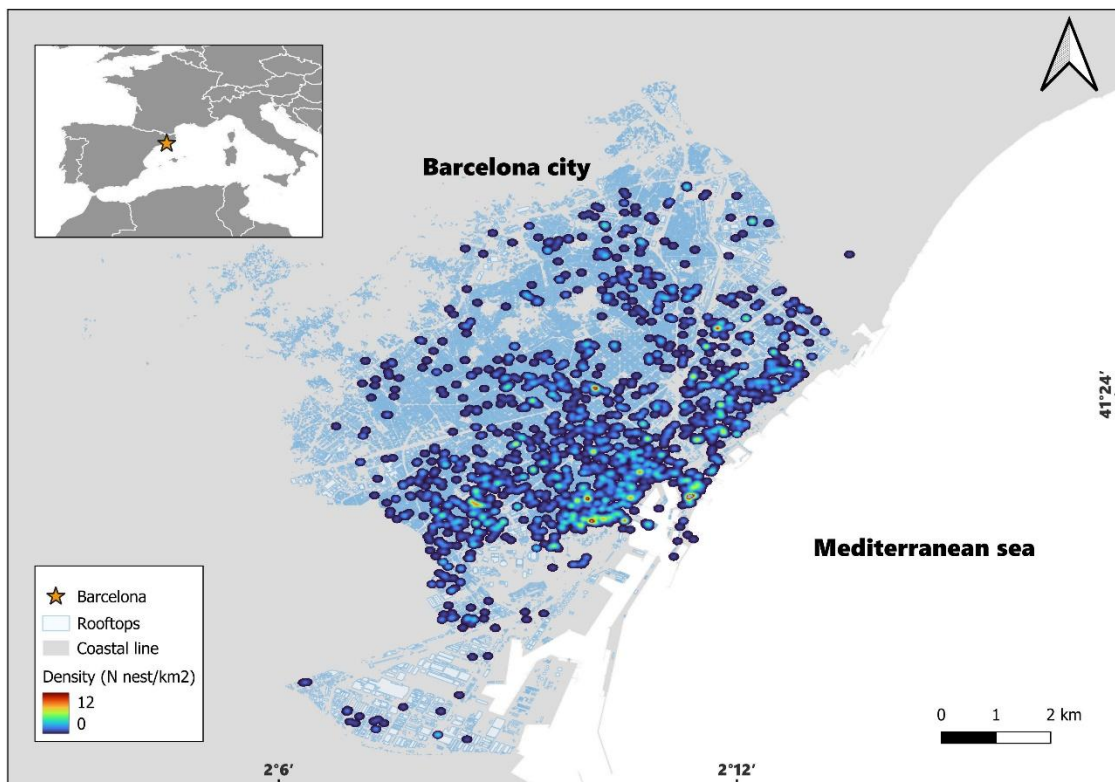


Figure 3. Spatial density of nests detected in Barcelona over 12 years (2013–2024). The upper-left corner shows the location of Barcelona within Europe (Martín-Vélez and Navarro, 2025).

2. Objectives of the master thesis

We investigated the impact of urban air pollution (PM₁₀, PM_{2.5} and NO_x) in Barcelona city on urban yellow-legged gull. For this, we measured the enzymatic activity of acetylcholinesterase (AChE), butyrylcholinesterase (BChE), carboxylesterases (CE), and glutathione S-transferase (GST) in the liver of 56 yellow-legged gull chicks collected through the city of Barcelona. The activity of these physiological biomarkers was related to cumulative concentrations of pollutants PM₁₀, PM_{2.5}, and NO₂ measured over the entire lifespan of each chick, based on the data corresponding to their nesting locations.

We hypothesized that air pollutants (PM_{2.5}, PM₁₀, and NO₂) affect the enzymatic activity of AChE, BChE, CE, and GST measured in gull's chicks. Based on previous studies, we anticipated that esterases will exhibit an inhibition response due to pollutant exposure (Laranjeiro *et al.*, 2020; Nos *et al.*, 2021), while GST activity may increase as a compensatory mechanism (Antognelli *et al.*, 2006). The behaviour of these enzymatic responses could serve as a reliable biomarker for urban air pollution, offering a fast, robust and cost-effective method for assessing environmental contamination in the future.

3. Material and Methods

3.1. Fieldwork procedures

From March to June 2023, 56 yellow-legged gulls chicks were sampled from nests located within the city of Barcelona (Figure 4). The sampled chicks are between three to four weeks old. The chicks were handled by the Public Health Agency of Barcelona, according to the Legislative Decree 2/2800, April 15, DOGC. Upon capture, morphological measures were taken, such as body weight (g), culmen length (cm) and tarsus length (cm). Newly grown body feathers were also collected for further isotope analysis (see below in “stable isotopes” section). The chicks were dissected and their livers removed and stored at $-70\text{ }^{\circ}\text{C}$ until biochemical analysis.

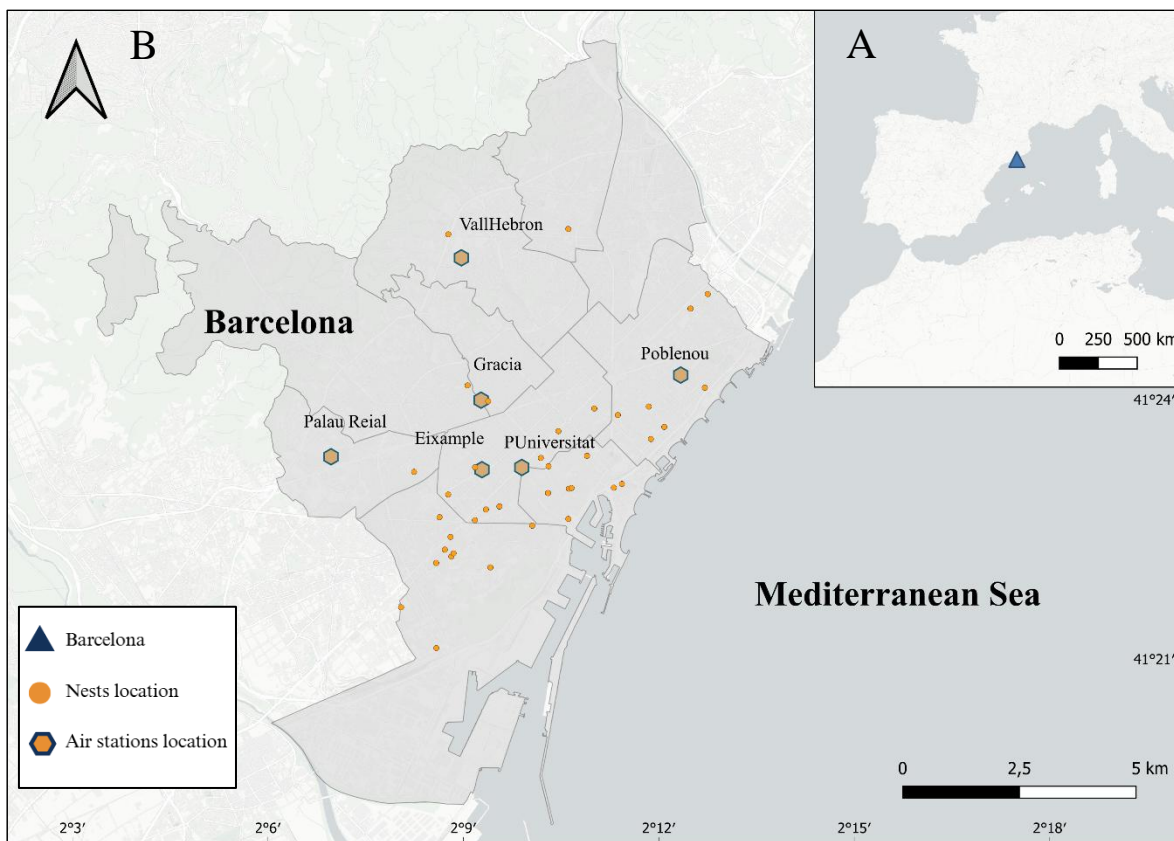


Figure 4. (A) Location of Barcelona in southern Europe (blue triangle). (B) Distribution of the air quality monitoring stations (orange hexagons) and the nests (orange dots) of the yellow-legged gull chicks where sampled in Barcelona during 2023.

3.2. Physiological analyses

The enzymatic activities of AChE, BChE were measured following the Ellman et al., (1961) protocol, CEs activities were measured according to the Hosokawa & Satoh, 2001 protocol and GST activities were measured following the Habig et al., (1974) protocol. Livers from 56 chicks were initially weighed, homogenized, and diluted at a 1:4 (tissue:buffer) ratio in 100 mM phosphate buffer, pH 8.0. AChE and BChE activities were measured using acetylthiocholine (ATC) and butyrylthiocholine (BTC) as substrates, respectively, with the reaction coupled to 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) for colorimetric detection of thiocholine. CEs were measured using pNPA (p-nitrophenyl acetate) and pNPB (p-nitrophenyl butyrate) substrates, which are hydrolyzed by CEs to release yellow p-nitrophenol. GST was quantified by incubating the samples with 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH). Absorbance changes were read in real-time using a TECAN spectrophotometer at 412 nm for AChE and BChE, at 405 nm for CE, and at 340 nm for GST activities and quantified using Magellan v6.0 software. Protein concentrations were determined using the Bradford method (Bradford, 1976). Enzyme activities were expressed in nmol/min/mg protein.

3.3. Stable Isotope Analysis (SIA)

Stable isotope analysis of nitrogen (^{15}N) and carbon (^{13}C) provides valuable information on trophic ecology (Inger and Bearhop, 2008). Trophic levels are identified using $\delta^{15}\text{N}$ values, with higher values reflecting a higher position within the food web (Inger and Bearhop, 2008). The $\delta^{13}\text{C}$ values relate to foraging habitats and diet. Higher $\delta^{13}\text{C}$ values correspond to feeding in coastal or benthic environments and lower $\delta^{13}\text{C}$ values suggest feeding on pelagic or oceanic food sources (Inger and Bearhop, 2008). The sampled chicks were from three to four weeks old, so the isotope values from their feathers reflect their diet throughout their entire lifespan. SIA values were obtained using feather samples. The feathers were initially cleaned, dried and powdered. The processed samples were then analysed at the Stable Isotopes Laboratory of Estación Biológica de Doñana (<https://www.ebd.csic.es/en>) to determine the ratios of stable carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) isotopes. Isotopic measurements were performed using a Flash HT Plus elemental analyser coupled with a Delta V Advantage mass spectrometer, following established

protocols.

3.5. Air pollution associated to each sampled chick

Air pollutants data were collected from air monitoring stations located throughout Barcelona and made publicly available in the Open Data BCN database (Open Data BCN, Servei de dades obertes de l'Ajuntament de Barcelona). Specifically, we extracted the daily concentrations of PM_{2.5}, PM₁₀, and NO₂ for each of the six monitoring stations within the urban area (see Figure 5) during April-May 2023 (corresponding to the chick-rearing period). Subsequently, we assigned the cumulative air pollution values from the station closest to each sampled nest. The cumulative PM_{2.5}, PM₁₀, and NO₂ values were calculated by summing the daily air pollution values over the entire over the entire period of each chick's age. The age of each chick was estimated based on their morphological measurements (Méndez *et al.*, 2020). The distance between the nest and the monitoring station as used as a control variable in the statistical analysis.

3.6. Statistical analysis

To assess the relationship between liver enzyme activities and atmospheric pollution, linear regression models were applied using the “lm” function from the R package “lmtest” (Zeileis and Hothorn, 2002). Due to high collinearity among some enzyme activity measures, the analyses were focused on BChE, CE (with substrate pNPB), and GST. Highly correlated response variables were excluded from the models (AChE was strongly correlated with BChE, and pNPA with pNPB (Figure S1); correlation > 0.90 (Dormann *et al.*, 2013)). Log transformations were applied to the response variables to achieve normality of the data (Curran-Everett, 2018). For graphical presentation of model predictions, values were back-transformed (exponentiated) to return to the original scale (nmol/min/mg protein), allowing for easier biological interpretation. The explanatory variables included in the models were body weight, distance from the nest to the closest station (Distance_nest_station), $\delta^{13}\text{C}$ values (dC), $\delta^{15}\text{N}$ values (dN), accumulated NO₂ (sumNO2_sampler), accumulated PM_{2.5} (sumPM2.5_sampler) and accumulated PM₁₀ (sumPM10_sampler). Due to low correlation values between explanatory variables, further statistical testing was not conducted (Figure

S1). Model selection was based on AIC values, with lower AIC values indicating better model fit. The “dredge” function, from the MuMIn package in R, was used to generate all possible subsets of the global model, allowing identification of the most parsimonious models (Table 3) (Chakrabarti and Ghosh, 2011). Model fit was assessed by the multiple R^2 statistic, which indicates the proportion of variance in the response variable that is explained by the set of predictors, making it useful for assessing overall model fit (Rodríguez del Águila and Benítez-Parejo, 2011). The linear model estimates were reported in table 4. All statistical analysis were performed using RStudio version 4.2.2.

4. Results

4.1. Morphological measures and stable isotopic results

Morphological measurements showed variation across individuals (Table 1). The mean culmen length was 38.85 ± 4.64 mm (95% CI: 37.61–40.09 mm), and the mean tarsus length was 65.85 ± 4.87 mm (95% CI: 64.54–67.15 mm). The average body weight was 741.25 ± 173.90 g (95% CI: 694.68–787.82 g), suggesting higher variability in size within the population.

The mean $\delta^{13}\text{C}$ value was $-20.49 \pm 0.78\text{‰}$ (median = -20.36‰ ; 95% CI = -20.69 to -20.28‰), while the mean $\delta^{15}\text{N}$ value was $9.88 \pm 0.59\text{‰}$ (median = 9.84‰ ; 95% CI = 9.72 to 10.03‰) (Table 1). The small range of the 95% confidence intervals indicate consistent isotopic signatures across individuals, suggesting similar diet sources or foraging environments within the sampled population.

Table 1. Mean and standard deviation (SD), median and 95% confidence interval of the morphology, stable isotopic values and enzymatic activity in liver of 56 yellow-legged gull chicks sampled in Barcelona during the breeding season 2023.

	Mean \pm SD	Median	95 % CI
Morphology			
Culmen Length (mm)	38.85 ± 4.64	38.48	[37.61–40.09]
Tarsus Length (mm)	65.85 ± 4.87	66.56	[64.54–67.15]
Body Weight (g)	741.25 ± 173.90	735.00	[694.68–787.82]
Stable isotopic values (‰)			
$\delta^{15}\text{N}$	9.88 ± 0.59	9.84	[9.72 - 10.03]
$\delta^{13}\text{C}$	-20.49 ± 0.78	-20,36	[20.69 –20.28]
Enzymatic activity (nmol/min/mg protein)			
AChE	13.07 ± 4.31	12.40	[11.92 - 14.23]
BChE	16.85 ± 5.38	16.25	[15.41- 18.29]
pNPA-CE	41.93 ± 18.13	36.14	[37.07 - 46.78]
pNPB-CE	86.20 ± 39.09	75.95	[75.73 - 96.67]
GST	106.58 ± 28.80	100.19	[98.86 - 114.29]

4.2. Enzymatic activity

The mean AChE activity was 13.07 ± 4.31 nmol/min/mg protein (95% CI: 11.92 - 14.23), while BChE activity averaged 16.85 ± 5.38 nmol/min/mg protein (95% CI: 15.41 - 18.29) (Table 1). The mean activities of carboxylesterases with substrates pNPA and pNPB were 41.93 ± 18.13 and 86.20 ± 39.09 nmol/min/mg protein, respectively (95% CIs: 37.07 - 46.78; 75.73 - 96.67) (Table 1). GST activity showed greater variability, with a mean of 106.58 ± 28.80 nmol/min/mg protein (95% CI: 98.86 - 114.29) (Table 1). Enzyme activity values ranged from 5.570 – 24.662 nmol/min/mg protein for AChE, 6.846 – 29.886 nmol/min/mg protein for BChE, 17.382 - 89.754 nmol/min/mg protein for pNPA-CE, 41.875 – 244.505 nmol/min/mg protein for pNPB-CE and 65.280 – 209.228 nmol/min/mg protein for GST.

4.3. Air pollutant concentrations

PM_{2.5} levels showed a mean of 392.75 $\mu\text{g}/\text{m}^3$ with a relatively narrow standard deviation (± 72.96) and 95% CI [373.21–412.29], indicating low variability (Table 2). PM₁₀ levels were higher, with a mean of 484.05 $\mu\text{g}/\text{m}^3$, a larger standard deviation (± 158.87) and wider 95% CI [441.50–526.59] indicating greater variability compared to PM_{2.5} (Table 2). NO₂ showed the highest concentration with a mean of 611.99 $\mu\text{g}/\text{m}^3$ and the widest variability (± 202.49) (Table 2). The corresponding 95% CI [557.76–666.21] suggests more uncertainty around the mean estimate.

Table 2. Mean and standard deviation (SD), median and 95% confidence interval of the cumulative concentrations of different air pollutants around the nest of the 56 yellow-legged gull chicks sampled in Barcelona during the breeding season 2023.

Air pollutant ($\mu\text{g}/\text{m}^3$)	Mean \pm SD	Median	95 % CI
PM _{2.5}	392.75 \pm 72.96	391.33	[373.21 – 412.29]
PM ₁₀	484.05 \pm 158.87	475.20	[441.50 – 526.59]
NO ₂	611.99 \pm 202.49	544.61	[557.76–666.21]

4.4. Relationship between atmospheric pollution and liver enzyme activities

For each enzyme (BChE, pNPB-CE, and GST), the retained explanatory variables and their corresponding Akaike Information Criterion (AIC) values are shown (Table 3). For BChE, the final model included PM₁₀ concentration and body weight, with an AIC of -64.63, indicating improved fit compared to the full model (AIC = -59.05). The pNPB-CE model retained both PM_{2.5} and PM₁₀, with an improved AIC of -40.10. The GST model included NO₂ exposure and weight, with the lowest AIC overall (-88.65), suggesting the best fit among the three models. These results confirm the importance of specific pollutants and body weight in explaining variations in enzymatic activity.

Regression coefficients (estimate ± SE) for body weight and air pollutants included in the final models are summarized in Table 4, indicating which variables had a statistically significant effect on enzyme activity. Specifically, body weight and PM₁₀ concentration were the retained variables in the BChE model based on selection criteria (Adjusted R² = 0.17) (Table 3). Since the correlation between weight and BChE activity was not statistically significant ($t = -1.72$, $p = 0.09$), this variable was not plotted against BChE activity. PM₁₀ showed a statistically significant positive association with BChE activity ($t = 3.42$, $p = 0.001$), suggesting increased enzymatic activity with higher PM₁₀ exposure (Figure 5A). Both PM₁₀ and PM_{2.5} were retained in the CE model (Adjusted R² = 0.16) (Table 3). PM_{2.5} exhibited a negative but statistically non-significant association with CE activity ($t = -1.88$, $p = 0.07$), therefore this variable was not plotted against CE activity. In contrast, PM₁₀ showed a significant positive effect ($t = 3.565$, $p = 0.0008$) on CE activity (Figure 5B). Both body weight and NO₂ were retained in the GST model (Adjusted R² = 0.089) (Table 3). Body weight showed a significant negative association with GST activity ($t = -2.10$, $p = 0.04$) (Figure 5C), while NO₂ had a significant positive effect ($t = 2.44$, $p = 0.02$), indicating opposing influences on enzyme levels (Figure 5D).

Table 3. Linear models assessing the effects of air pollutants and chick weight on enzyme activity.

Enzymes	Selected Model Explanatory Variables	AIC total model	AIC
BChE	sumPM10_sampler + weight	-59.05	-64.63
pNPB-CE	sumPM10_sampler + sumPM2.5_sampler	-34.73	-40.10
GST	sumNO2_sampler + weight	-85.20	-88.65

Table 4: Linear model estimates (\pm SE) for enzyme activities in relation to pollution predictors, degrees of freedom and Multiple R² values.

Enzyme	Weight (Estimate \pm SE)	SumPM _{2.5} (Estimate \pm SE)	Sum PM ₁₀ (Estimate \pm SE)	SumNO ₂ (Estimate \pm SE)	d.f.	R ²
BChE	-0.03 \pm 0.02	–	0.06 \pm 0.02**	–	53	0.20
pNPB-CE	–	-0.05 \pm 0.03	0.09 \pm 0.03***	–	53	0.19
GST	-0.03 \pm 0.01 *	–	–	0.04 \pm 0.01 *	53	0.12

Significance: * $p < 0.05$ ** $p < 0.01$ = **, $p < 0.001$ = ***

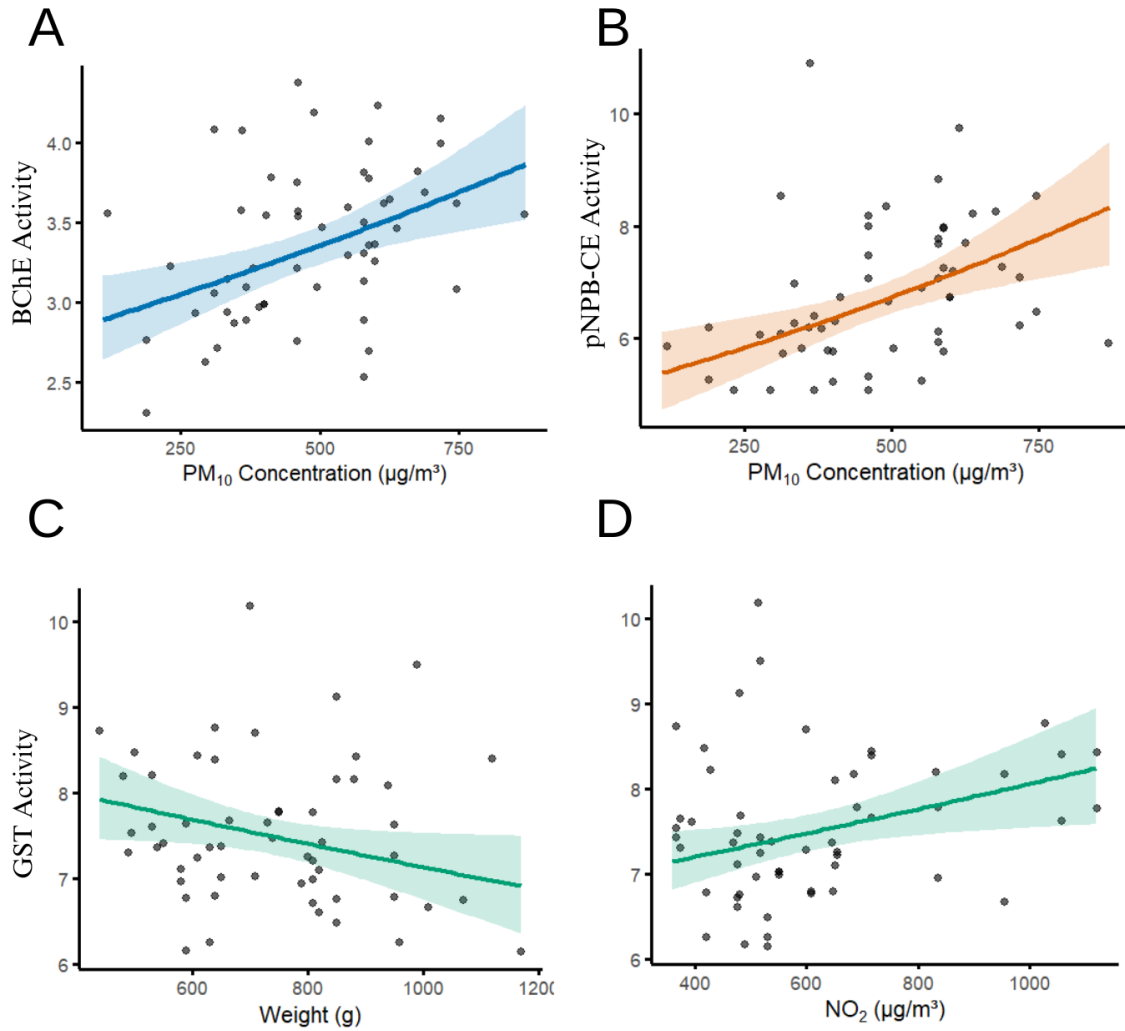


Figure 5. Relationships between liver enzyme activity and selected explanatory variables in urban yellow-legged gull chicks from Barcelona, based on model-derived estimates. (A) Predicted BChE activity in relation to PM₁₀ concentration. (B) Predicted pNPB-CE activity in relation to PM₁₀ concentrations. (C–D) Predicted GST activity in relation to body weight and NO₂ concentration. Y-axis values represent back-transformed fitted values from models with log-transformed enzyme activity, and are shown on the original scale (nmol/min/mg protein). Explanatory variables were not transformed.

5. Discussion

This study examined how urban air quality affects enzyme activities in urban yellow-legged gull chicks inhabiting the overpopulated city of Barcelona. We analysed the activity of five biomarkers commonly used to assess detoxification and neurophysiological stress in vertebrates - cholinesterases (AChE, BChE), carboxylesterases (pNPA-CE, pNPB-CE), and glutathione S-transferase (GST) - in relation to air pollutants cumulative levels of PM_{2.5}, PM₁₀, and NO₂ from fixed monitoring stations, during the entire life of each gull chick. We formerly hypothesized that enzyme activity would reflect local air pollution levels, with chicks serving as particularly sensitive indicators due to their stationary behaviour during their nesting time and continuous exposure to the surrounding urban atmosphere. Our findings partially confirmed our expectations, as we observed significant responses in enzyme activity in relation to varying pollution levels. The results revealed that the enzymatic activity of BChE and CE in yellow-legged gull chicks situated across the whole urban area of the city of Barcelona were positively associated with PM₁₀ levels, while GST activity increased in the presence of atmospheric NO₂. Additionally, results are discussed in relation to individual chick traits. To account for the individual influence of chick size, developmental stage and diet, we minimized the biological traits as much as possible but still considered the influence of body weight and trophic markers (stable isotope values) of yellow-legged gulls chicks in the statistical models.

Chick body weight was retained as a significant explanatory variable in the GST model, indicating its influence on enzyme activity. Body weight is known to influence physiological responses and can add substantial variability to biomarker-based ecotoxicological studies. For example, Rainio et al. (2013) found that body condition and size significantly modulated oxidative stress biomarkers in passerines exposed to metal pollution. Our results showed that GST activity decreases with increasing weight, suggesting that smaller or younger chicks exhibit higher GST activity. This may reflect a higher sensitivity to oxidative stress in early developmental stage or a more efficient detoxification response during growth (Metcalf and Alonso-Alvarez, 2010). This finding is consistent with observations in other species; for instance, some studies in rats indicate dynamic regulation of GSTs during development, with specific isoforms peaking in activity during early growth stages (Xu *et al.*, 2018).

We also accounted for the influence of trophic ecology in modulating biomarker responses using stable isotope mean values ($\delta^{13}\text{C} = -20.49 \pm 0.78\text{‰}$; $\delta^{15}\text{N} = 9.88 \pm 0.59\text{‰}$), which shows that yellow-legged gull chicks consumed a mixed diet consisting of both anthropogenic food sources and natural prey. When compared to isotopic baselines reported by Ramos *et al.* (2009), the $\delta^{13}\text{C}$ values of our samples are lower than those associated with marine and terrestrial invertebrates (-18.38‰), and closer to values from refuse tips (-21.67‰). This suggests a substantial intake of anthropogenic food waste in urban chicks. However, the relatively high $\delta^{15}\text{N}$ values—similar to those of marine prey (9.64‰) and freshwater invertebrates (9.91‰)—indicate a diet rich in animal protein, likely from higher trophic levels. These patterns align with the well-known opportunistic feeding strategies of yellow-legged gulls (Ramírez *et al.*, 2020; Carmona, Aymí and Navarro, 2021).

Both hepatic BChE and pNPB-CE activities showed significant positive associations with PM_{10} concentrations while GST activity was positively associated with airborne NO_2 levels but negatively correlated with chick body weight. In order to reduce multicollinearity among enzymatic variables, we excluded highly correlated measurements from the models—specifically AChE (highly correlated with BChE) and pNPA-CE (highly correlated with pNPB-CE). Nonetheless, preliminary models indicated that while AChE did not show a statistically significant response to air pollution, pNPA-CE activity was also significantly positively associated with PM_{10} levels ($\beta = 0.00043$, $p = 0.005$), showing a consistent pattern with pNPB-CE activity retained in the final models.

BChE activity was predicted to decrease under increasing pollution load, particularly due to its sensitivity to neurotoxic compounds such as organophosphates (OPs), carbamates (CBs), heavy metals and persistent organic pollutants (POPs) (Van Dyk and Pletschke, 2011; Nos *et al.*, 2024; Shabnam *et al.*, 2024). These xenobiotics are known to directly inhibit BChE, a hepatic enzyme involved in detoxification reactions (Pohanka, 2013), by binding to its active site or disrupting its enzymatic co-factors and compromising its activity (Van Dyk and Pletschke, 2011). Surprisingly, against our expectations, our results revealed a positive relationship between PM_{10} concentrations and BChE activity. Rather than a direct neurotoxic consequence, exposure to urban air pollutants such as PM_{10} may trigger both systemic inflammation (Shen *et al.*, 2023) and oxidative stress (Li *et al.*, 2003). Previous studies in humans have shown that serum BChE levels typically drop during systemic inflammation

(Zivkovic *et al.*, 2016), although this relationship has not been properly explored in birds and should be addressed in future studies. On the other hand, PM₁₀ exposure can also induce oxidative stress, which originates from the redox-active components of the particles (*e.g.*, heavy metals, PAHs) ((Li, Xia and Nel, 2008). Oxidative stress can stimulate hepatic metabolic pathways (Reyes-Caballero *et al.*, 2019), potentially influencing the synthesis of BChE. Thus, the final response of BChE enzymatic activity may depend on the balance of oxidative versus inflammatory signals (Neu *et al.*, 2024). Supporting our hypothesis, Giovanetti, 2024 observed a similar trend in wild prey birds (*Falco tinnunculus* and *Parus major*), where plasmatic BChE activity also increased with higher atmospheric PM₁₀ concentrations. The author also interpreted this as a potential adaptive physiological response to oxidative stress (Giovanetti, 2024). Another possible explanation is that higher PM₁₀ levels enhance the organisms' response, possibly triggering the synthesis of BChE as a bioscavenger to neutralize these toxic compounds, since BChE has previously been shown to act as a stoichiometric bioscavenger against organophosphates (Nachon *et al.*, 2013; Berglund *et al.*, 2014). This discrepancy with our results could be either species-specific or reflect different stages of stress response, where an initial compensatory upregulation is followed by enzymatic depletion in cases of chronic damage. These contrasting patterns highlight the complexity of BChE metabolic pathways and the need for future studies to understand its potential as an airborne pollution biomarker.

Carboxylesterases are known for their ability to hydrolyse environmental esters and serve as valuable sentinel biomarkers in toxicological assessments (Wheelock and Nakagawa, 2010; Nos *et al.*, 2021; Giovanetti *et al.*, 2024). We found that carboxylesterase activity (pNPB-CE) together with BChE activity, exhibited a significant positive relationship with PM₁₀ exposure. An increase in carboxylesterase's enzymatic activities in birds has been previously reported from natural to urban areas (Giovanetti *et al.*, 2024), coincident with our results. The increase in CE activity observed in relation to PM₁₀ levels may be related to a physiological response to air pollutants. PM₁₀ often contains a range of lipophilic organic pollutants such as polycyclic aromatic hydrocarbons (PAHs) (Chirino *et al.*, 2015) and phthalates (Ma *et al.*, 2014), which in humans can activate nuclear receptors including the constitutive androstane receptor (CAR), pregnane X receptor (PXR) and aryl hydrocarbon receptor (AhR) (Hurst, 2004; Nakayama Wong *et al.*, 2011; Begum and Carpenter, 2022).

These receptors are known to upregulate detoxification pathways, including carboxylesterase expression, as part of an adaptive response to xenobiotic stress (Nakayama Wong *et al.*, 2011). As previously stated, PM₁₀ can trigger oxidative stress, which contributes to CE activation through reactive oxygen species (ROS) signalling pathways. Moderate ROS levels can function as secondary messengers that activate protective enzymes, including hepatic esterases, as part of the overall antioxidant defence response (Nakayama Wong *et al.*, 2011). Therefore, the observed concurrent increase of BChE and CE activities might reflect an integrated detoxification and stress response to PM₁₀ exposure.

Glutathione S-transferase enzymes are involved in detoxification by conjugation and antioxidant defence mechanisms and the breakdown of lipid peroxides (Leaver and George, 1998). Our results showed a positive correlation between NO₂ concentration and GST activity in yellow-legged gull chicks, suggesting an oxidative stress defence response. This finding aligns with our initial prediction that GST activity would increase as a compensatory mechanism to counteract higher oxidative stress levels in polluted environments. Urban pollutants such as nitrogen dioxide (NO₂) contribute significantly to oxidative stress by increasing ROS and RNS levels in tissues (Bocedi *et al.*, 2019). GSTs help detoxify ROS and RNS products, including NO₂, a known environmental oxidant generated during fossil fuel combustion (Bocedi *et al.*, 2019; Raj Rai *et al.*, 2021). In our study, an increased GST activity in liver may reflect an adaptive response of yellow-legged gull chicks to urban air pollution, caused by NO₂-derived radicals. This hypothesis is supported by a study with passerine birds that found that NO_x exposure was correlated with increased plasmatic antioxidant capacity, although responses were species dependent (Salmón *et al.*, 2018). Various studies consistently report increased GST activity in response to various types of environmental pollutants. In avian species, increased GST and GSH peroxidase (GP_x) activities have been reported in birds exposed to heavy metals (Mateo *et al.*, 2003; Berglund *et al.*, 2014). *GSTM3* gene expression, which encodes a GST enzyme from the Mu class in double-crested cormorants was found to positively correlate with mercury (Hg) exposure, supporting that GST activation is a general response to face oxidative stress in birds (Gibson *et al.*, 2014). Similar responses have also been observed in other taxa: studies in fish (Santana *et al.*, 2018) and invertebrates (Braga *et al.*, 2018) observed that increased GST activity, following exposure to polycyclic aromatic hydrocarbons (PAHs) and pharmaceutical

pollutants (Pusceddu *et al.*, 2018), is a common feature. Other markers of oxidative stress support the sensitivity of glutathione-related pathways to environmental pollution in avian species. Reduced glutathione (GSH) is oxidized to GSSG during oxidative stress, and the GSH:GSSG ratio is considered a reliable index of redox balance (Isaksson, 2010). Lower GSH:GSSG ratios with higher GSSG levels have been observed in urban great tits and Eurasian kestrels (Isaksson *et al.*, 2005; Wemer *et al.*, 2021). Our study adds to a growing body of evidence suggesting that well recognised urban air pollutants like NO₂ induce oxidative stress in wildlife, triggering a response in enzymatic antioxidants such as GST.

Broader implications and future perspectives

Our results indicate that urban air pollutants, specifically PM₁₀ and NO₂, are significantly associated with physiological changes in yellow-legged gull chicks, as reflected in the activity of three key detoxification enzymes. Both BChE and pNPB-CE showed positive associations with PM₁₀ concentrations, while GST was positively associated with NO₂ levels but negatively with chick's body weight. These findings support that these parameters could serve as sensitive physiological biomarkers for assessing the impact of air pollution on bird chicks health. Yellow-legged gull chicks in cities are particularly valuable sentinels of urban air pollution due to their stationary behaviour during the nesting period, their continuous exposure to the local air conditions and their young age (three to four weeks), which allows for the early detection of physiological responses at an early stage. The association between air pollutant levels and enzymatic responses observed in this study highlights the potential of gull chicks—and the proposed biomarkers—as sensitive early indicators to alert on the consequences of air pollution in urban ecosystems.

While our study provides novel insights, several limitations must be acknowledged to guide future research. On one site, pollutants exposure was estimated based on monthly average concentrations at nesting sites, without accounting for the spatial and temporal variability of air contaminants. Given that pollutant dispersion is influenced by wind patterns, urban topography, and meteorological conditions (Raluy-López *et al.*, 2025), the actual exposure levels experienced by the chicks in the nests may have varied significantly over time and space. Incorporating fine-scale air dispersion models, such as GIS-based tools that consider air currents and urban structure, would improve the accuracy of exposure

assessments. On the other site, the explanatory power of our statistical models was limited, since other environmental/physiological factors such as parasites incidence, exposure to other environmental pollutants and genetic variation (Seewagen et al., 2022; Vizuete et al., 2022; Martín-Vélez et al., 2024) may contribute to modulate enzymatic activities. Future research should focus on enhancing model robustness by increasing the sample size, improving pollution exposure estimates, and including additional biological variables. It will also be important to test these biomarker responses in controlled or repeated studies to confirm their robustness. This will enhance the suitability of yellow-legged gull chicks as sentinels for environmental monitoring in urban ecosystems.

Our study is also valuable within the framework of the One Health approach, which emphasizes the connexion between human and animal health and with that of the environment quality (Bègue *et al.*, 2025). The same airborne pollutants affecting chick physiology are likely to equally harm human health, particularly children and vulnerable populations (WHO, 2006; Cao, Liang and Niu, 2017; Zhang *et al.*, 2017). Therefore, tracking physiological biomarkers in urban fauna contributes to early warning systems that benefit public health, ecosystem's resilience, and sustainable urban development alike. Moreover, from a conservation and urban planning perspective, this study emphasizes the need for pollution mitigation strategies in overpopulated cities like Barcelona. Reducing emissions of fine particulates and nitrogen oxides — through transportation regulation, green infrastructures, and emission's monitoring — can have common benefits for human and wildlife health. Our analysis identifies Eixample and Poblenou as the most polluted neighbourhoods across multiple pollutants (PM_{2.5}, PM₁₀, and NO₂), with Gràcia also showing high NO₂ levels, highlighting these areas as priority targets for mitigation action. Integrating physiological biomonitoring into urban biodiversity management could help guide targeted actions, track progress, and ensure that conservation measures address not only habitat loss, but also the invisible toxic threat of air pollution in urban ecosystems.

6. Conclusions

Our study shows that common urban air pollutants, such as PM₁₀ and NO₂, are associated with significant physiological changes in yellow-legged gull chicks during their early development. We demonstrated that air pollutants modulate enzymatic activity of BChE, pNPB-CE and GST, enzymes involved in detoxification and oxidative stress response. Our results revealed that both hepatic BChE and pNPB-CE activities showed a significant positive association with PM₁₀ air concentrations, while GST activity was significantly and negatively correlated with chick's body weight, but positively associated with airborne NO₂ levels. Our findings highlight the potential of yellow-legged gull chicks as effective and sensitive sentinel organisms for urban air quality monitoring. Unlike mobile adults, nestling chicks provide a temporally and spatially stable model for assessing cumulative pollutant exposure at fine scales. Sentinel species such as gull chicks offer a cost-effective and ecologically grounded approach to environmental surveillance. This study is also relevant under the One Health framework. Besides serving as indicators of wildlife health, gull chicks also reflect environmental risks that may equally affect human populations living in the same urban areas. Urban air pollutants such as NO₂ and PM₁₀, shown here to influence chick physiology, are well-documented threats to human health—particularly affecting children, the elderly, and individuals with respiratory conditions. The shared exposure environment between wildlife and humans in cities highlights the relevance of monitoring wildlife biomarkers, which can contribute to early warning systems for environmental degradation and public health risk. While our study has some limitations, such as reliance on ambient pollution data from fixed stations and a limited set of biological variables, it lays a strong foundation for future biomonitoring programs. Future research should aim to improve exposure assessment through more refined spatial models, increase sample sizes for stronger statistical power, and include a wider range of physiological and environmental variables to better understand cause-effect relationships and generalize findings to other urban wildlife. From a conservation and urban management perspective, our findings emphasize the need for air quality monitoring in polluted Barcelona neighbourhoods, such as Eixample and Poblenou, where pollution levels were highest. Incorporating physiological biomonitoring of sentinel species such as gull chicks into urban biodiversity and sustainability programs can

offer a practical means of evaluating the success of air quality regulations and ecological restoration initiatives.

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Supplementary Data

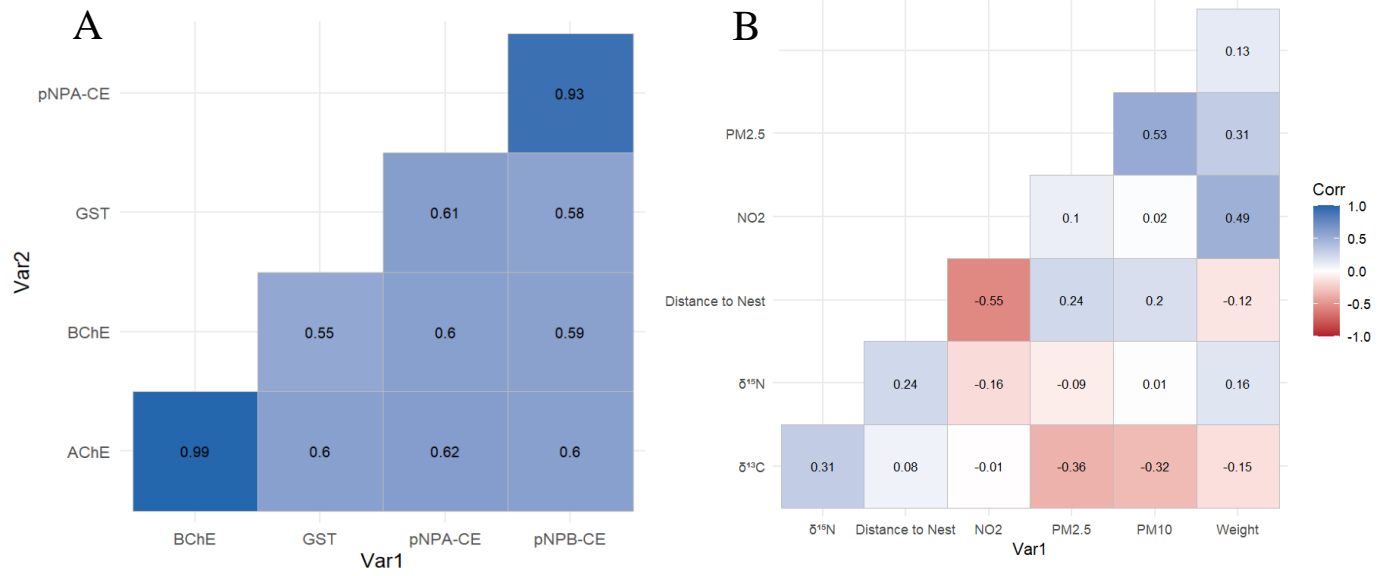


Figure S1: A) Correlation matrix of enzymatic biomarkers (AChE, BChE, GST, pNPA-CE and pNPB-CE variables). B) Correlation matrix of explanatory variables (distance to nest, accumulated PM₁₀, accumulated PM_{2.5}, accumulated NO₂, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$).

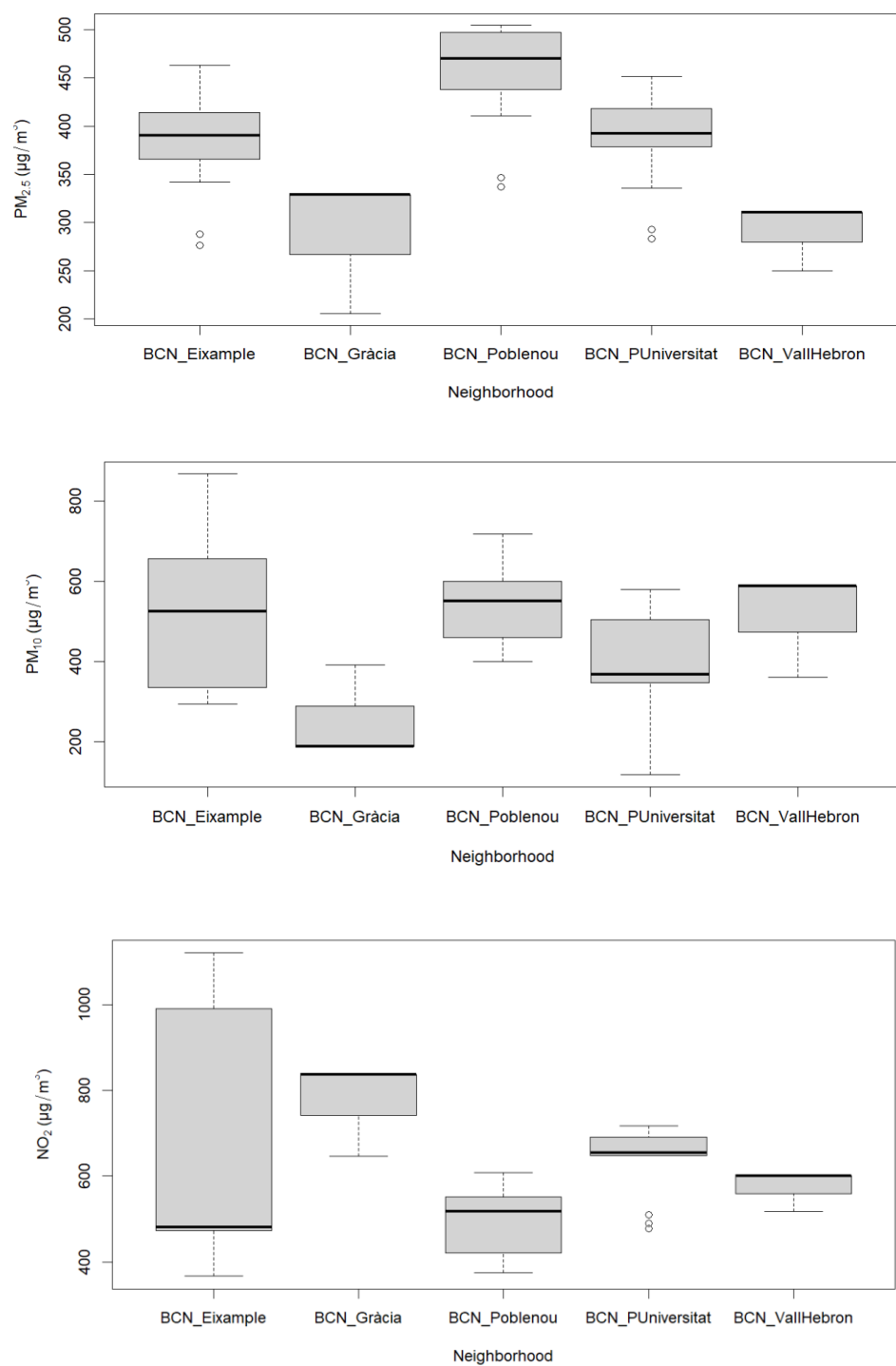


Figure S2: Boxplots showing the distribution of PM_{2.5}, PM₁₀ and NO₂ concentrations ($\mu\text{g}/\text{m}^3$) across five neighborhoods in Barcelona (Eixample, Gràcia, Poble Nou, Universitat and Vall d'Hebron).