Temporal variability measurements of $PM_{2.5}$ and its associated metals and microorganisms on a suburban atmosphere in the Central Iberian Peninsula

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ABSTRACT

A novel and multidisciplinary observational analysis of atmospheric components in the Central Iberian Peninsula is presented here. $PM_{2.5}$ concentrations and both populations of cultivable and non-cultivable microorganisms and concentrations of a wide range of trace elements associated have been simultaneously studied during multiple events along one year. The aim has been to characterize their potential relations and dependencies, and their seasonal, daily and hourly evolution. Tools that could explain the atmospheric mechanisms and sources from all these elements have been also evaluated. As it would be expected from a suburban environment, absolute levels obtained were not close to legislation limits. Anthropogenic and natural sources, such as heating home, soil resuspension, or Sahara dust intrusion; and atmospheric factors are responsible for higher $PM_{2.5}$ and metals concentrations in months with both low and high temperatures. Daily and hourly evolution depends on University Campus activity, especially on traffic flow and resuspended dust due to human transit. No statistical significant differences on daily or seasonal scales between cultivable counts of fungi and bacteria were displayed. However, using the q-PCR technique, the bacterial population was lower in winter. Positive correlations between $PM_{2.5}$ and relative humidity; and $PM_{2.5}$ and cultivable microorganism have been established. It was also the case among 7 of the 11 trace elements, indicating then common natural or anthropogenic sources. In summary, this work illustrates the interest of a combined inspection of elements, interactions and dependencies when studying the unique and continuous atmospheric environment, which are typically analyzed separately.

Keywords: environmental chemistry; particles; trace elements; suburban area; airborne microbiota

1. **Introduction**

Atmospheric particulate matter (PM) is a key parameter in air quality monitoring, not only because of its potential health and environmental impacts (WHO,2016; EEA, 2018), but also for its important role in global climate and atmospheric chemistry (IPCC, 2013). The atmospheric PM is a complex mixture containing various abiotic and biotic particles with different size distribution. There is reliable evidence of the effects of short-term exposure to PM on respiratory health. Mortality, especially as a consequence of long-term $PM_{2.5}$ (<2.5 µm aerodynamic diameter) exposure, is a stronger risk factor than the coarse particles (size

2.5 µm - 10 µm diameter) (WHO, 2013). Estimates of the health impacts attributable to long-term exposure to ambient $PM_{2.5}$ indicate that this type of pollution is responsible for about 422 000 premature deaths in Europe (EAA, 2018) and 2.1 million worldwide every year (Silva et al., 2013). So, an increase of 10 µg m^{-3} in ambient $PM_{2.5}$ has been shown to be statistically associated with a 9% increase in mortality risk for non-accidental causes (Yin et al., 2017). Furthermore, the European Environment Agency (EEA) has recently reported that 5% of the urban population in the total European area studied was exposed to $PM_{2.5}$ levels above the EU limit value (25 µg m^{-3}) in 2016, and approximately 68% was exposed to concentrations exceeding the World Health Organization (WHO) air quality guidelines (10 µg m^{-3}) (EEA, 2018).

On the basis of epidemiological and toxicological studies, the PM composition has an essential role when considering their health impacts (Schwarze et al., 2006; Strak et al., 2012; Happo et al., 2014). On one hand, it is well known that the chemical PM composition (such as organic carbon, element carbon, insoluble minerals and transition metals) is strongly associated with an increase of the oxidative potential in human cells (Schwarze et al., 2006; Strak et al., 2012; Sotty et al., 2019). The consistency between epidemiological and experimental findings for specific PM-components appears to be more clearly stated for metals, which seem to be important for the development of both pulmonary and cardiovascular diseases, and may also be involved in PM-induced allergic sensitization (Schwarze et al., 2006). For example, Sorensen et al., (2003) revealed that water-soluble V and Cr from $PM_{2.5}$ were significantly associated with the increase of DNA damage measured in blood. Moreover, the chemical composition of these particles is also affected by several factors, such as the annual cycle, meteorological conditions, geographical location, photochemical transformation and vicinity of emission sources. Toxicological studies have also shown that the seasonal variation in the PM composition and their emission sources have a major effect on the toxicological properties (Lippmann et al., 2013; Mcwhinney et al., 2013, Manzano-León et al., 2016). On the other hand, $PM_{2.5}$ is also formed by biological material suspended in the air (bioaerosols) such as bacteria, fungi, viruses, microbial toxins, proteins, enzymes, mites, pollen and their fragments, being its composition variable and dependent on several factors (Gat et al., 2017; Mu et al., 2020; Xie et al., 2018). In most terrestrial environments, bioaerosols constitute a substantial fraction of the atmospheric aerosol load, typically accounting for around 30% in urban air. They have important effects on public health and epidemiology and can serve as nuclei for cloud droplets, ice crystals, and precipitation, thus influencing the whole hydrological cycle and even the climate (Fröhlich-Nowoisky et al., 2016). Besides, the products of their biological activities or some cellular components can cause allergies, intoxications or infections to humans and animals. It has also been reported that PM-borne bacteria and fungi could modify the PM oxidative potential by interplaying with its contents. For example, Samake et al. (2017) confirmed a cumulative effect on oxidative potential by fungal spores with airborne PM, copper and 1,4-naphthoquinone (1,4-NQ), in contrast to a strong reductive effect from bacterial cells. Also, there are studies reporting that individual bacterial taxa could influence cloud formation and ice nucleation, and so the role of PM-borne microorganisms on the atmospheric processes can not be overlooked (Fröhlich-Nowoisky et al. 2016; Smets et al., 2016).

In the light of the above, the health effects of $PM_{2.5}$ depend not only on its biological composition but also on its chemical characteristics. In this context, considering only $PM_{2.5}$ mass concentration, as a guideline in the current air quality assessment by particles, would be a limited approach, since it ignores its sources, constituents, seasonal variations and biological activity. Previous studies about the chemical and biological composition of the $PM_{2.5}$ and the influence of the annual cycle, the atmospheric conditions that control the dispersion mechanisms and its composition origin, are scarce, and mainly located in heavy polluted regions, specifically in China (Zhong et al, 2019; Huang et al., 2017; Zhang et al., 2016; Liu et al., 2020). The global current knowledge does not allow for a precise quantification of the health and environmental effects of individual components associated to

$PM_{2.5}$ or the interplay between them, without forgetting the effect of meteorology or geographical location of the emission sources in the particles composition (WHO, 2016).

In Spain, a large portion of the population lives in small cities: 47% in villages of < 50 000 inhabitants and 24% in small cities from 50 000 to 200 000 inhabitants. Only 29% of the population lives in great cities (>200 000 inhabitants) (INE, 2019). The atmospheric plume in big cities may be quite different from that of smaller towns. For large cities, external contributions may be very small due to the huge urban emissions over a large territory, which may be responsible for the overall behaviour. In contrast, small cities may be more sensitive to the effect of given emissions in their surrounding areas. Thus, the characterization of PM at different places, especially in small cities, is crucial. The chemical composition associated to $PM_{2.5}$, mainly metals, has been studied in some urban - industrialized areas of Spain (Querol et al., 2007 and 2008; Santacatalina et al., 2010; Sanchez de la Campa et al., 2013; Morillas et al., 2019), but fewer studies have been focused on small cities or rural areas (Querol et al., 2007; Pey et al., 2009; Arruti et al., 2011). Regarding microbiological composition associated with $PM_{2.5}$, to the best our knowledge, no study has been carried out in Spain.

With this in mind, the aim of this study was to characterize the $PM_{2.5}$ concentrations and its associated metals and microbiological populations, during a whole annual cycle, taking into account the atmospheric conditions over a small city (84 000 inhabitants) located in the centre of the Iberian Peninsula. Although it is not a heavily polluted location, it is close to Madrid (70 Km), a megacity with a large industrial area. To achieve this holistic approach, we sampled air during a year performing the following tasks: analysis temporal variations (monthly, daily and hourly) of the $PM_{2.5}$ concentration; characterization and quantification of its associated metals; microbiological quantification by using culture dependent and culture independent techniques; and description of the air mass movements that reach the sampling site and their relation with the mean levels of these particles. Therefore, the main aspects of novelty when compared with previous studies are the combination of several environmental elements (atmospheric chemistry and physics, chemical and microbiological analysis, biochemistry), some of them usually analysed, but others not frequently measured, and likely not simultaneously, which leads to a very challenging, multidisciplinary and heterogeneous approach.

2. Methods

2.1. Sampling site, calendar and meteorological conditions

Toledo, declared a World Heritage site in 1986, is one of the most important touristic cities in Spain. It is placed in the Tagus valley in the centre of the Iberian Peninsula in Castilla–La Mancha region at 529 m above sea level. It is approximately 70 km south away from Madrid (Fig. 1) and has about 84 000 inhabitants. At this location, one suburban background site was selected for this study, the Castilla-La Mancha University Campus (39º51´50´´ N 4º02´25´´ O), at 1.5 Km West from Toledo city (Fig. 1). The sampling site is located close to Tagus riverbanks, in a pedestrian area surrounded by abundant riparian vegetation and soil with a gravel surface layer. This area presents no significant direct vehicle emissions, although it is surrounded by neighbourhoods with some road traffic.

Toledo presents typical Mediterranean climate conditions, with very hot and dry summers, cold and dry winters, and intermediate more rainy seasons (Kottek et al., 2006). Wind speed is relatively low during the whole year (Troen and Petersen, 1989) and radiation conditions correspond to an amount of sunshine hours (when averaged hourly direct solar irradiation exceeds 120 W m^{-2} (WMO, 2010)) of nearly 2900 h/year (Sanchez-Lorenzo et al., 2007). Some meteorological variables were also measured during the sampling dates (see Table S1).

The air sampler was positioned on the rooftop of the Faculty of Environmental Sciences and Biochemistry (University of Castilla-La Mancha , UCLM) at 10 m above the ground. Samplings were performed from September 2017 to July 2018. To analyse the seasonal evolution of concentration and composition of $PM_{2.5}$, 24 h samples were collected during 8-10 weeks of each season. In addition, during four full weeks (a week per each season), daily samples (Monday to Sunday) were also collected to investigate if there were differences in the analysed parameters between days with (weekdays) and without (weekend) activity in the University Campus.

Meanwhile, for another six weeks, samples were collected during different short time periods. Specifically, these time periods in local time were H1: 8:00–10:30; H2: 10:30–13:00; H3: 13:00–16:00; H4: 16:00–18:00; H5: 18:00–20:30; H6: 20:30 – 8:30, in order to assess and compare hourly variations. During these short period samplings, filters were replaced in the sampler for the appropriate hourly time interval to create one weekly sample for each interval. The same filter was used for the same time period to increase the particle's mass. This hourly analysis was designed to capture the variation in emission sources such as the increasing traffic emissions in rush hours or the rise of soil resuspension in hours with more activity in the University Campus.

All the specific sampling days were indicated in the 2017-2018 calendar, differentiating between monthly, daily and hourly samplings (See Fig. S1).

2.2. Sampling methodology

$PM_{2.5}$ were sampled on quartz fiber filters (Whatman, 47 mm) using low-volume sampler (Derenda, 2.3 $m^3 h^{-1}$) (Fig. 1), which measures temperature and RH simultaneously (See Table S1). Before sampling, all filters were decontaminated by baking at 500°C for 5 hours. When the sampling was completed, $PM_{2.5}$ content of the filters was determined by gravimetry according to UNE-EN 12341:2014, employing a Sartorius LA130 S-F balance (0.1 mg sensitivity). Filters were equilibrated under the same temperature and humidity conditions before the weighting process. Next, each filter was cut into four equal parts. Two opposite parts were packed in plastic bags covered with aluminium foil and stored in a freezer at -80°C until metals analysis was performed, and the remaining two parts were immediately submitted to microbiological analysis.

2.3. Chemical analysis by the ICP-MS

A quarter of each filter was digested following the method described by UNE-EN 14902: 2006. Briefly, a quarter of filter (0.025 g, approximately) were digested with 8 mL HNO_3 (65% Suprapur) and 2 mL H_2O_2 (30% v/v Suprapur) in Milestone EthosPlus microwave oven. The microwave oven program consisted in three steps: step 1, a 20 min linear ramp from room temperature to 180ºC; step 2, a 10 min linear ramp from 180ºC to 220ºC; step 3, from 20 min to 220ºC; and step 4, cooling at room temperature. Control filter samples were processed in each batch of digestion. Then, the digestion solution was diluted to 50 mL with H_2O (ultrapure water,18.2 MΩ cm). The total Ni, As, Cd, Pb, Cr, Co, Mn, Cu, Mg, Hg and Se analyses were carried out with an inductively coupled plasma mass spectrometer (ICP-MS) equipped with a collision cell (CCT; Thermo Electron Model X Series II). Solutions used for calibration were prepared from commercial certified stocks standards with 1 g L^{-1} of each element. Rh (15 µg L^{-1}) was simultaneously aspirated during the ICP-MS data acquisition and it was used to correct signal drifts.

The detection limits (LODs, in µg g^{-1}, back calculated to 0.025 g in filter sample) and metal concentrations (µg g^{-1}) in the blank filter sample are shown in Table S2. For control analysis, the control filter samples were spiked with standard solution of Pb, As, Ni and Cd in concentration of 10 µg L^{-1} to check the accuracy of the analysis of the acidic digestion. The recovery for the determination of these metals were close to 100% in all cases.

2.4. Microbiological analysis

Half of the quartz fiber filter was bathed in 20 mL of sterile saline solution (9 g L^{-1} sodium chloride) and shaked for 24 h. Aliquots were used to count culturable microorganisms and the remaining (15 mL approximately) was stored in a freezer at -80ºC to DNA extraction.

2.4.1. Cultivable bacteria and fungi counts

Both the suspension from filter and serial dilutions were spread-plated, in duplicate, onto the surface of trypticase soy agar (TSA), supplemented with 100 ppm cycloheximide, and rose bengal agar (RBA), supplemented with 50 ppm chloramphenicol, to count culturable bacteria and fungi fractions in ambient $PM_{2.5}$, respectively. The incubation conditions were 30ºC for 3 days and 25ºC for 5 days for bacteria and fungi, respectively (Rodríguez et al. 2018). The growing colonies were counted and the mean count was calculated, expressing concentration as colony forming units per cubic meter of air (CFU m^{-3}).

2.4.2. Bacterial and fungi concentration determined by quantitative PCR

Genomic DNA from $PM_{2.5}$ was extracted from 15 mL of suspension from the filter previously obtained using a DNeasy Blood & Tissue Kit (Quiagen, USA) according to the manufacturer's instructions. The nucleic acid concentration sensor NanoDrop Biotek (Synergy HT. USA) was used to check DNA quality and to determine DNA concentration, being the range of that from 3.3 to 9.8 ng μL^{-1} of DNA. To quantify the total bacteria and fungi, quantitative PCR (qPCR) was performed in triplicate with an ABI PRISM 7500 Fast Sequence Detection System (Applied Biosystems, Foster City, CA. USA) and the software Applied Biosystems using the SYBR Green system was used. The abundance of airborne bacteria was estimated by the quantification of the number of copies of the gene 16S rRNA using the universal bacterium primers 27f/1492r: the forward primer 5´-AGAGTTTGATCCTGGCTCAG-3´ and the reverse primer 5´-CTACGGCTACCTTGTTACGA-3´. The abundance of airborne fungal was estimated by the quantification of the number of copies of the gene 18S rRNA, the self-designed primers used were the forward primer 5´-CGGCTACCACATCCAAGGAA-3´ and the reverse primer 5´-GCTGGAATTACCGCGGCT-3´ All primers were supplied by Bonsai Technologies (USA). The qPCR was performed in a total volume of 20 µL reaction mixture including 10 µL SYBR-

Green PCR Master Mix (Applied Biosystem, Foster City, CA. USA), 0.6 µL forward primer, 0.6 µL reverse primer, 5 µL DNA and 3.8 µL distilled H_2O. It was performed under thermal cycling conditions consisting of an initial 1-min denaturation at 50ºC and 10 min of further denaturation at 95ºC, followed by 40 cycles of 15s of denaturation at 95ºC and 60s of annealing/extension at 60ºC. Standard curves were developed for each qPCR bacterial and fungal species using dilutions from a known concentration of genomic DNA. Then, according to the threshold cycle values of the standard curve, the number of gene copies of bacteria and fungi were calculated, expressing concentration as gene copy number per cubic meter of air (Gene copy number m^{-3}).

2.5. Back-trajectories analysis

For each of the sampling dates, with the aim to analyse the origin of the air masses, HYSPLIT-NOAA dispersion model was used (Stein et al., 2015; Rolph et al., 2017; Hu et al., 2020). There are plenty of studies that have employed this methodology, and in particular, over the region of study (Notario et al., 2014; Diaz de Mera et al., 2015). A height of 100 m and 48 h back trajectories at 12 UTC (and every 3h after during the whole 24 hours, to capture the variability along the measurement period) values are used to perform the analysis of trajectories for each of the measurement days. This ensemble of trajectories would be likely to represent the overall atmospheric boundary layer conditions where the air masses would come from. Meteorological data to compute those trajectories were obtained from NCAR/NCEP reanalysis (Kalnay et al., 1996). SplitR (Iannone, 2020) and OpenAir (Carslaw and Ropkins, 2012) packages based on R software language (R Core Team, 2020) were used to compute and represent clustered and individual trajectories.

2.6. Statistical analysis

All statistical analyses were made using SPSS (IBM SPSS Statistics 23). Descriptive analysis was made using mean, median, standard deviation, and range. In the case of normally distributed variables, ANOVA with Tukey post hoc test was performed for the comparisons among seasons and months; and Student's t-test to compare values between weekdays and weekends collected samples. If the measurements were not normally distributed, then Kruskal-Wallis test and U-Mann Whitney test were performed for seasonal/monthly and daily measurements, respectively. Spearman's rank correlation test was used to determine the relationships between $PM_{2.5}$ concentrations, microorganisms and metals associated with PM, and meteorological parameters. P values less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. Data overview

Table 1 shows the annual mean, standard deviation, median, and minimum and maximum 24-h average of all measured variables during the entire study period in Toledo (Spain). The annual average $PM_{2.5}$ concentration in Toledo was 15.5 ± 5.7 µg m^{-3}, being 32.6 µg m^{-3} the maximum measured 24-h average. Therefore, it is important to note that all samples analysed corresponded to "fair air" according to IAQ by UE (10-20 µg m^{-3}) (EEA, 2020), unlike other recent studies focusing on more contaminated air (Du et al., 2018; Wei et al., 2020). Otherwise, the annual average culturable bacteria and fungi counts were 45.5 and 42.4 CFU m^{-3}; being 420.3 and 268.1 CFU m^{-3} respectively, the maximum measured 24-h averages.

Table 1 also summarizes the annual average (±SD, ng m^{-3}) concentrations of the metals studied in $PM_{2.5}$, together with the annual mean of total concentration of trace elements (43.5±27.9 µg m^{-3}). The highest concentrations (ng m^{-3}) were observed for Pb (11.8±10.8); Cr (8.4±13.3); Mg (7.0±5.8); Ni (6.3±7.6); Mn (6.1±4.5) and Cu (2.7±2.4). All other metals were in concentrations below 1 ng m^{-3}.

3.2. $PM_{2.5}$ variability and air-mass back trajectories

Fig. 2 presents temporal evolutions for $PM_{2.5}$ concentrations at the sampling site. An ANOVA test displayed significant differences between the mean monthly $PM_{2.5}$ concentration. The consequent post-hoc test revealed that $PM_{2.5}$ levels in November, February and March were significantly lower than those of the rest of months, while the largest values were obtained in January ($p \leq 0.05$). Thereby, the average value measured in January was 22.8 ± 5.1 µg m^{-3}, followed by July with 19.8 ± 3.1 µg m^{-3} (Fig. 2a). Several factors can contribute to this annual cycle, being both the origin and the atmospheric factors that can enhance or reduce the dispersion, and so the measured concentrations.

On one hand, during months with lower temperatures, when usage of home heating is extended, PM emissions to the atmosphere increase (Chen et al., 2016; García et al., 2019; Morillas et al., 2019). These periods are typically characterised by anticyclonic synoptic patterns in the area, that, together with the large night periods, lead to strong thermal inversions and finally, to a significant reduction of the mixing over the boundary layer, due to such very stable conditions (Querol et al., 2008; Chen et al., 2016). Therefore, dispersion capacity is low, due to such reduced atmospheric turbulence, and so particles can be accumulated. January back trajectories frequencies (Fig. 3a) confirm that during previous hours, atmospheric movements are short and mainly over the land regions surrounding the area of study, hardly coming from the oceanic areas, which are too far. These low temperature periods are also characterized by small precipitation amounts, as already mentioned, due to the mediterranean climate of this area (Kottek et al., 2006).

On the other hand, the summer displayed high values (July), this feature can also serve as a partial explanation, together with the higher average global irradiance, that involves formation of secondary particles by photochemical processes. Sometimes in summer (although not only during that season, being also relevant in spring or autumn), Sahara dust intrusions can contribute to increased $PM_{2.5}$ concentrations (García et al., 2019, Russo et al., 2020). But also, reduced atmospheric movement and so increased concentrations can be due to the dominant Azores summer high pressure synoptic pattern. These high pressure conditions lead to reduced vertical and horizontal mixing for the whole troposphere, and in particular on the boundary layer. In our results this is reflected, again, as shorter 48h back trajectories frequencies (Fig. 3b). Minimum values were obtained in February and the beginning of the spring, March and April, with mean values around 11-13 μg m^{-3}. Several atmospheric mechanisms could play a role in those results: first, rain during the spring season (typical of the climate of the area), as precipitation is a very efficient particle removal process (Xu et al., 2017). In addition, not only low levels of particles were recorded during rain events but also in a later period since processes, such as resuspension were inhibited (Garcia et al., 2019). Second, more unstable synoptic conditions compared with winter patterns, and so larger wind speeds are typically obtained during late winter and early spring over the region (Lorente-Plazas et al., 2015). Wind conditions can be related to obtained particle's concentration with quite complex relations (Wang and Ogawa, 2015). It can favour or reduce their concentrations, depending on several meteorological factors. These are, among others, humidity conditions, location of sources that can add a large variety of particles (for example, from the Atlantic ocean, as it seems to be the case here for March values (Fig. 3c) with the potential addition of marine particles), or also wind direction and not only wind speed, that can play, for sure, a major role. Even size of particles has also not a simple relation with wind speed (Zhang et al., 2017).

Daily samples during the same week were collected to investigate changes in $PM_{2.5}$ concentrations between both weekdays and weekends. The mean particle concentration decreased at weekends compared to the average values of weekdays (Fig. 2b). An analysis of these means displayed statistically significant differences at 95.0% confidence level. In addition, air samples collected daily at different time periods allowed us to compare the hourly evolution (Fig. 2c). This analysis could provide important information for identifying potential emission sources and the time of day when maximum levels were recorded. The highest particle concentrations were observed throughout the morning and early afternoon, the time interval in which the educational activity in the Campus was maximum and, in consequence, a larger number of people is present. As the afternoon went on, the levels decreased, until reaching the minimum value during the night. According to these results, the impact of human activity at the university campus was evident on the hourly and daily $PM_{2.5}$ levels. Greater anthropogenic activities around the sampling point, such as traffic or the resuspension dust particles coming from the people movement in a mainly gravel soil, during the weekdays and along main scholar schedule, would be responsible for the $PM_{2.5}$ concentrations increase.

Finally, it is important to highlight that the annual average concentration for $PM_{2.5}$ (15.5 ± 5.7 μg m^{-3}) did not exceed the annual limit values recommended by EU regulations (25 μg m^{-3}) (Directive 2008/50 / CE), indicating that $PM_{2.5}$ pollution in the Toledo suburban area is relatively acceptable. This value is similar to other studies that measured in suburban sites in Spain, where the mean concentration ranged between 10 and 20 μg m^{-3} (Querol et al., 2008, Santacatalina et al., 2010; García et al., 2019). However, the annual value recorded here is higher than the international standards, 12 μg m^{-3}, established by the National Ambient Air Quality Standards (NAAQS) (EPA, 2020), or the 10 μg m^{-3} established by the WHO (2015); so it would be advisable that fine particles concentrations could be reduced.

3.3. Trace elements associated to $PM_{2.5}$

Fig. 4 shows different time evolutions for metal concentrations at the sampling site. The monthly evolution presents different patterns for each analysed metals (Fig. 4a) and a Kruskal-Wallis and ANOVA analysis of variance, displayed statistically significant differences between the monthly mean concentration of all metals, except for Cd, Pb and Mg, with a confidence level of 95.0%. The most relevant trace metals in the monthly evolution were Pb, Cr, Mg, Ni, Mn and Cu. It has been well documented that trace elements like Pb, Mn and Cu are the tracers of traffic emissions (Pey et al., 2009; Zhang et al., 2009); Cr and Ni can be emitted from industrial sources and fossil fuel combustion (Qi et al., 2016); and Mg and Mn are identified as tracers of dust or soil (Querol et al., 2004; Rivas et al., 2014). The minority elements were Hg, As, Se, Cd and Co, which can be emitted from fossil fuel combustion (Pey et al., 2009; Qie et al., 2018). The highest total metal concentrations were observed in April (125.1 ng m^{-3}), followed by December and January (72.4 and 50.2 ng m^{-3}, respectively), and summer months (60 ng m^{-3} on average). The observed increases in December, January and the warmest months agree with the higher measured $PM_{2.5}$ concentrations; so that a particle concentration increase causes a rise of the associated metals, which would be due to anthropogenic emissions from the home heating in December and January (Chen et al., 2016; Garcia et al., 2019; Morillas et al., 2019); an increase of metals from Sahara dust intrusions (Garcia et al., 2019) or a stronger resuspension of the soil, in the hot season (Meresová et al., 2008); and a low atmospheric dispersion in both periods, as the back trajectories indicated. High metal concentrations were measured in April, as the Cr and Ni levels were very high. Unusual pollution from an industrial source may have caused this rise, although further analysis would be necessary to find the specific source.

Regarding daily evolution, unlike what was observed for $PM_{2.5}$, no statistically significant differences ($p>0.05$) were observed between weekdays and weekends (Fig. 4b). The main metals detected both daily and monthly were Pb, Mg, Ni, Cr, Mn and Cu, being all of them in the range of 2.1-9.4 ng m^{-3}.

In order to know the diurnal evolution of metal concentrations, air samples were collected daily at different time intervals (Fig. 4c). The concentrations of predominant metals (Pb (39.5 ng m^{-3}), Mg (39.1 ng m^{-3}), Cr (26.4 ng m^{-3}), Mn (25.9 ng m^{-3}) and Ni (20.0 ng m^{-3})) were kept almost constant during the diurnal range. As expected, metals levels decreased during nocturnal range recording values below 8.0 ng m^{-3} in all cases. This might be associated with the reduction of anthropogenic activity in the surroundings of the university campus at the night time. Therefore, as commented above, traffic and resuspension mineral dust could control daily and hourly variations in metallic emissions.

No air quality guidelines have been set for any of these metals in $PM_{2.5}$, however limit values have been established by the EU for some heavy metals (As, Pb, Cd and Ni) associated to PM_{10} (Directive 2008/50/CE). Taking into account this legislation, among the regulated heavy metals detected, Pb and Ni were the most abundant, with levels below the limits of 500 and 20 ng m^{-3} respectively, recommended by EU regulations. Furthermore, the low levels of As and Cd (below 1 ng m^{-3}) are remarkable. Consequently, in this studied area there are no major problems to fulfil the requirements from the EU air quality directives concerning levels of metals. Regarding the other trace metals, the concentrations lie within the range of 0.1-10 ng m^{-3}, values that do not imply a risk for human health or the environment.

Ambient airborne PM is a complex mixture of particles originated from a wide range of sources, and their identification is an arduous task. However, to know the chemical composition of these particles can be useful to address this difficulty. In order to find a similar composition and, therefore, common sources, we compared the annual average concentration of trace metals in Toledo with other suburban cities in Spain. Annual mean concentrations of the main elements (Pb, Cr, Ni, Mn and Cu) measured in this work were slightly higher than those of other suburban cities (Querol et al. 2007, 2008; Pey et al., 2009); but the concentrations of minor elements (As, Se, Cd, Co) were akin to those of other Spanish cities. These values were measured over a decade ago, so the comparisons may not be very accurate. However, it is important to note that in suburban environments, the origin of the measured metals is practically the same, standing out anthropogenic sources such as fuel oil combustion, traffic emissions or even resuspended dust. For example, some main elements associated to $PM_{2.5}$ measured in this work (Pb, Cd, Cu or Mn), are emitted from tyre wear debris (Adachi and Tainosho, 2004), coal combustion, vehicle emissions or the resuspension of crustal dust (Thorpe et al., 2008; Qie et al., 2018). However, other European cities exhibited different and highly variable profiles in terms of mixtures of trace elements (Vasilakos et al., 2007; Slezakova et al., 2007; Mooibroek et al., 2011; Cesari et al., 2018). Based on these comparisons, it may be concluded that the concentrations of metals associated with $PM_{2.5}$ mainly depend on the meteorology and the sampling site, and this in turn on the influence of sources of metal emissions nearby, such as vehicles or industries. Therefore, it is difficult to compare results from different studies, since each place would be influenced by the local meteorological features, the orography and the anthropogenic emissions typical of the area. This study would help to fill the picture and improve our understanding of suburban environments over Europe and the importance of linking such atmospheric studies with meteorological parameters.

3.4. $PM_{2.5}$-borne bacteria and fungi quantification

Urban regions are affected by different microorganism loads depending on their structure and local sources (Köck et al., 1998). Quantification of bacterial and fungal populations related to airborne particles is a complex task and therefore the use of both dependent and independent culture methods would be an adequate strategy in order to obtain global information of culturable and non-culturable microorganisms. Traditionally, these studies have been carried out so far by classical microbiological methods, based on plate counts (dependent of culture). But using exclusively this approach has been repeatedly criticized because only easily culturable microorganisms can be detected and counted, while those that no need selective enrichments or are in a particular physiological condition (in a sublethal or injured state) are lost. Therefore, the combination of the classic methods with independent approaches of culture, such as those based on the analysis of the total DNA extracted from the sample, is quite advisable. To date, both culture-independent and-dependent methods have been rarely used together to quantify microorganisms from airborne-PM.

The seasonal, daily and hourly bacteria and fungi counts obtained from cultivation methods and qPCR, are shown in Fig. 5. On one hand, variability in counts using culture dependent method among seasons was observed (Fig. 5a), although these differences were not statistically significant ($p>0.05$) neither for fungi nor for bacteria. Likewise, no differences between fungi and bacteria counts were displayed in any season. Comparisons between measured data and other similar studies are complicated due to spatiotemporal variability and lack of standardization in air collection and sample-processing methods, but there is an evident fact that airborne microbial quantity vary during the daily and annual cycle, and with location, as described in other studies (Lighthart, 2000; Raisi et al., 2010; Hu et al., 2020). Although culturable microorganisms may represent only a small fraction of the total microbial populations in the air, cultivation methods still remain the most widely used technique for collection and identification of airborne fungi and bacteria (Parat et al., 1999).

On the other hand, the ANOVA test from qPCR data displayed significant differences in microbial biomass among seasons (Fig. 5b) and a post-hoc test revealed that $PM_{2.5}$ from winter samples contained significantly less bacterial biomass than those from summer samples. In addition, no differences were observed in the quantification of fungi in any season. Lang-Yona et al. (2020) showed quite comparable results, over a region with similar features to the one studied here. The lower abundance of bacteria in winter could be due to low temperatures in that season, which are less favourable for bacterial growth. Otherwise, there are many studies reporting that there is no relationship between bacterial abundance and season (Maron et al., 2005; Lee et al., 2010).

In order to investigate the changes in PM-borne bacteria and fungi concentrations between weekdays and weekends, daily samples were collected. As Fig. 5 c and d show, there were no significant differences ($p>0.05$) between weekdays and weekends in the counts of bacteria or fungi regardless of the determination technique used.

To compare the hourly evolution for airborne bacteria and fungi quantification, air samples at different time intervals were daily collected (Fig. 5 e and f). Regardless of the technique used for the quantification of bacteria or fungi, the highest amount of them was observed throughout the morning and early afternoon, the interval of time in which there were more people at the University Campus, reaching the minimum value during the night, as well as in $PM_{2.5}$ concentrations (Fig. 2c), confirming that the more intense activity in the Campus during the morning and the early afternoon, as well as other activities, such as dial cycle of plants, animals and fungi, increased the microbial biomass.

Unlike what happens with metals or particles concentrations, currently there is no legislation in Europe about maximum values of airborne microorganisms in outdoor spaces due to the difficulty of controlling biological agents in the environment. However, several countries like the United States, Canada and France have already established standards and guidelines with the safe maximum number of fungal spores in indoor environments (Fujiyoshi et al., 2017), and set limits about indoor biological contaminants necessary to take into account usual outdoor levels. So, this kind of studies can provide important data that can be useful to assist the authorities to propose future recommendations concerning microbiological quality of air.

3.5. Correlations

With the aim to integrate all the previously shown results, correlation analysis was applied to examine the relationships between $PM_{2.5}$ concentration; some meteorological variables; and PM-borne metals concentrations and cultivable microorganisms counts. As shown in Fig. 6, there was no relationship between $PM_{2.5}$ and temperature, however, the fine particles concentrations displayed a negative relation with RH. In the analysed area, an increase in humidity is usually related to precipitation, which entails the particles deposition and thus the reduction of their concentration in the air.

Regarding correlations of trace elements, up to 7 of the 11 were positively correlated among them. Regulated heavy metals correlated each other, except Ni and Pb. The association of Ni, As and Cd is coincident with the characteristic tracers of coal and fossil fuel combustion.

Pb is a characteristic tracer of road traffic (Qi et al., 2016), which correlates with As, Cd, Co, Mg and Mn, and it would be indicative of a common source for all of them, such as vehicle emissions, as previously mentioned. Moreover, Mg and Mn are identified as tracers of dust or soil (Querol et al., 2004; Rivas et al., 2014), and the correlation between both has been also observed, thus, very probably, this source could also be present.

Positive correlations between $PM_{2.5}$ and culturable PM-borne bacteria and fungi counts were revealed. Previous studies in other European cities have also shown that maximum suspended dust particle concentrations in urban areas correlate positively with maximum number of colony-forming airborne microorganisms (Köck et al., 1998). This is in contrast with other studies in the urban ambient air where no correlation between $PM_{2.5}$ and PM-borne microorganism was displayed (Hass et al., 2013). Therefore, there is a mixture of results related to dust/microorganism correlations on previous literature. The effects of air pollutants and meteorological factors on microorganisms associated PM are complex. The low viable biological fraction associated PM may be attributed to many factors such as: PM composition, meteorological parameters, air pollution, physical and chemical transformation, and geographical characteristics. Besides, PM may contain toxic compounds which could kill or affect microbial viability (Alghamdi et al., 2014). Although it has been previously described that meteorological conditions, including temperature and RH, are among the most important factors influencing the concentrations of outdoor microorganisms (Jones and Harrison 2004; Mouli et al., 2005), culturable airborne microorganism counts do not correlate ($p>0.05$) with any of these variables in our study. However, this is controversial, some papers state that there is a correlation while others say there is none. It is unclear if the 2.5 cutoff size might impact and have a role on these results. The air serves primarily as a transport medium for microorganisms and not as habitat. Their counts are highly variable and mostly depend on factors such as vegetation, annual cycle, time of day, traffic volume and environment. Moreover, their biological stability can be affected by the "open-air-factor" (OAF) which depends on radiation, ozone and other air factors. Since OAF is not a single molecule, but a collection of highly reactive chemical species unidentified which can adversely affect their survival, it was described that this factor significantly reduced the number of viable microorganisms (Bailey et al., 2007).

4. Conclusions

The study presents the results of an observational campaign in the center of the Iberian Peninsula, measuring, on different time scales, $PM_{2.5}$, metals and biota (viable, viable non-cultivable and dead microorganisms) pollutants.

The main findings can be summarized as follows: i) Highest measured $PM_{2.5}$, Pb, Cr, Mg, Ni, Mn and Cu concentrations would point to the influence of some anthropogenic sources, such as home heating or traffic emissions. ii) Correlations between some trace elements were obtained, and combined correlations of $PM_{2.5}$ with RH and microorganism counts was also found. iii) The meteorological analysis points to several and far-from-simple mechanisms, such as Saharan dust events, soil resuspension, or the complex relation of wind strength, dispersion capability and particles sources. iv) No statistically significant differences were displayed between microorganism counts for all periods studied except for the bacterial seasonal results obtained by qPCR, due to summer higher count. Differences between fungi and bacteria counts were not observed, independently of the used method. v) Measured concentrations of particles and metals are not close to legislation limits. As no full legislation (only some indoor regulations) about microbial contaminants levels exists, this study points to the interest of establishing limit values as quality air indicators, not only of the microbial contaminants analyzed here, but also others such as pathogenic or anthropogenic-related microbes, and establish limit values as quality air indicators.

Several limitations and open questions arise from this pioneer work towards an integrated atmospheric environmental assessment. Among them: the representativity of the obtained results when more polluted areas are studied; the extrapolation of the shown role of time cycles when longer periods are considered; the need of consistent integration of methodologies that usually are with an isolated and quite heterogeneously usage; or a deeper characterization of the microbiota full composition.

Finally, an important implication of the study is that it provides information that could be used to assist the authorities and policy makers when defining local plans for air quality management and to increase the awareness of the population.

Declaration of Competing Interest

The authors declare that they have not known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Fig. 1. Sampling site and low volume sampler used. Toledo location in the centre of the Iberian Peninsula plateau, and, in more detail, the position of the sampler was at the University of Castilla-La Mancha (UCLM) Campus.

Fig. 2. Temporal evolution of average $PM_{2.5}$ concentrations: a) monthly variation; b) daily variation and c) time periods variation.

Fig. 3. Back trajectories of selected months (3a: January, 3b: July, 3ct: March). Colours indicate the relative amount or frequency of trajectories at each cell. Red colours indicate high frequencies and blue colour low frequencies in a relative value compared with the total possible trajectories obtained with HYSLPLIT and SplitR library software.

Fig. 4. Temporal evolution of average trace elements concentration: a) monthly variation; b) diary variation and c) hourly variation.

Fig. 5. Temporal evolution of average results for bacterial and fungal counts from both dependent and independent of culture methods: a) and b) seasonal variation; c) and d) daily variation and e) and f) time periods variation

Fig. 6. Spearman correlation coefficients of the parameters measured. Colour scale indicates positive/negative correlation. Only values with p-value < 0.05 are shown.

Fig. 1

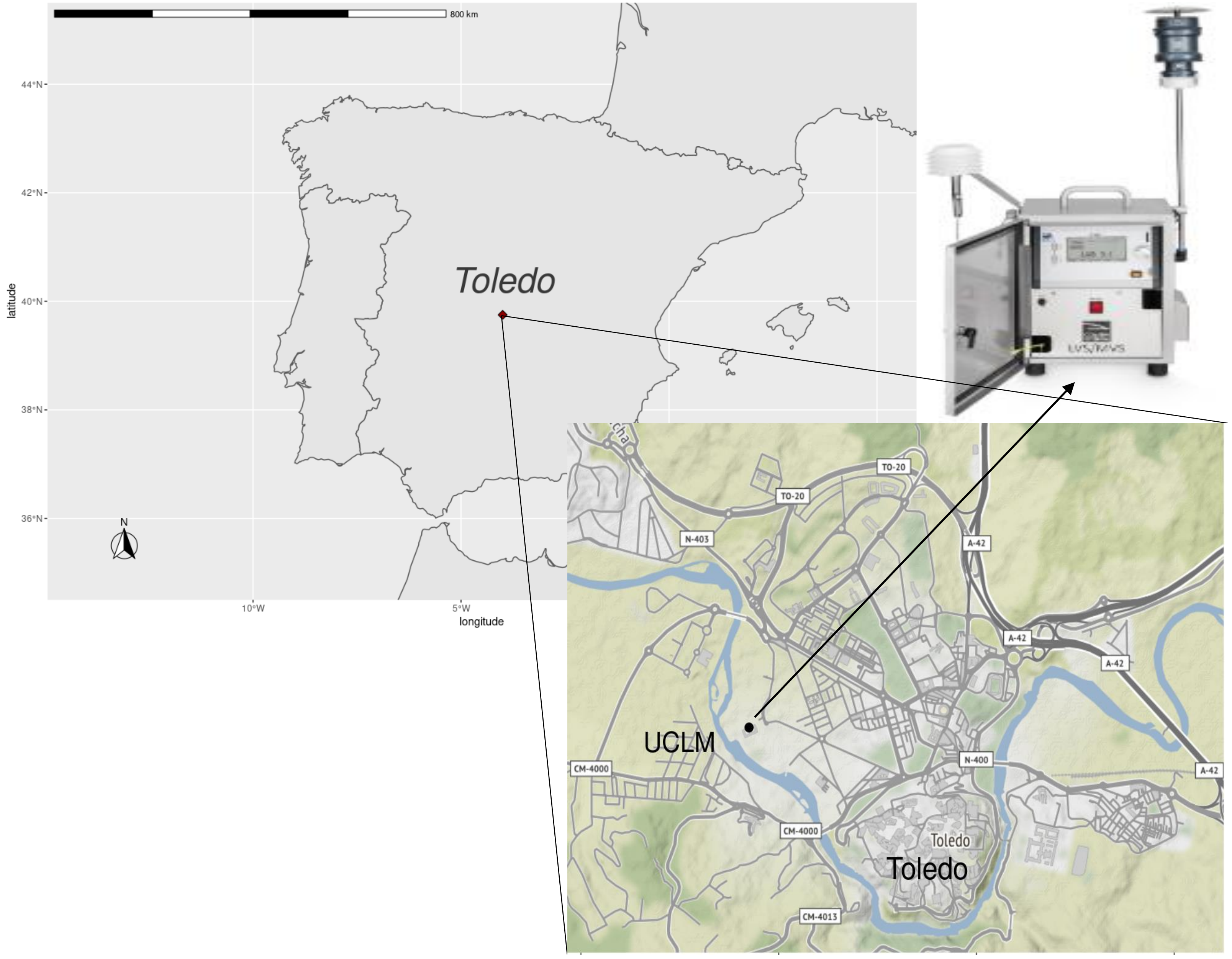
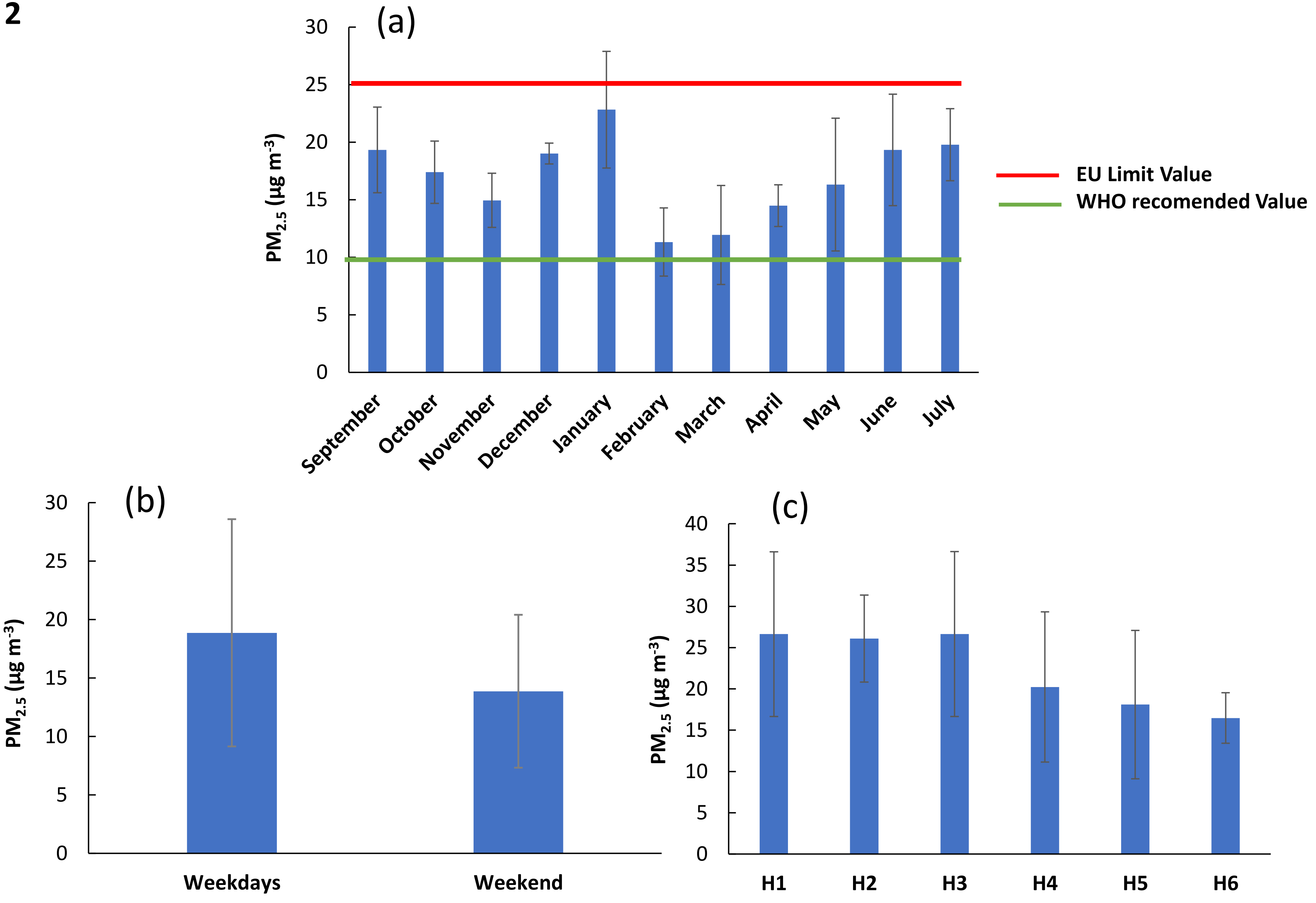

800 km
44°N
42°N
40°N
38°N
36°N
latitude
Toledo
N
10°W
5°W
longitude
LVS/MVS
TO-20
TO-20
N-403
A-42
A-42
A-42
UCLM
CM-4000
N-400
A-42
CM-4000
Toledo
Toledo
CM-4013

Fig. 2



(a)
30
25
20
15
10
5
0
PM2.5 (µg m-3)
September
October
November
December
January
February
March
April
May
June
July
EU Limit Value
WHO recomended Value
(b)
30
25
20
15
10
5
0
PM2.5 (µg m-3)
Weekdays
Weekend
(c)
40
35
30
25
20
15
10
5
0
PM2.5 (µg m-3)
H1
H2
H3
H4
H5
H6

Fig. 3

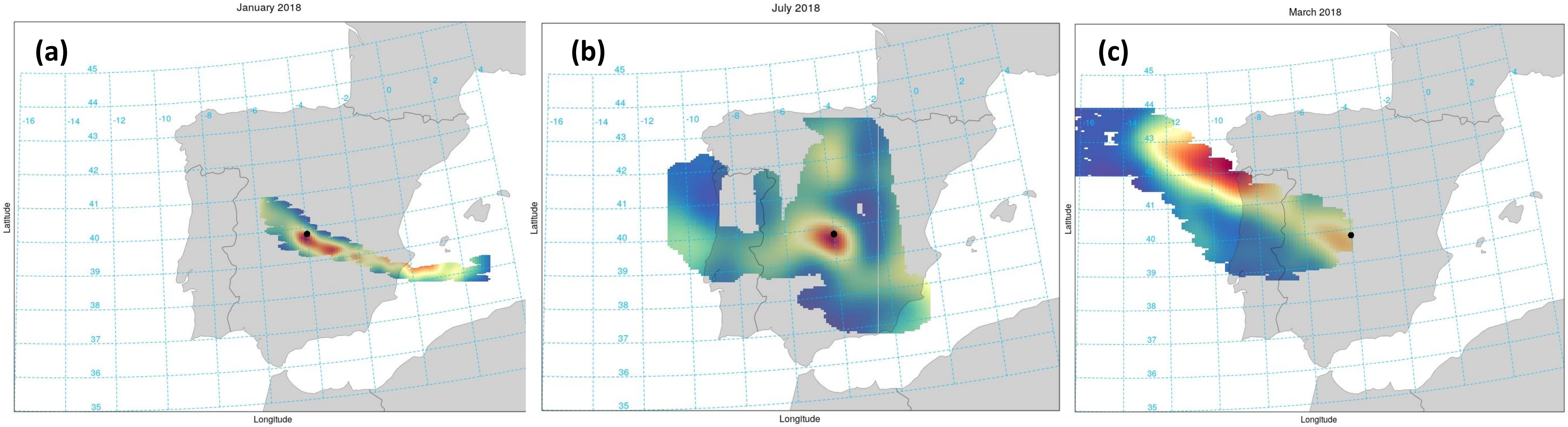
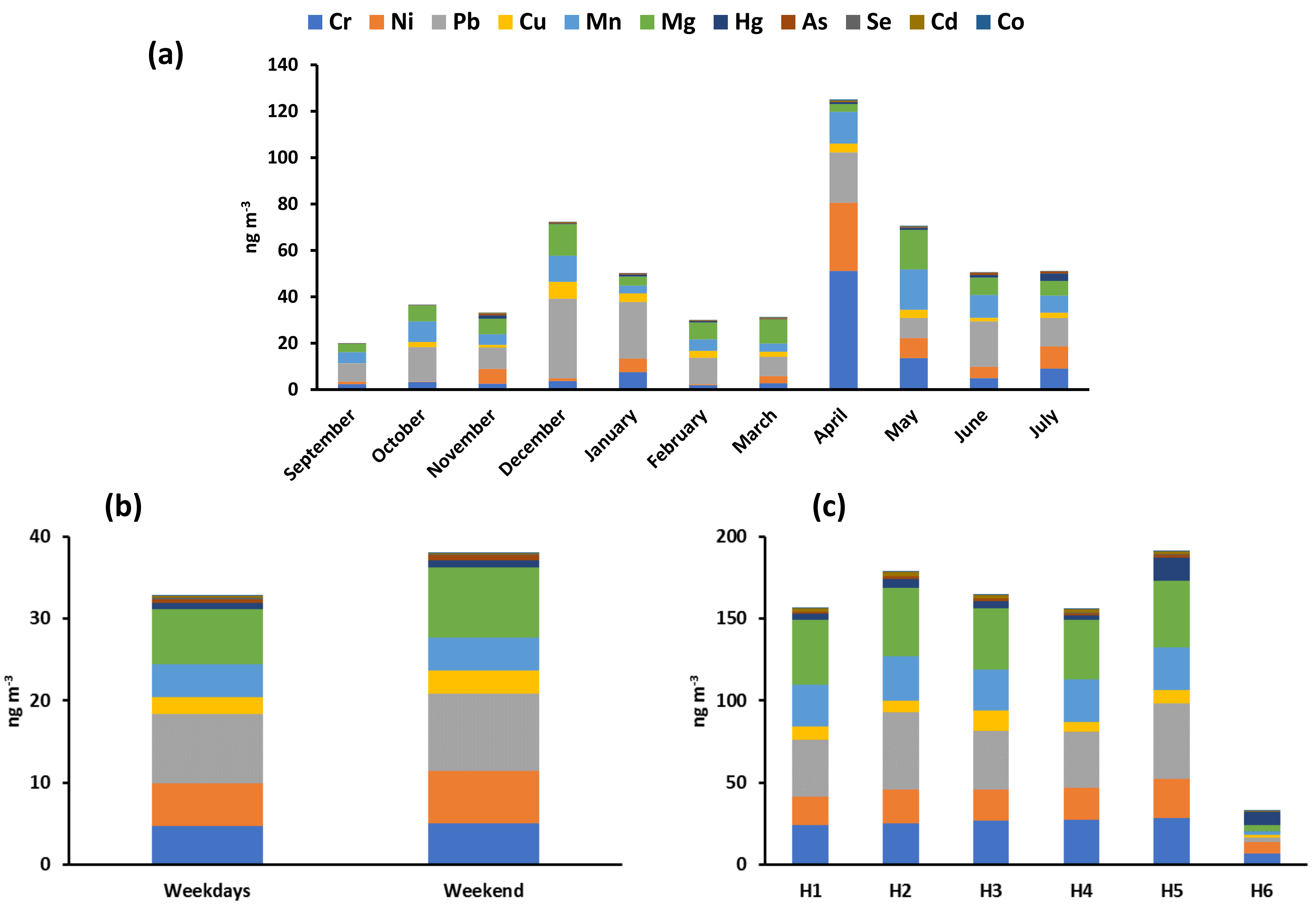
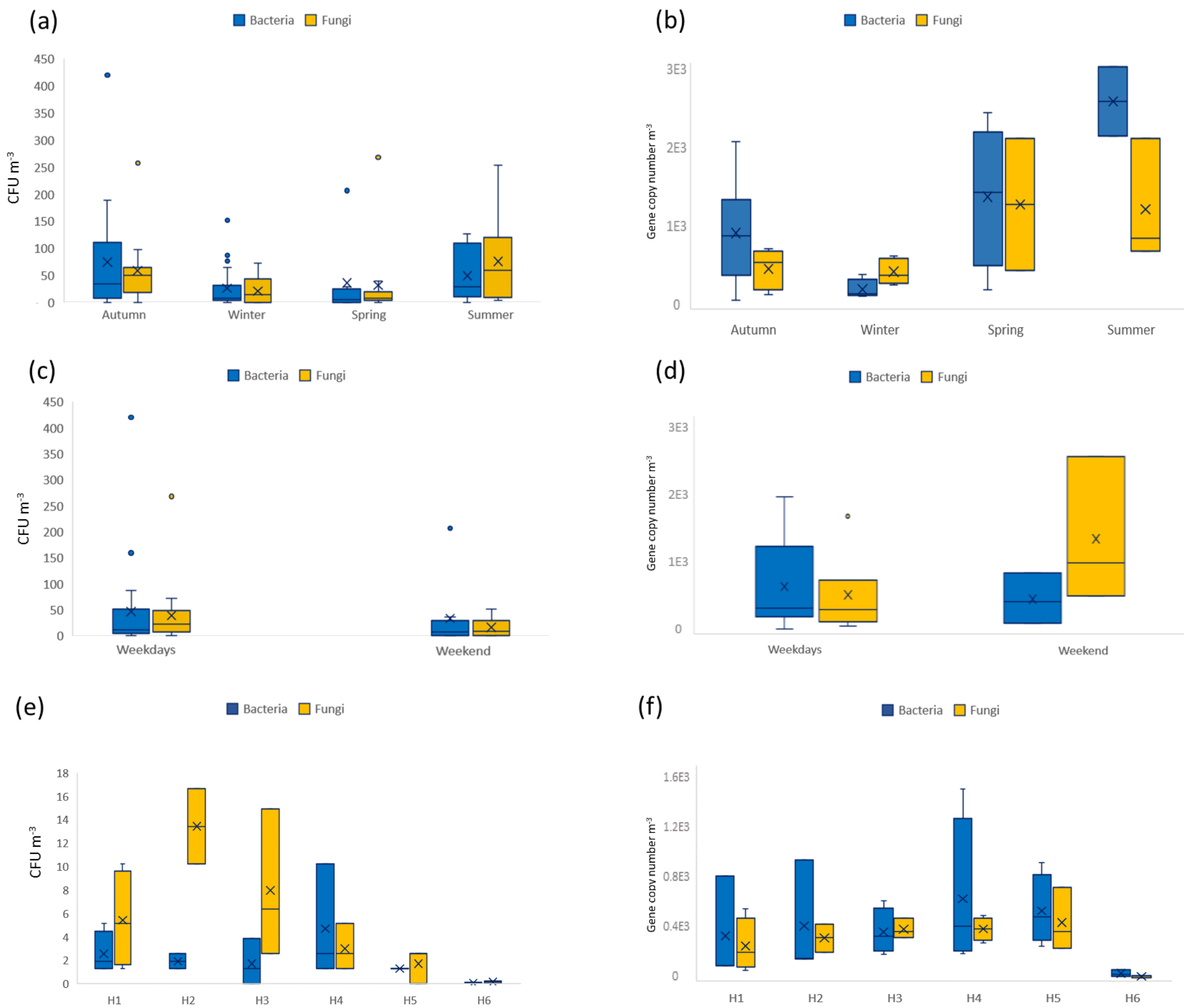


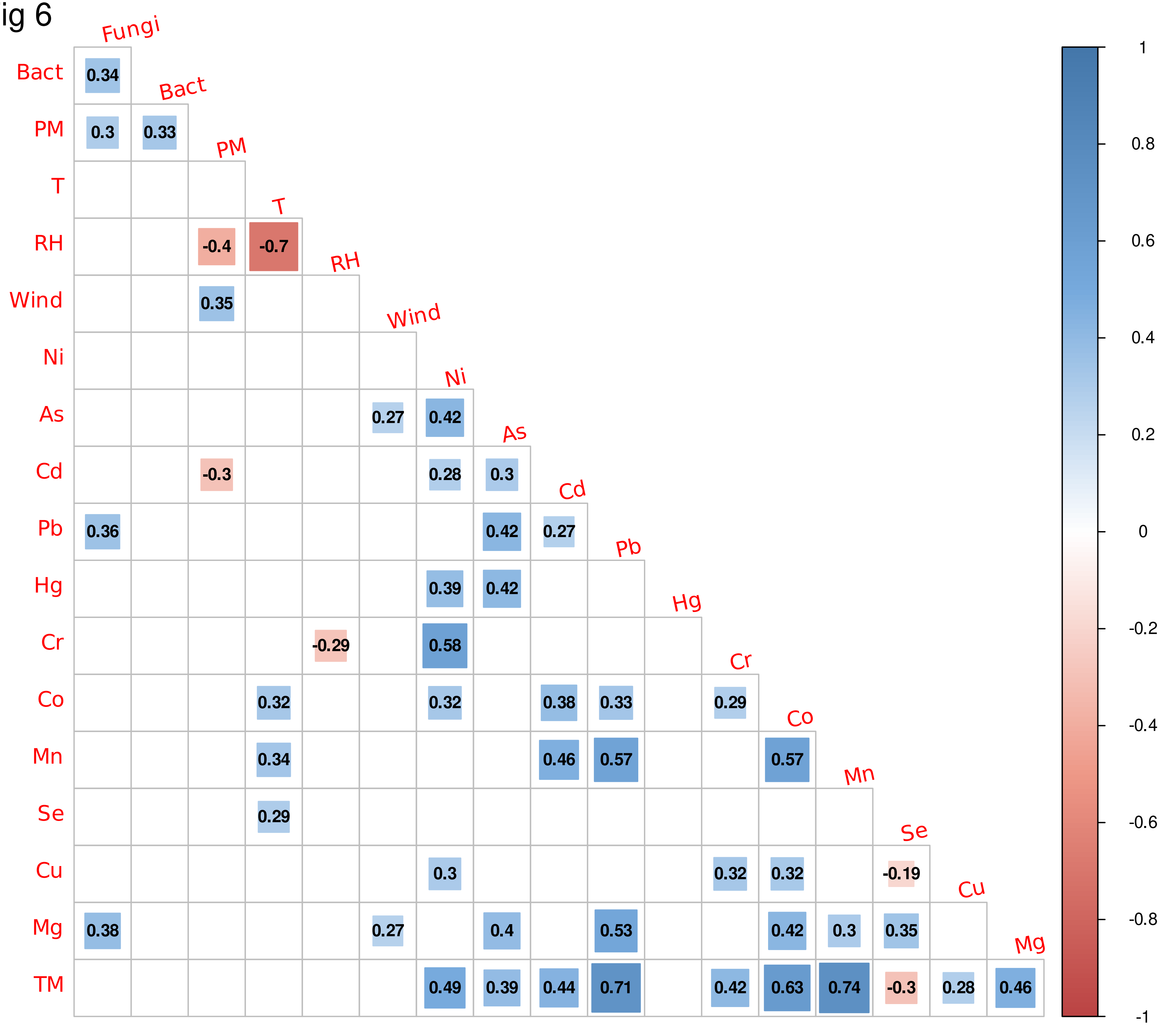
Fig. 4


Cr Ni Pb Cu Mn Mg Hg As Se Cd Co
(a)
ng m-3
0
20
40
60
80
100
120
140
September
October
November
December
January
February
March
April
May
June
July
(b)
ng m-3
0
10
20
30
40
Weekdays
Weekend
(c)
ng m-3
0
50
100
150
200
H1
H2
H3
H4
H5
H6



(a)
Bacteria
Fungi
CFU m-3
450
400
350
300
250
200
150
100
50
0
Autumn
Winter
Spring
Summer
(b)
Bacteria
Fungi
Gene copy number m-3
3E3
2E3
1E3
0
Autumn
Winter
Spring
Summer
(c)
Bacteria
Fungi
CFU m-3
Weekdays
Weekend
(d)
Bacteria
Fungi
Gene copy number m-3
Weekdays
Weekend
(e)
Bacteria
Fungi
CFU m-3
18
16
14
12
10
8
6
4
2
0
H1
H2
H3
H4
H5
H6
(f)
Bacteria
Fungi
Gene copy number m-3
1.6E3
1.2E3
0.8E3
0.4E3
0
H1
H2
H3
H4
H5
H6

Fig 6

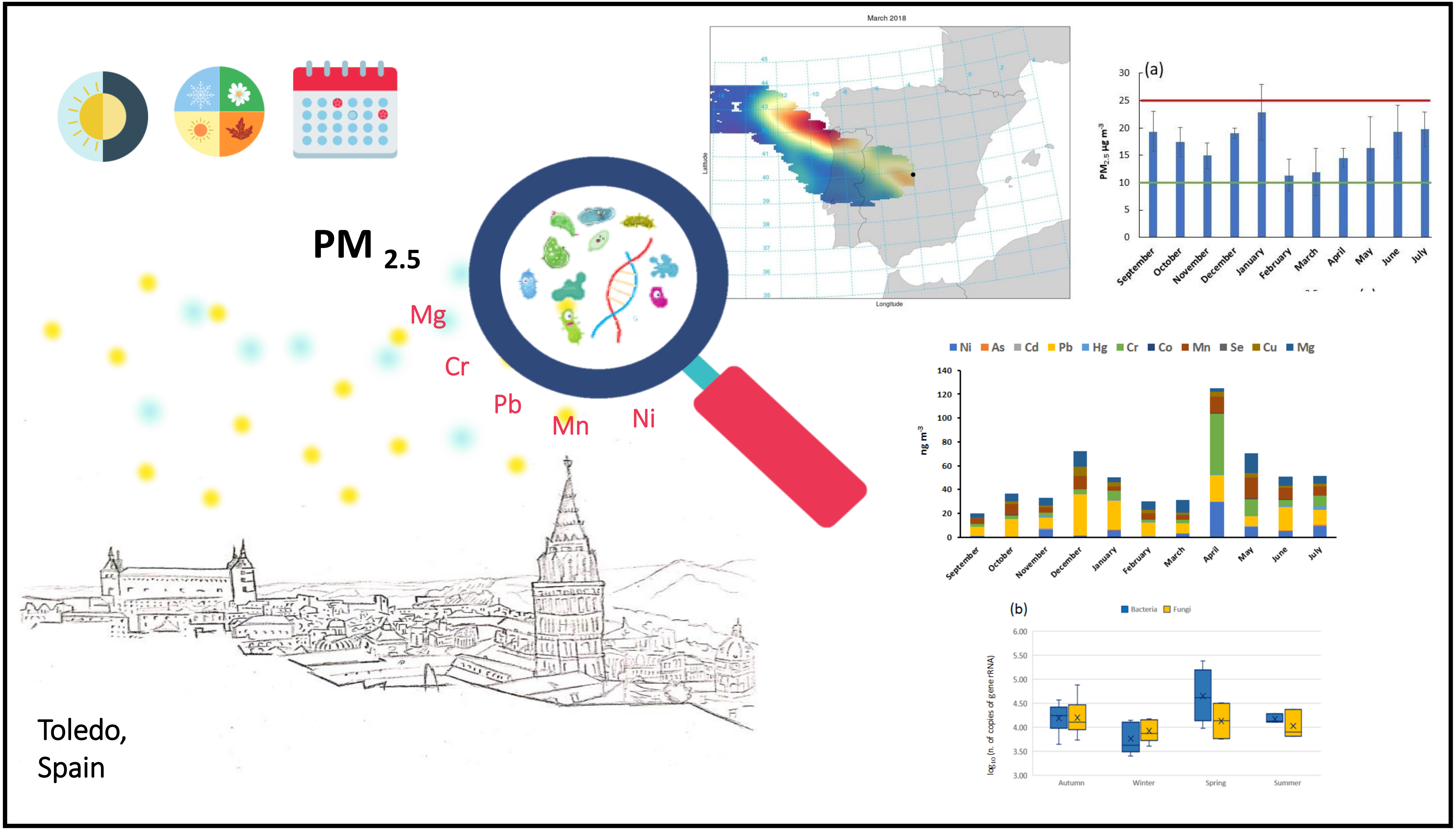


Fungi
Bact
PM
T
RH
Wind
Ni
As
Cd
Pb
Hg
Cr
Co
Mn
Se
Cu
Mg
TM
0.34
0.3
0.33
-0.4
-0.7
0.35
0.27
0.42
-0.3
0.28
0.3
0.36
0.42
0.27
0.39
0.42
-0.29
0.58
0.32
0.32
0.38
0.33
0.29
0.34
0.46
0.57
0.57
0.29
0.3
0.32
0.32
-0.19
0.38
0.27
0.4
0.53
0.42
0.3
0.35
0.49
0.39
0.44
0.71
0.42
0.63
0.74
-0.3
0.28
0.46
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1

Table 1
Statistical summary for the daily average measurements of chemical physical and microbiological parameters during the entire studied period.

Variable	Parameter				
	Min	Max	Mean	SD	Median
$PM_{2.5}$ (μg m^{-3})	3.6	32.6	15.5	5.7	15.3
Bacteria associated $PM_{2.5}$ (CFU m^{-3}) *	0.0	420.3	45.5	73.9	10.9
Fungi associated $PM_{2.5}$ (CFU m^{-3}) *	0.0	268.1	42.4	60.1	21.7
T (ºC)	4.3	31.0	16.5	7.3	16.2
R.H. (%)	22.0	91.7	57.5	17.0	59.7
Ni (ng m^{-3})	ND	39.5	6.3	7.6	4.6
As (ng m^{-3})	ND	1.3	0.4	0.3	0.3
Cd (ng m^{-3})	ND	0.8	0.1	0.2	0.1
Pb (ng m^{-3})	ND	35.4	11.8	10.2	7.2
Hg (ng m^{-3})	ND	6.1	0.8	1.3	0.2
Cr (ng m^{-3})	ND	68.7	8.4	13.3	4.1
Co (ng m^{-3})	ND	1.0	0.1	0.2	0.1
Mn (ng m^{-3})	ND	20.4	6.1	4.5	5.5
Se (ng m^{-3})	ND	0.5	0.2	0.2	0.1
Cu (ng m^{-3})	ND	11.2	2.7	2.4	2.1
Mg (ng m^{-3})	ND	22.5	7.0	5.8	5.6
Total Metals (ng m^{-3})	1.2	134.6	43.5	27.9	35.5

*Cultivable microorganism counts
ND: not detectable


PM 2.5
Mg
Cr
Pb
Mn
Ni
Toledo,
Spain
March 2018
Latitude
Longitude
(a)
PM2.5 µg m-3
September October November December January February March April May June July
Ni As Cd Pb Hg Cr Co Mn Se Cu Mg
ng m-3
(b)
Bacteria Fungi
log10 (n. of copies of gene rRNA)
Autumn Winter Spring Summer