First nation-wide estimation of tobacco consumption in Spain using wastewater-based epidemiology

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Abstract

Wastewater-based epidemiology (WBE) has become a very useful tool to monitor a population's drug consumption or exposure to environmental and food contaminants. In this work, WBE has been applied to estimate tobacco consumption in seven Spanish regions. To this end, 24 h composite wastewater samples were taken daily for one week in 17 wastewater treatment plants, covering altogether a population of ca. 6 million inhabitants. The samples were treated by enzymatic deconjugation and the wastewater content of two human-specific nicotine metabolites (namely, cotinine and trans-3′-hydroxycotinine) was measured to estimate the daily consumption of nicotine. The population-weighted average nicotine consumption in the seven analyzed regions was 2.2 g/(day·1000 inh.), without any daily pattern. This average estimated nicotine consumption value agreed with the value derived from official tobacco sales data. Differences in consumption among the seven studied regions were found, being Galicia, the region with the lowest rate, and the Basque Country and Catalonia those with the highest rates. However, no conclusive correlation was found between those values and the prevalence data taken from two different national surveys, nor sociodemographic and health data. This study demonstrates that this tool can complement other indicators in order to accurately assess tobacco consumption rates at regional and national levels and provides the most extensive application of the approach in the Spanish territory.

Keywords: nicotine, nationwide monitoring, sewage surveillance, addiction

1. Introduction

Wastewater-based epidemiology (WBE) has become a well-established technique for assessing the consumption of several legal and illicit drugs [1, 2]. This approach is based on the measurement of the excreted biomarkers (parent compounds or metabolites) of the substances of interest in wastewater from a specific population. Subsequently, some back-calculations are performed to estimate consumption rates [3]. WBE has been applied so far to estimate consumption of illicit drugs [4], new psychoactive substances [5], caffeine [6], alcohol [7], nicotine [8, 9], some pharmaceuticals and personal care products (PPCPs) [10], and also to evaluate the exposure to different contaminants, e.g. phthalates [11] or pesticides [12].

Nicotine (NIC) is the most consumed substance of licit abuse after alcohol worldwide [13]. According to the World Health Organization (WHO), smoking is one of the main risk factors for several chronic diseases, such as cancer, and respiratory and cardiovascular diseases [14]. Although legislation on its consumption and sale has been tightened in recent years, its use is still very prevalent worldwide [13]. In Spain, ca. 22% of the adult population consumes tobacco daily [15]. Thus, identifying areas and regions with the highest incidence of consumption, and potential reasons for these figures, is crucial to design strategies to reduce tobacco use and evaluate the success of the actions undertaken [16]. Furthermore, other sociodemographic factors, such as the average age or the unemployment rate, and the relationship with factors usually associated with tobacco use, such as the incidence of pulmonary or cardiovascular diseases need to be explored.

So far, the main sources of information to determine the prevalence of tobacco use are self-reported surveys, sales statistics, and medical diagnosis records. However, conventional approaches typically underestimate the actual prevalence due to non-response bias and bias in the selection of the sampled populations (case of surveys), the illicit trade markets that are not taken into account (case of sales records), and incomplete medical reports [2]. WBE provides additional information to these traditional sources, and it is considered as a fast and not overly expensive technique to complement and validate consumption figures [17, 18].

WBE has been used to estimate tobacco consumption in different countries including the United States [19], Australia [20, 21], China [22, 23], Maldives [24], Italy [6, 8], Spain [9, 17], and other European countries [17, 25-27]. In most published studies, the metabolites of NIC, cotinine (COT), trans-3′-hydroxycotinine (OH-COT), and their glucuronides have been analyzed, and back-calculations performed to estimate the amount of NIC consumed by the population. To a lesser extent, other biomarkers of tobacco consumption, such as the alkaloids anabasine and anatabine, have also been used [28]. These compounds are exclusively found in tobacco and not in the products used for nicotine replacement therapies (NRT), so they can be used to discern nicotine metabolites in wastewater coming from such products. However, their concentrations in tobacco are much lower than NIC and their determination in wastewater far more difficult, while there is no data on metabolism for back-calculating tobacco use [21].

The aim of this study was to perform the most ambitious assessment of tobacco consumption in Spain by means of WBE. To this end, 17 wastewater treatment plants (WWTPs) from 13 cities belonging to seven different regions were sampled every day during a whole week. NIC metabolites, COT and OH-COT, were measured in the wastewater samples to estimate NIC consumption. The results obtained through the WBE approach were compared with consumption estimates derived from the official data on tobacco sales and with survey data on smoking prevalence. Moreover, potential correlations between tobacco consumption and socio-economic and health conditions of the population were explored.

2. Material and Methods

2.1 Reagents and materials

High-performance liquid chromatography (HPLC)-grade methanol and acetic acid were supplied by Merck (Darmstadt, Germany); the enzyme β-glucuronidase (from Helix pomatia, Type H-2), ammonium acetate, sodium acetate, and sodium chloride were obtained from Sigma–Aldrich (Steinheim, Germany). GHP polypropylene syringe filters (0.22 μm) were acquired from Merck. Ultrapure water was obtained from a Milli-Q water generator (Millipore, Bedford, MA, USA). Standards of COT and OH-COT, as well as their deuterated analogs cotinine-d3 (COT-d3) and trans-3′-hydroxycotinine-d3 (OH-COT-d3), used as surrogate internal standards (IS) in the quantification process, were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Individual stock solutions (ca. 1 mg/mL) were prepared in methanol (Merck). Two mixture solutions containing the analytes (10 μg/mL) or the IS (0.5 μg/mL) were prepared in methanol and used as working solutions. For the enzymatic deconjugation of samples, sodium acetate buffer (1 M, pH 5) for pH adjustment, and a solution containing 3000 units of β-glucuronidase in 0.2% NaCl were used.

2.2 Samples

The wastewater sampling campaign was done in 2018, at seventeen WWTPs located in thirteen cities of seven Spanish regions: Galicia (one WWTP in Santiago de Compostela), the Basque Country (one WWTP serving Bilbao and its metropolitan area), Community of Madrid (two WWTPs in Madrid and one in Móstoles), Castile-La Mancha (one WWTP in Guadalajara and one in Toledo), Catalonia (Barcelona, Lleida, Reus and Tarragona; one

WWTP in each location), Valencian Community (three WWTPs covering Valencia and its metropolitan area, and one WWTP in Castellón), and Balearic Islands (two WWTPs covering Palma de Mallorca), see map in Figure 1). The entire population of all main cities was covered by the catchment area of the WWTPs selected, except for Madrid, where the two WWTPs sampled covered up to 30% of the city population, and Barcelona, where the only WWTP sampled covered 35% of the city population (SEM, Table S1). In the case of Palma de Mallorca (Balearic Islands), part of the flow entering one of the WWTPs was directed to a second one, and hence, for NIC load and back calculations (section 2.5), these two WWTPs were considered together. Thus, hereinafter, the original 17 sampling sites will be referred to as 16. The names of the cities in each region and specific details regarding WWTPs sampling, such as number of inhabitants served, daily wastewater flow rates, etc., are given in the SEM, Table S1. The selected WWTPs serve a population of ca. 6 million people, which represents 12.8% of the total population of Spain in 2018 [15]. Raw wastewater composite samples (24 h) were collected daily over a week that did not coincide with local festivities or special events. Samples were transferred into glass bottles and shipped frozen to Santiago de Compostela for analysis.

2.3 Sample preparation

The samples were processed following the protocol developed by Rodríguez-Álvarez et al. [9]. Briefly, 5 mL of wastewater were filtered through 0.22 µm GHP filters, and an aliquot of 1.14 mL was spiked with 60 µL of the IS mixture (0.5 µg/mL) and adjusted to pH 5 with 0.15 mL of a sodium acetate buffer (1 M). Then, 300 units/mL of β-glucuronidase (0.15 mL of a 3000 units solution in 0.2% NaCl) were added and the samples were heated at 37 ºC for

5 h to trigger the complete deconjugation of COT and OH-COT glucuronides. Thus, the total COT and OH-COT contained in the samples were subsequently measured by LC-MS/MS.

2.4 Instrumental analysis

Samples were analyzed using a Varian (Walnut Creek, CA, USA) liquid chromatograph composed of two ProStar 212 high-pressure mixing pumps, an autosampler and a ProStar 410 thermostated column compartment, coupled to a Varian 320-MS triple quadrupole mass spectrometer with an electrospray interface (ESI). The experimental parameters used for the determination of analytes and internal standards, such as quantification and qualification transitions, voltages, ratios, etc. are reported in Rodríguez-Álvarez et al. [9] and provided here in SEM, Table S2.

2.5. Back calculations for the estimation of NIC intake

The estimation of NIC consumption was performed using the concentrations found for COT and OH-COT (after deconjugation) independently [9]. Subsequently, the results obtained from both metabolites were compared. These calculations consider the excretion rate of each metabolite (sum of free and conjugated forms, i.e., 27% for COT and 44.5% for OH-COT on a molar basis [9, 29]) and the ratio between the molecular weights (MW) of NIC and each metabolite. The estimated concentration of NIC is then multiplied by the daily flow rate of the WWTP to calculate the daily loads, as indicated by Eq.1 and Eq. 2.

$$\text{Daily load NIC } (g/day) = [COT] \times \left(\frac{MW_{NIC}}{MW_{COT}}\right) \times \left(\frac{100}{27}\right) \times flow \qquad \text{Eq. 1}$$

$$\text{Daily load NIC } (g/day) = [OH-COT] \times \left(\frac{MW_{NIC}}{MW_{OH-COT}}\right) \times \left(\frac{100}{44.5}\right) \times flow \qquad \text{Eq. 2}$$

where [COT] and [OH-COT] are, respectively, the total concentrations of COT and OH-COT (in g/L); MW_{NIC}, MW_{COT}, and MW_{OH-COT} are the molecular weights of NIC, COT, and OH-COT, respectively; and flows are expressed in terms of L/day. The average value for the NIC daily loads estimation using COT and OH-COT was finally calculated, since both metabolites provided equivalent results (see section 3.1). Finally, to get population-normalized consumption rates, the values were divided by the number of inhabitants (n. inh) served by each WWTP (SEM, Table S1) and multiplied by 1000 as indicated in Eq.3.

$$\text{Daily NIC consumption } \left(\frac{g}{day \cdot 1000\ inh}\right) = \text{Daily load NIC } (g/day) \times \left(\frac{1000}{n.inh}\right) \quad \text{Eq.3}$$

2.6 Official data sources

Several sources were consulted to obtain official information regarding the different variables affecting tobacco consumption. Data related to tobacco sales were taken from the website of the Spanish Tobacco Market Commission ("Comisionado para el Mercado de Tabacos", CMT) [30]. Survey data on prevalence were taken from two different reports: the Spanish Observatory of Drugs and Addictions ("Observatorio Español de las Drogas y las Adicciones", OEDA) [31] publishes every second year the report "EDADES" (last data from yr. 2017) and the National Institute of Statistics ("Instituto Nacional de Estadística", INE) provides every five years data on several health and lifestyle issues, including smoking prevalence, in the National Health Survey ("Encuesta Nacional de Salud de España", ENSE) (last data from yr. 2017) [32]. Some socioeconomic variables of the population from the seven Spanish regions included in this study were tested to search for potential correlations with tobacco consumption, viz., death rate, unemployment rate, educational level, mean age, and average economic family income. These variables were taken from the website of the

INE (data from yr. 2018) [15]. Finally, the number of hospital admissions with a diagnosis of any disease related to tobacco use in each region was obtained from the database CMBD Discharges Record on Hospitalization and Specialized Out-Patient Care from the Spanish Ministry of Health (data from yr. 2017) [33]. The investigated diseases were nicotine addiction, heart stroke, lung and oral cancer, chronic bronchitis, and chronic obstructive pulmonary disease (COPD).

2.7 Statistical analyses

Statistical analyses were performed with the IBM SPSS statistics 25 software. Firstly, the normal distribution of data was tested with the *Kolmogorov-Smirnov (K-S)* test. Then, a *Pearson*'s correlation test was applied to compare the NIC estimation obtained based on the concentration of the two different metabolites. Analysis of variance (ANOVA) with a *Tukey post-hoc* correction was performed to compare the results obtained between the different days of the week and WWTPs and regions. A *paired t*-test was selected to search for differences between the NIC consumption estimated in this work and the NIC consumption calculated from the official sales published by the government. *Pearson*'s test (or *Spearman*'s rank test, depending on the data distribution) was finally used to assess the strength of the correlation between the WBE-derived NIC consumption results with the smoking prevalence (survey data), tobacco-related diseases incidence, and the aforementioned socioeconomic variables, at the 95% confidence level. The American Psychological Association (APA) style is used along the manuscript for reporting the results of statistical tests.

3. Results and discussion

3.1 Levels of NIC metabolites in wastewater

COT and OH-COT were detected in all samples analyzed at average concentrations per WWTP ranging from 0.6 to 7.7 μg/L and from 0.9 to 13.3 μg/L, respectively, being the lowest values found in the WWTP of Santiago de Compostela and the highest ones in Móstoles and some sampling points in Catalonia (Table S3). Some authors performed the back-calculations for the estimation of NIC using both metabolites (as sum) [8], however, other authors preferred to use only COT, due to high variability found in OH-COT determinations [21]. In our case, such problems were not encountered. The correlation between the concentration values obtained for COT and OH-COT was studied through a *Pearson*'s test, since the distribution of both variables was found to be normal. This correlation was positive, strong and statistically significant ($r(110)=0.961$, $p<0.001$). This relationship is graphically shown in SEM, Figure S1. The slope of the COT vs OH-COT linear regression model is 1.62 (standard deviation = 0.04), which is close to the theoretical value of 1.80 obtained from the average ratio of excretion rates reported for COT and OH-COT metabolism, after glucuronide deconjugation and molecular weight adjustment [29] (theoretical range: 1.36-2.42, considering the extreme metabolic excretions reported). The concentration profiles detected in wastewater match metabolism reports and both metabolites provide similar results for NIC consumption, as reported by Lai et al. [20]. Thus, the daily NIC loads were calculated as the average of the estimations obtained from COT and OH-COT individually. The fact that some authors [21] reported a high variability in OH-COT concentration determination can be attributed to an incomplete deconjugation of OH-COT, depending on wastewater and storage conditions as reported in Rodriguez-Alvarez et al. [9], which was avoided here by implementing an enzymatic deconjugation.

3.2 NIC consumption

Measured concentrations of COT and OH-COT were converted to daily NIC loads and to population-normalized consumption as previously explained in section 2.5 (Figure 1). The daily average NIC consumption was 2.2 ± 0.7 g/(day·1000 inh). The differences found between WWTPs ($F(15, 96)=18.75$, $p<0.0001$) were analyzed by a *post-hoc Tukey* test and the results are presented in Table S4. The WWTPs with the lowest values of NIC consumption were Santiago de Compostela, Madrid (I) and (II), Toledo, Lleida, and Valencia (I) and (III) (Figure 1). The NIC use levels in the city of Santiago de Compostela reported in a previous study were 1.7-1.9 g/(day·1000 inh.) [9]. This study included samples from three different years (2012-2014) taken during the same period of the year. These values are slightly higher than the levels estimated by the present study (1.4 ± 0.3 g/(day·1000 inh.)). Although the differences are not statistically significant ($F(3, 24) = 1.55$, $p = 0.22$). From these data, a slight diminution of consumption in the city can be assumed from 2014 to 2018, but, obviously, such trend should be confirmed in future campaigns. The results obtained for Móstoles, 3.7 ± 0.8 g/(day 1000 inh.), were the highest, and differ significantly from all the other cities (Table S5).

NIC consumption was also evaluated by region since its comparison with other consumption indicators cannot be performed at local scale. In those regions where more than one WWTP was analyzed, the weighted average was calculated considering the population covered by each WWTP (Table 1). Following this classification, Galicia (represented only by the WWTP of Santiago de Compostela) remains with the lowest NIC use values (1.4 ± 0.3 g/(day·1000 inh.)), while the Basque Country (represented by the WWTP of Bilbao metropolitan area) and Catalonia (represented by the WWTP of Barcelona, Reus, Lleida and Tarragona) become the regions with the highest consumption rates, 2.8 and 2.6 g/(day·1000 inh.) of NIC, respectively.

As shown in Figure S2, the daily overall consumption average ranged between 2.2 and 2.4 g/(day·1000 inh.), and no trends of tobacco consumption were observed within the different sampling days ($F(6, 105)=0.26$, $p=0.95$). This is consistent with previous results reported for one of the studied WWTPs (case of Santiago de Compostela) [9] and also in other locations in the world [8, 20, 21], indicating that tobacco is consumed on a regular daily basis. Conversely, some authors have reported higher NIC consumption during the weekend [26], but they attribute such results to a large concentration of entertainment and nightlife locals, where increased tobacco use is expected.

3.3 Comparison of WBE with sales and survey data

The CMT publishes official data regarding tobacco sales in the different Spanish regions (Table 1) [30]. The main sales belong to cigarettes (ca. 84% of the total tobacco sales in economic terms). Deeming that data are provided as boxes of 20 cigarettes and that for back-calculations we assumed 0.8 mg NIC absorbed per cigarette that would be equivalent to 16 mg per box of 20 cigarettes. The amount of NIC per cigarette (0.8 mg) was estimated through the average of the NIC content reported for 20 different brands using a smoking machine [34], according to our former publication considering Spanish tobacco information [9], and by means of the data reported by the 5 most consumed brands in the country in 2018. If in Spain, ca. 22% of the adult population (≥ 15 years, 84.0% of total population [15] consumes tobacco daily [32], this means that on average an adult smoker consumes 15.1 cigarettes per day.

Furthermore, considering the amount of cigarette boxes sold during 2018 and the population of each region, the average NIC officially consumed daily per 1000 inh. in each region was calculated (Table 1). A *paired t*-test showed no statistically significant differences between the sales- and the WBE-derived estimations of NIC consumption by region ($t(6)$= -0.86, p=0.41). Furthermore, the WBE national estimate calculated as population-weighted mean of all sites was 2.2 g/(day·1000 inh.), which is in agreement with the sales-derived NIC intake of 2.3 g/(day·1000 inh.) for the same regions. Moreover, this value is close to the figure obtained from sales for the whole of Spain: 2.1 g/(day·1000 inh.), Table 1. Thus, this study shows that there is a very good agreement between sales data and WBE data at national scale. However, the similarity is not that good at regional scale, particularly in Galicia and Balearic Islands, where WBE data is lower than sales statistics (Table 1). Different reasons may explain this finding, e.g. only one week was sampled at each location, which may not be representative of the NIC consumption in the whole year and also the limited amount of population covered by the sampled WWTPs being extrapolate to the whole community. Furthermore, different values for absorbed NIC have been used in back-calculations by other authors, ranging from 0.8 to 1.25 mg/cigarette [8, 9, 20, 21, 23, 25-27], which could affect the final estimations of tobacco consumption. These sources of uncertainty will be discussed in detail in section 3.5.

For further comparison between experimental data derived from wastewater analysis and reported prevalence data, two official general population surveys, namely, EDADES and ENSE were consulted (section 2.6). In both cases, the data are reported by region and the surveyed population was over 15 years old. No significant correlation was found between the NIC daily consumption estimated in our work and the data obtained from surveys (Figure 2). Yet, it is also noteworthy the lack of significant correlation between both surveys (Figure S3). According to EDADES and ENSE surveys, the mean Spanish daily smoking last month prevalence was 34.2% and 21.9%, respectively. In both surveys, the region presenting the lowest smoking prevalence is Galicia (30.9% and 17.8%, respectively), which is also the region with the second lowest sales (Table 1). These results are in agreement with the daily consumption estimates obtained by WBE, according to which Galicia presents also the minimum daily consumption, 1.4 ± 0.3 g/(day 1000 inh). It has been observed also in other studies [21, 27] that survey data differ from the experimental WBE estimations and the official sales data i.e., up to 50% differences [21]. In the first case, the differences between survey data and WBE estimates may be attributed to the distinct parameters being compared, prevalence versus NIC absorbed. These parameters do not necessarily need to go hand-by-hand since, for instance, a large proportion of heavy smokers can account for high NIC consumption levels but relatively low prevalence, and the other way around. On the other hand, the differences between sales data and WBE estimates can derive from tobacco purchases not actually being consumed.

3.4 Tobacco consumption, related diseases, and socioeconomic factors

Thirteen variables related with health condition and lifestyle were tested for correlations with tobacco consumption (WBE estimates of NIC) in the different regions assessed. These variables were created using data from governmental entities (see section 2.6) and included death and unemployment rates, educational level (divided in three variables, primary studies or less, secondary studies, and university studies), mean age, average economic family income and annual rates (number per million of inhabitants) of diagnosis of: nicotine addiction, heart stroke, lung and oral cancer, chronic bronchitis, and COPD. All these variables, except the number of bronchitis diagnosis were normally distributed ($p>0.05$) (Table S6). Thus, a *Pearson*'s correlation test was performed for the normal variables, and correlations with bronchitis were evaluated through a *Spearman*'s rank test. Table 2 shows the correlation coefficients obtained for weighted average NIC consumption and each of the socioeconomic factors tested. NIC consumption was only correlated with the educational level (in the category of primary studies or less) showing a negative correlation ($r(5)=-0.773$, $p=0.041$), which means that the higher the ratio of inhabitants with a low educational level, the lower the NIC daily loads measured in the studied regions (Figure 3). This correlation could, however, be biased by the low consumption value obtained in Galicia (which, on the other hand, is only represented by one city). In fact, if Galicia is excluded from this calculation, this negative correlation would not be statistically significant, meaning that these data are not conclusive. None of the other variables showed a statistically significant correlation with NIC consumption. The correlation between these variables and NIC consumption derived from the official sales and prevalence data (EDADES and ENSE surveys) was also evaluated, and no significant correlations were found (data not shown).

3.5 Uncertainties in tobacco estimates

There are already several publications discussing the uncertainties associated to WBE derived calculations [1, 35]. These include contributions from population estimations, stability of the selected biomarkers, sampling strategy, back-calculations, and analytical measurements. In this work, we have used different population estimation methods, since each WWTP responsible entity provided us with the values calculated by the method that better reflected its specific casuistry. This has been considered the best practice approach already adopted at international level in WBE estimation of illicit drug usage [4, 36]. Also, COT and OH-COT have been proven to be stable in lab-scale experiments simulating a sewer system [37]. In a real sewer, COT and OH-COT concentrations increased [38] due to the (partial) deconjugation of their glucuronides. Bearing in mind that we totally deconjugated both analytes enzymatically, transformation does not seem to be a major source of uncertainty in this study. Within the uncertainties derived from the study design, the selection of a specific sampling week in each region (avoiding festivities, etc.) could not be representative of the entire year. Also, the selected regions, covering ca. 13% of the Spanish population, could not be representative of the whole country. To minimize this latter aspect, regions with a wide range of population densities were included (i.e Community of Madrid and Castile-La Mancha have population densities of 830 and 25.5 inh./Km^2, respectively). Monte Carlo simulations indicate, however, that such aspects play a minor effect in the overall uncertainty of WBE-derived tobacco estimates [39]

A relevant aspect is, however, the amount of NIC absorbed by the human body for each tobacco unit, which differs within the published methods, as explained in section 3.3. Thus, working with the different reported values (from 0.8 to 1.25 mg) the variability could be up to 40%. There is no a simple method to calculate this parameter, hence the variability in the figures used by various authors [8, 9, 20, 21, 23, 25-27]. This value depends on the NIC content of the tobacco brands consumed in each country, the style of each individual smoking, etc. In fact, the Monte Carlo simulation study by Wang et al. [39] concluded that this is the factor related to a higher degree of uncertainty, followed by the variability of excretion rates of the metabolites. For this reason, we decided to use the values obtained using smoking machines and the reported by brands sold in Spain, which agreed quite accurately. As regards excretion ratios, the pioneer publication by Castiglioni et al. [8] used a correction factor based on the sum of COT and OH-COT masses, which is not completely correct and was based on a single publication [40]. Conversely, we used the correction factors defined in Rodríguez-Álvarez et al. [9] by using the data compiled by Hukkanen et al. [29] who reviewed 9 different publications. At the same time, we performed calculations on each of the metabolites independently (and the averaged them) which allows to check discrepancies between both biomarker estimates. As shown in 3.1, the ratio between both metabolites in wastewater is close to that proposed in [29] and [9] which provides further confidence and minimizes the contribution of excretion rates to overall uncertainty.

A further source of uncertainty in tobacco estimation from NIC metabolites is contribution due to the usage of NRT products (e.g. patches, chewing gum, etc.) or electronic cigarettes with NIC [21]. There are, however, no statistics about NRT products in Spain, while a question about electronic cigarettes has only been included in the last EDADES survey [31]. This survey reflected that 0.7-1.4 % of the (15-65 years old) Spanish population use them on a daily basis, from which 58.6% use electric cigarettes with NIC cartridges and 14.5% alternate NIC-containing and non-containing cartridges. Therefore, the prevalence of NIC consumption through this type of products is ca. 1%, compared to the reported prevalence of tobacco consumption (34.0%) in the same survey.

There is in general a good agreement, if sales and WBE figures are considered at an overall scale. Yet, traditional survey methods generally tend not to agree between them. This points out the fact that different figures are reached from different indicators, and that it is necessary to have several sources of tobacco estimations to get the most accurate picture.

4. Conclusions

The results estimated in this study of NIC consumption using both metabolites (COT and OH-COT) are comparable, with no specific trend of consumption observed between the different days of the week. The results of tobacco use differ between the different cities and regions, but they are not correlated with the results of prevalence of consumption reflected in the official surveys. However, these estimated values are a better approximation to the actual consumption reflected by the official sales than the prevalence records taken from the different national surveys, since the available surveys do not correlate with sales nor between them. No clear correlation has been found between tobacco use in the different Spanish regions and the prevalence of related diseases or the socio-economic characteristics. This study demonstrates that the WBE approach is a fast and relatively inexpensive approach to accurately estimate tobacco consumption and to guide public health policies.

Acknowledgments

Financial support. This study was supported by MINECO/AEI projects (CTM2016-81935-REDT, CTM2017-84763-C3-2-R, CTM2017-84763-C3-1-R, CTM2017-84763-C3-3-R), MICINN project (CEX2018-000794-S), Galician Council of Culture, Education and Universities (ED481D 2017/003 and ED431C2017/36), cofunded by FEDER/ERDF. **Sampling and analytical support**: Viaqua and Concello de Santiago, Jordi Palatsi from AQUALIA (Lleida WWTP), Cristian Mesa and Angela Vidal from Aigues de Barcelona (Barcelona WWTP), Santiago Querol and Sara Gargallo from Sociedad de Fomento Agrícola Castellonense (FACSA, Castellón), Subdirección General de Gestión del Agua, Ayuntamiento de Madrid, the Public Entity of Wastewater Treatment (EPSAR) of the

Generalitat Valenciana and especially to Fernando Llavador. Luis Aceiton, Enrique Albors, Angel Jiménez, Maria José Tarrega, Sonia Tristante and all the staff of the WWTPs (Aguas de Valencia, Spain). **Data provision:** “Subdirección General de Información Sanitaria, Ministerio de Sanidad”

Captions

Supplemental Electronic Material

Figure S1: Scatter plot that shows the relationship between COT and OH-COT concentrations ($r(110)=0.961$, $p<0.001$)

Figure S2: Overall NIC daily loads by day of the week.

Figure S3: Representation of prevalence data from EDADES *vs* ENSE surveys ($r(5)=0.673$, $p>0.05$)

Table S1: WWTP details and sampling conditions

Table S2: LC-MS/MS instrumental parameters

Table S3: Average measured concentration levels of COT and OH-COT by WWTP

Table S4: Daily NIC consumption estimated in each WWTP.

Table S5: Significance (p) of the HSD Tukey multiple (pair-wise) comparisons for NIC loads g/(day 1000 inh) in each WWTP.

Table S6: Results of K-S normality test for data on socio-economic [15] and health condition [33].

Tables:

Table 1: Comparison of NIC consumption estimated from WBE and official sales data by region.

Table 2: Data on socio-economic [15] and health condition [33] by region and correlation with WBE-measured NIC consumption.

Figures:

Figure 1: Map of Spain summarizing the WWTPs covered and estimated NIC consumption. N.B.: two WWTPs were sampled in Palma de Mallorca, but they were considered as one for calculations (see details in section 2.2)

Figure 2: Representation of daily NIC loads *vs* prevalence data from (a) EDADES and (b) ENSE surveys.

Figure 3: Graphical representation of NIC consumption *vs* educational level (primary or less).

References

1. Lorenzo M, Picó Y. "Wastewater-based epidemiology: current status and future prospects". Current Opinion in Environmental Science & Health. **2019**; 9:77-84. DOI:10.1016/j.coesh.2019.05.007
2. Subedi B, Burgard D. "Wastewater-Based Epidemiology as a Complementary Approach to the Conventional Survey-Based Approach for the Estimation of Community Consumption of Drugs". Wastewater-Based Epidemiology: Estimation of Community Consumption of Drugs and Diets. **2019**; 1319(1319):3-21. DOI:10.1021/bk-2019-1319.ch001

3. Choi PM, Tscharke BJ, Donner E, O'Brien JW, Grant SC, Kaserzon SL, et al. "Wastewater-based epidemiology biomarkers: Past, present and future". TrAC Trends in Analytical Chemistry. **2018**; 105:453-69. DOI:10.1016/j.trac.2018.06.004
4. González-Mariño I, Baz-Lomba JA, Alygizakis NA, Andrés-Costa MJ, Bade R, Bannwarth A, et al. "Spatio-temporal assessment of illicit drug use at large scale: evidence from 7 years of international wastewater monitoring". Addiction. **2020**; 115(1):109-20. DOI:10.1111/add.14767
5. Bijlsma L, Celma A, López FJ, Hernández F. "Monitoring new psychoactive substances use through wastewater analysis: current situation, challenges and limitations". Current Opinion in Environmental Science & Health. **2019**; 9:1-12. DOI:10.1016/j.coesh.2019.03.002
6. Senta I, Gracia-Lor E, Borsotti A, Zuccato E, Castiglioni S. "Wastewater analysis to monitor use of caffeine and nicotine and evaluation of their metabolites as biomarkers for population size assessment". Water Research. **2015**; 74:23-33. DOI:10.1016/j.watres.2015.02.002
7. Boogaerts T, Covaci A, Kinyua J, Neels H, van Nuijs ALN. "Spatial and temporal trends in alcohol consumption in Belgian cities: A wastewater-based approach". Drug and Alcohol Dependence. **2016**; 160:170-6. DOI:10.1016/j.drugalcdep.2016.01.002
8. Castiglioni S, Senta I, Borsotti A, Davoli E, Zuccato E. "A novel approach for monitoring tobacco use in local communities by wastewater analysis". Tobacco Control. **2015**; 24(1):38-42. DOI:10.1136/tobaccocontrol-2014-051553
9. Rodríguez-Álvarez T, Rodil R, Rico M, Cela R, Quintana JB. "Assessment of Local Tobacco Consumption by Liquid Chromatography–Tandem Mass Spectrometry Sewage Analysis of Nicotine and Its Metabolites, Cotinine and trans-3′-Hydroxycotinine, after Enzymatic Deconjugation". Analytical Chemistry. **2014**; 86(20):10274-81. DOI:10.1021/ac503330c
10. Lopardo L, Adams D, Cummins A, Kasprzyk-Hordern B. "Verifying community-wide exposure to endocrine disruptors in personal care products – In quest for metabolic biomarkers of exposure via in vitro studies and wastewater-based epidemiology". Water Research. **2018**; 143:117-26. DOI:10.1016/j.watres.2018.06.028
11. González-Mariño I, Rodil R, Barrio I, Cela R, Quintana JB. "Wastewater-Based Epidemiology as a New Tool for Estimating Population Exposure to Phthalate Plasticizers". Environmental Science & Technology. **2017**; 51(7):3902-10. DOI:10.1021/acs.est.6b05612
12. Rousis NI, Zuccato E, Castiglioni S. "Monitoring population exposure to pesticides based on liquid chromatography-tandem mass spectrometry measurement of their urinary metabolites in urban wastewater: A novel biomonitoring approach". Science of The Total Environment. **2016**; 571:1349-57. DOI:10.1016/j.scitotenv.2016.07.036
13. Global Drug Survey. GDS 2019 key findings report. **(2019)**. Available from: https://www.globaldrugsurvey.com/. Last Accessed: 05 March, 2020.
14. World Health Organization. Health topics: Tobacco. **(2020)**. Available from: https://www.who.int/health-topics/tobacco. Last Accessed: 20 February, 2020.
15. Instituto Nacional de Estadística. **(2018)**. Available from: https://www.ine.es/. Last Accessed: 20 February, 2020.
16. Drope J, Schluger N, Cahn Z, Drope J, Hamill S, Islami F, et al. "The Tobacco Atlas. Atlanta: American Cancer Society and Vital Strategies". https://tobaccoatlasorg/. **2018**:Last accessed: 20 February 2020.

17. Baz-Lomba JA, Salvatore S, Gracia-Lor E, Bade R, Castiglioni S, Castrignanò E, et al. "Comparison of pharmaceutical, illicit drug, alcohol, nicotine and caffeine levels in wastewater with sale, seizure and consumption data for 8 European cities". BMC Public Health. **2016**; 16(1):1035. DOI:10.1186/s12889-016-3686-5

18. Bijlsma L, Celma A, Gonzalez-Mariño I, Postigo C, Andreu V, Andres-Costa MJ, et al. "Wastewater-based epidemiology: applications towards the estimation of drugs of abuse consumption and public health in general. The Spanish network ESAR-Net". Revista espanola de salud publica. **2018**; 92.

19. Chen J, Venkatesan AK, Halden RU. "Alcohol and nicotine consumption trends in three U.S. communities determined by wastewater-based epidemiology". Science of The Total Environment. **2019**; 656:174-83. DOI:10.1016/j.scitotenv.2018.11.350

20. Lai FY, Gartner C, Hall W, Carter S, O'Brien J, Tscharke BJ, et al. "Measuring spatial and temporal trends of nicotine and alcohol consumption in Australia using wastewater-based epidemiology". Addiction. **2018**; 113(6):1127-36. DOI:10.1111/add.14157

21. Mackie RS, Tscharke BJ, O'Brien JW, Choi PM, Gartner CE, Thomas KV, et al. "Trends in nicotine consumption between 2010 and 2017 in an Australian city using the wastewater-based epidemiology approach". Environment International. **2019**; 125:184-90. DOI:10.1016/j.envint.2019.01.053

22. Gao J, Zheng Q, Lai FY, Gartner C, Du P, Ren Y, et al. "Using wastewater-based epidemiology to estimate consumption of alcohol and nicotine in major cities of China in 2014 and 2016". Environment International. **2020**; 136:105492. DOI:10.1016/j.envint.2020.105492

23. Zheng Q-D, Lin J-G, Pei W, Guo M-X, Wang Z, Wang D-G. "Estimating nicotine consumption in eight cities using sewage epidemiology based on ammonia nitrogen equivalent population". Science of The Total Environment. **2017**; 590-591:226-32. DOI:10.1016/j.scitotenv.2017.02.214

24. Fallati L, Castiglioni S, Galli P, Riva F, Gracia-Lor E, González-Mariño I, et al. "Use of legal and illegal substances in Malé (Republic of Maldives) assessed by wastewater analysis". Science of The Total Environment. **2020**; 698:134207. DOI:10.1016/j.scitotenv.2019.134207

25. Lopes A, Silva N, Bronze MR, Ferreira J, Morais J. "Analysis of cocaine and nicotine metabolites in wastewater by liquid chromatography–tandem mass spectrometry. Cross abuse index patterns on a major community". Science of The Total Environment. **2014**; 487:673-80. DOI:10.1016/j.scitotenv.2013.10.042

26. Mackuľak T, Birošová L, Grabic R, Škubák J, Bodík I. "National monitoring of nicotine use in Czech and Slovak Republic based on wastewater analysis". Environmental Science and Pollution Research. **2015**; 22(18):14000-6. DOI:10.1007/s11356-015-4648-7

27. van Wel JHP, Gracia-Lor E, van Nuijs ALN, Kinyua J, Salvatore S, Castiglioni S, et al. "Investigation of agreement between wastewater-based epidemiology and survey data on alcohol and nicotine use in a community". Drug and Alcohol Dependence. **2016**; 162:170-5. DOI:10.1016/j.drugalcdep.2016.03.002

28. Tscharke BJ, White JM, Gerber JP. "Estimates of tobacco use by wastewater analysis of anabasine and anatabine". Drug Testing and Analysis. **2016**; 8(7):702-7. DOI:10.1002/dta.1842

29. Hukkanen J, Jacob P, Benowitz NL. "Metabolism and Disposition Kinetics of Nicotine". Pharmacological Reviews. **2005**; 57(1):79-115. DOI:10.1124/pr.57.1.3

30. Comisionado para el mercado de tabacos. Ministerio de Hacienda. **(2018)**. Available from: https://www.hacienda.gob.es/es-ES/Areas%20Tematicas/CMTabacos/Paginas/Default.aspx. Last Accessed: 20 February, 2020.
31. Observatorio Español de las Drogas y las Adicciones. Ministerio de Sanidad. **(2017)**. Available from: http://www.pnsd.mscbs.gob.es/profesionales/sistemasInformacion/home.htm. Last Accessed: 20 February, 2020.
32. Encuesta Nacional de Salud. Ministerio de Sanidad. **(2017)**. Available from: https://www.mscbs.gob.es/estadEstudios/estadisticas/encuestaNacional/encuesta2017.htm. Last Accessed: 20 February, 2020.
33. Hospital Discharge Records in the National Health System (CMBD). Ministerio de Sanidad. **(2017)**. Available from: https://www.mscbs.gob.es/en/estadEstudios/estadisticas/cmbdhome.htm. Last Accessed: 20 February, 2020.
34. St.Charles FK, Kabbani AA, Borgerding MF. "Estimating tar and nicotine exposure: Human smoking versus machine generated smoke yields". Regulatory Toxicology and Pharmacology. **2010**; 56(1):100-10. DOI:10.1016/j.yrtph.2009.08.011
35. Castiglioni S, Bijlsma L, Covaci A, Emke E, Hernández F, Reid M, et al. "Evaluation of Uncertainties Associated with the Determination of Community Drug Use through the Measurement of Sewage Drug Biomarkers". Environmental Science & Technology. **2013**; 47(3):1452-60. DOI:10.1021/es302722f
36. Thomas KV, Bijlsma L, Castiglioni S, Covaci A, Emke E, Grabic R, et al. "Comparing illicit drug use in 19 European cities through sewage analysis". Science of The Total Environment. **2012**; 432:432-9. DOI:https://doi.org/10.1016/j.scitotenv.2012.06.069
37. Gao J, Li J, Jiang G, Shypanski AH, Nieradzik LM, Yuan Z, et al. "Systematic evaluation of biomarker stability in pilot scale sewer pipes". Water Research. **2019**; 151:447-55. DOI:10.1016/j.watres.2018.12.032
38. Gao J, Li J, Jiang G, Yuan Z, Eaglesham G, Covaci A, et al. "Stability of alcohol and tobacco consumption biomarkers in a real rising main sewer". Water Research. **2018**; 138:19-26. DOI:10.1016/j.watres.2018.03.036
39. Wang D-G, Dong Q-Q, Du J, Yang S, Zhang Y-J, Na G-S, et al. "Using Monte Carlo simulation to assess variability and uncertainty of tobacco consumption in a city by sewage epidemiology". BMJ Open. **2016**; 6(2):e010583. DOI:10.1136/bmjopen-2015-010583
40. Byrd GD, Chang KM, Greene JM, deBethizy JD. "Evidence for urinary excretion of glucuronide conjugates of nicotine, cotinine, and trans-3'-hydroxycotinine in smokers". Drug Metabolism and Disposition. **1992**; 20(2):192-7.

Table 1: Comparison of NIC consumption estimated from WBE and official sales data by region.

Year 2018				
Region	Boxes of 20 cigarretes sold per year[(1)]	Region population (inh.)[(2)]	Sales derived NIC consumption g/(day1000inh.)	WBE derived NIC consumption g/(day1000inh.) [(3,4)]
Galicia	125587069	2701743	2.0	1.4±0.3 [(a)]
Basque Country	117312935	2199088	2.3	2.8±0.4 [(d)]
Community of Madrid	285251875	6578069	1.9	2.0±1.2 [(b)]
Castile-La Mancha	101741005	2026807	2.2	2.3±0.6 [(b,c)]
Catalonia	420229481	7600065	2.4	2.6±0.5 [(b,c,d)]
Valencian Community	276757572	4963703	2.4	2.1±0.5 [(b,c)]
Balearic Islands	88262042	1128908	3.4	2.3±0.6 [(b,c)]
Total analyzed regions	1415141979	27198383	2.3	2.2±0.7
Total Spain	2231204734	46693630	2.1	

[(1)] Data taken from the Spanish Tobacco Market Comission [30]
[(2)] Data taken from the Spanish National Institute of Statistics [15]
[(3)] Population-weighted average
[(4)] Same letter codifies homogeneous groups after *HSD Tukey post-Hoc* test.

Table 2: Data on socio-economic [15] and health condition [33] by region and correlation with WBE-measured NIC consumption.

	Galicia	**Basque Country**	**Community of Madrid**	**Castile-La Mancha**	**Catalonia**	**Valencian Community**	**Balearic Islands**	**Correlation. NIC consumption (1,2)**
Death rate(%) year 2018	12.0	10.0	7.0	9.6	8.8	9.2	7.1	-0.366
Unemployment rate (%) year 2018	13.3	9.9	11.5	15.6	12.2	18.2	11.7	-0.315
Education level (%) Primary or less	23.8	17.2	21.0	17.9	14.5	23.7	16.3	-0.773*
Education level (%) Secondary	49.3	44.5	47.0	54.4	47.3	54.5	59.0	-0.166
Education level (%) University	27.0	38.2	32.0	27.7	38.2	21.7	24.6	0.564
Mean age (years) 2018	47.0	45.3	42.7	43.2	42.2	42.8	41.3	-0.432
Average economic family income (€/year) 2018	27658	35049	32763	25207	33055	24401	34007	0.495
Hospital admissions any diag."nicotine addiction" year 2017 (nº/million inh.)	8367	6849	4900	8052	7936	9789	7422	-0.145
Hospital admissions any diag."heart stroke" year 2017 (nº/million inh.)	729	469	522	405	1155	699	506	-0.002
Hospital admissions any diag."lung cancer" year 2017 (nº/million inh.)	1116	1068	1604	1340	1296	1690	1613	-0.066
Hospital admissions any diag."oral cancer" year 2017 (nº/million inh.)	8.1	10.8	17.0	7.0	6.0	9.8	10.5	-0.088
Hospital admissions any diag."bronchitis" year 2017 (nº/million inh.)	24.2	15.3	12.1	12.3	131.4	59.7	24.6	0.290
Hospital admissions any diag."COPD" year 2017 (nº/million inh.)	6141	5604	6700	6211	6485	6067	7352	-0.128

(1) Correlation coefficient between each variable and WBE-derived nicotine estimates.

(2) Statistically significant correlations (*p-value<0.05)* are highlighted with an asterisk (*).

Correlations were assessed by Pearson test for all variables except bronchitis, which was studied through the ρ-Spearman test since the data were not normally distributed

Figure 1

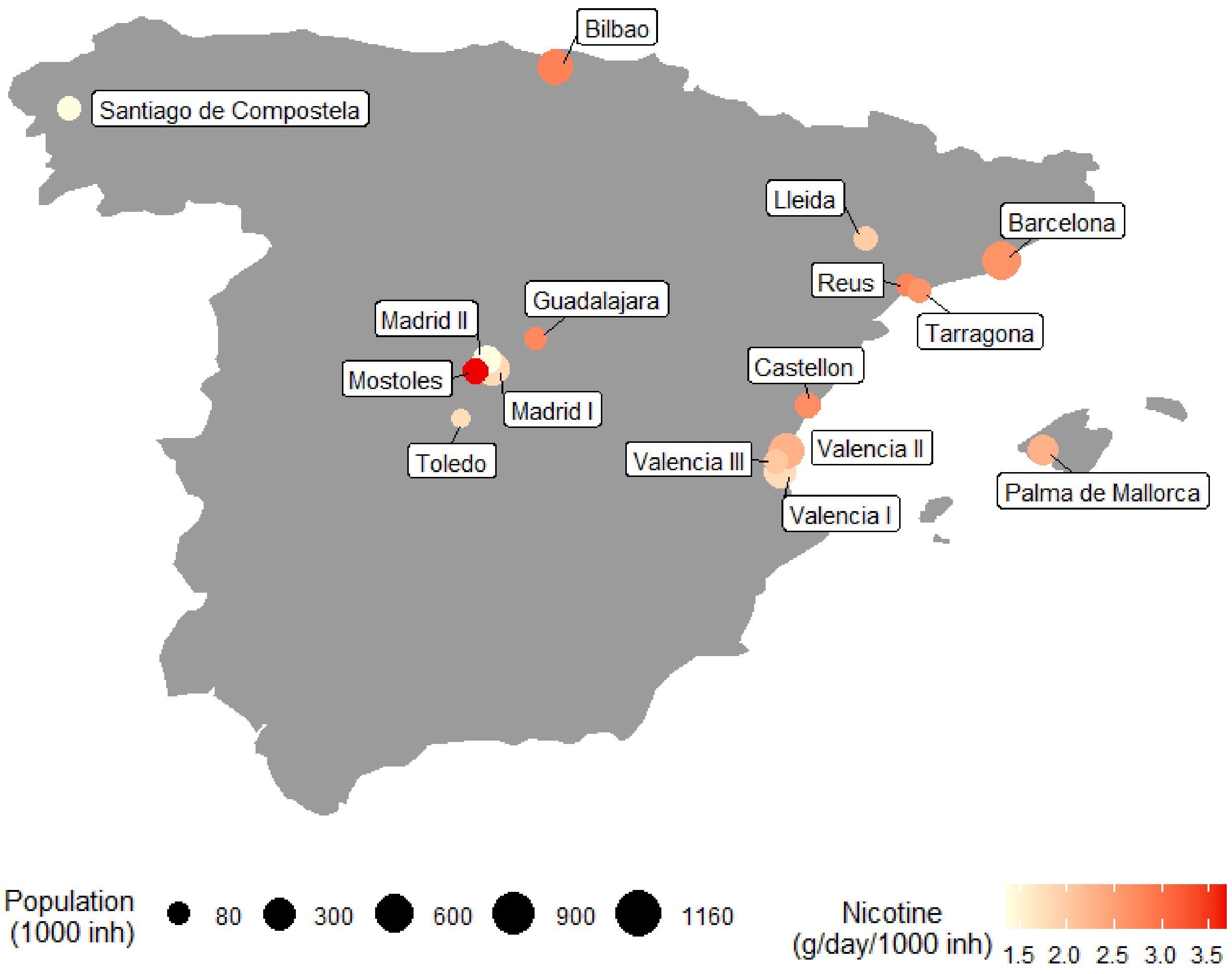
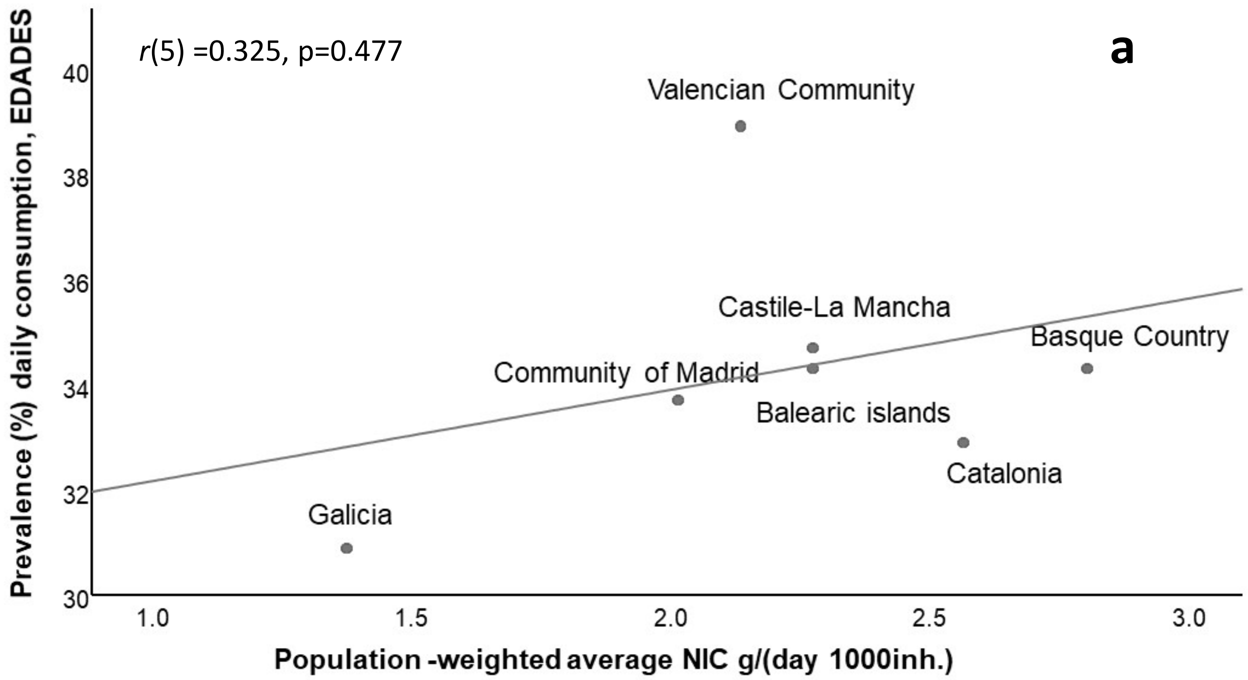
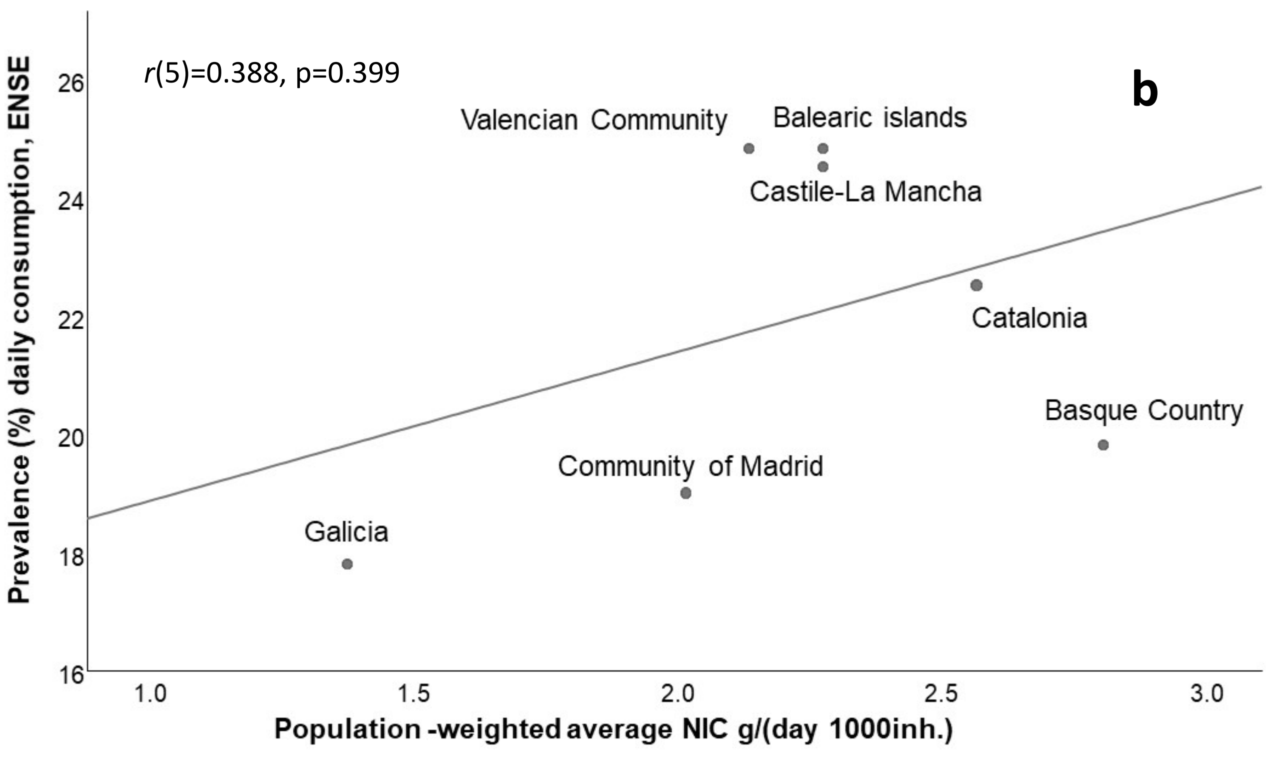

Bilbao
Santiago de Compostela
Lleida
Barcelona
Reus
Tarragona
Guadalajara
Madrid II
Mostoles
Madrid I
Toledo
Castellon
Valencia III
Valencia II
Valencia I
Palma de Mallorca
Population
(1000 inh)
80
300
600
900
1160
Nicotine
(g/day/1000 inh)
1.5
2.0
2.5
3.0
3.5

Figure 2

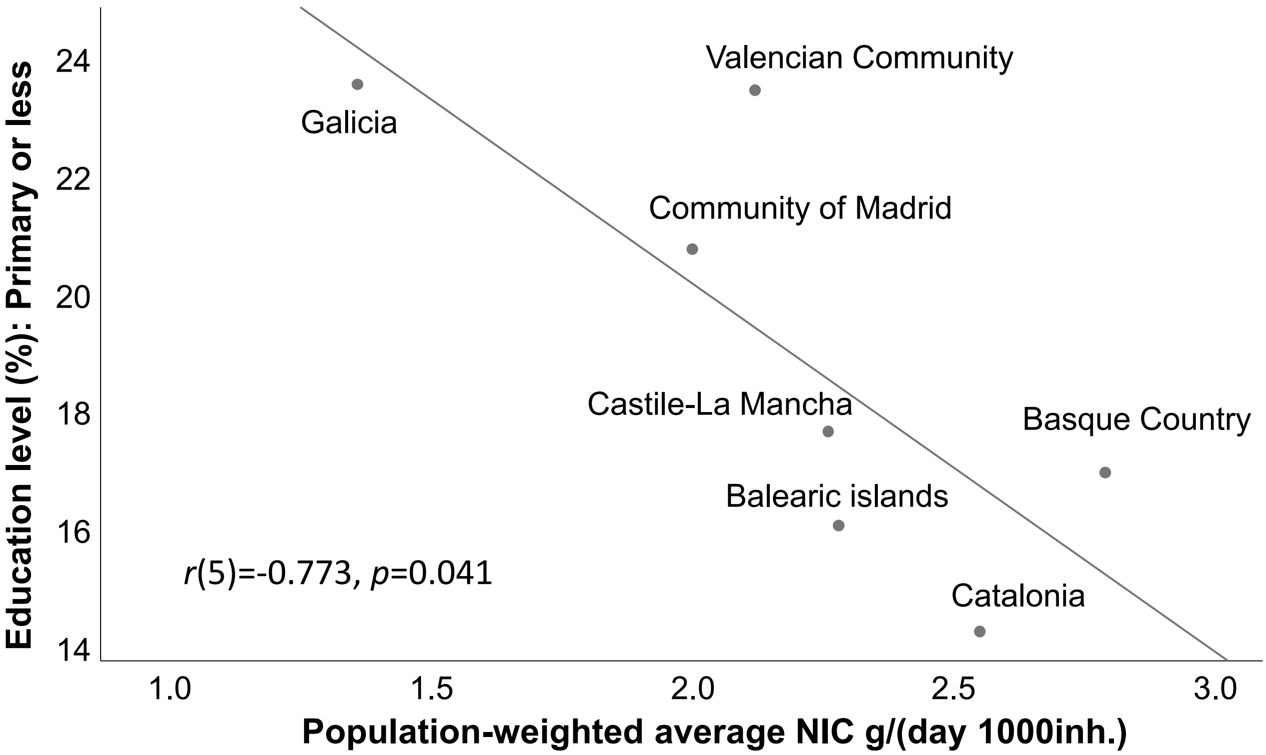


r(5) =0.325, p=0.477
a
Valencian Community
Castile-La Mancha
Basque Country
Community of Madrid
Balearic islands
Catalonia
Galicia
Prevalence (%) daily consumption, EDADES
30
32
34
36
38
40
1.0
1.5
2.0
2.5
3.0
Population -weighted average NIC g/(day 1000inh.)



r(5)=0.388, p=0.399
b
Valencian Community
Balearic islands
Castile-La Mancha
Catalonia
Basque Country
Community of Madrid
Galicia
Prevalence (%) daily consumption, ENSE
16
18
20
22
24
26
1.0
1.5
2.0
2.5
3.0
Population -weighted average NIC g/(day 1000inh.)

Figure 3

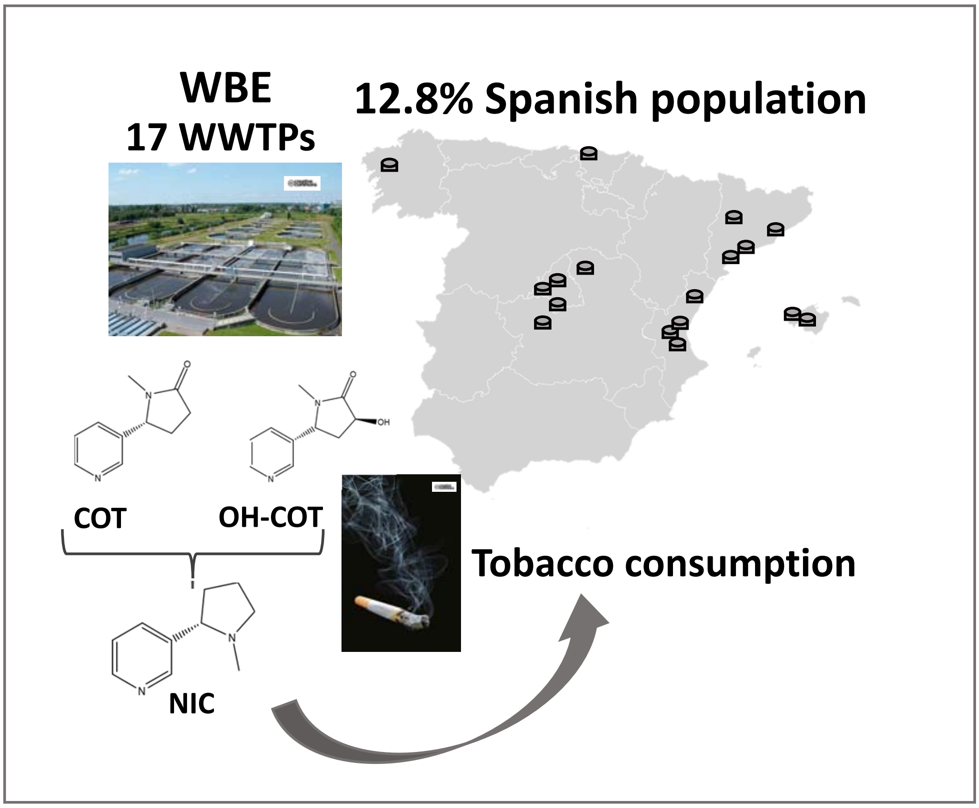


Education level (%): Primary or less
24
22
20
18
16
14
Galicia
Valencian Community
Community of Madrid
Castile-La Mancha
Basque Country
Balearic islands
Catalonia
r(5)=-0.773, p=0.041
1.0
1.5
2.0
2.5
3.0
Population-weighted average NIC g/(day 1000inh.)

Highlights:

- Nicotine metabolites measured in 17 wastewater treatment plants (WWTPs)
- The nicotine consumed was estimated through wastewater-based epidemiology (WBE)
- The mean per capita nicotine consumed daily was 2.2 mg (ca. 2.8 cigarettes)
- Largest Spanish study on nicotine by WBE
- The consumption estimates through WBE are consistent with official sales data

TOC



WBE
17 WWTPs
12.8% Spanish population
COT
OH-COT
NIC
Tobacco consumption

Supplementary electronic material to:

First nation-wide estimation of tobacco consumption in Spain using wastewater-based epidemiology

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Figure S1: Scatter plot that shows the relationship between COT and OH-COT concentrations ($r(110)=0.961$, $p<0.001$)

Figure S2: Overall NIC daily loads by day of the week.

Figure S3: Representation of prevalence data from EDADES *vs* ENSE surveys ($r(5)=0.673$, $p>0.05$)

Table S1: WWTP details and sampling conditions

Table S2: LC-MS/MS instrumental parameters

Table S3: Average measured concentration levels of COT and OH-COT by WWTP

Table S4: Daily NIC consumption estimated in each WWTP.

Table S5: Table S5: Significance (p) of the HSD Tukey multiple (pair-wise) comparisons for NIC loads g/(day 1000 inh) in each WWTP.

Table S6: Results of K-S normality test for data on socio-economic [15] and health condition [33].

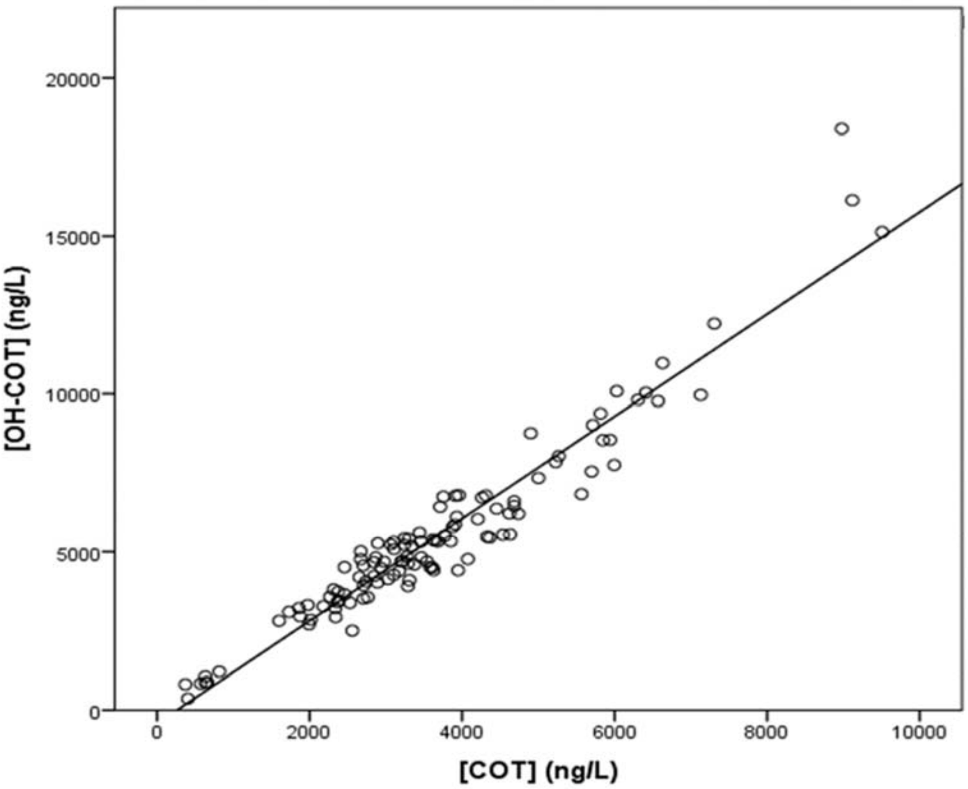

Figure S1: Scatter plot that shows the relationship between COT and OH-COT concentrations (*r*(110)=0.961, *p*<0.001)

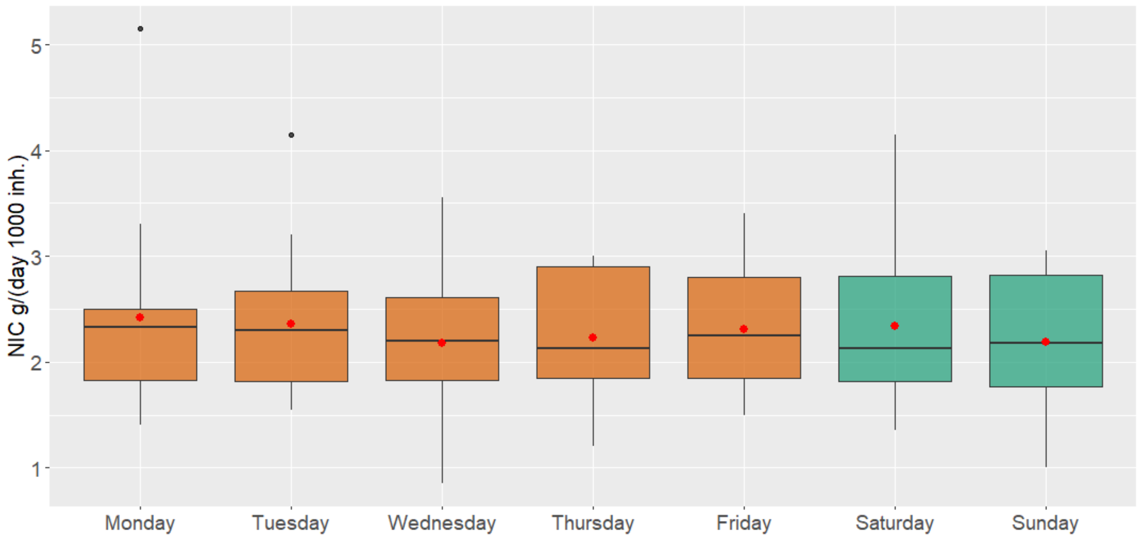

Figure S2: Overall NIC daily loads by day of the week.

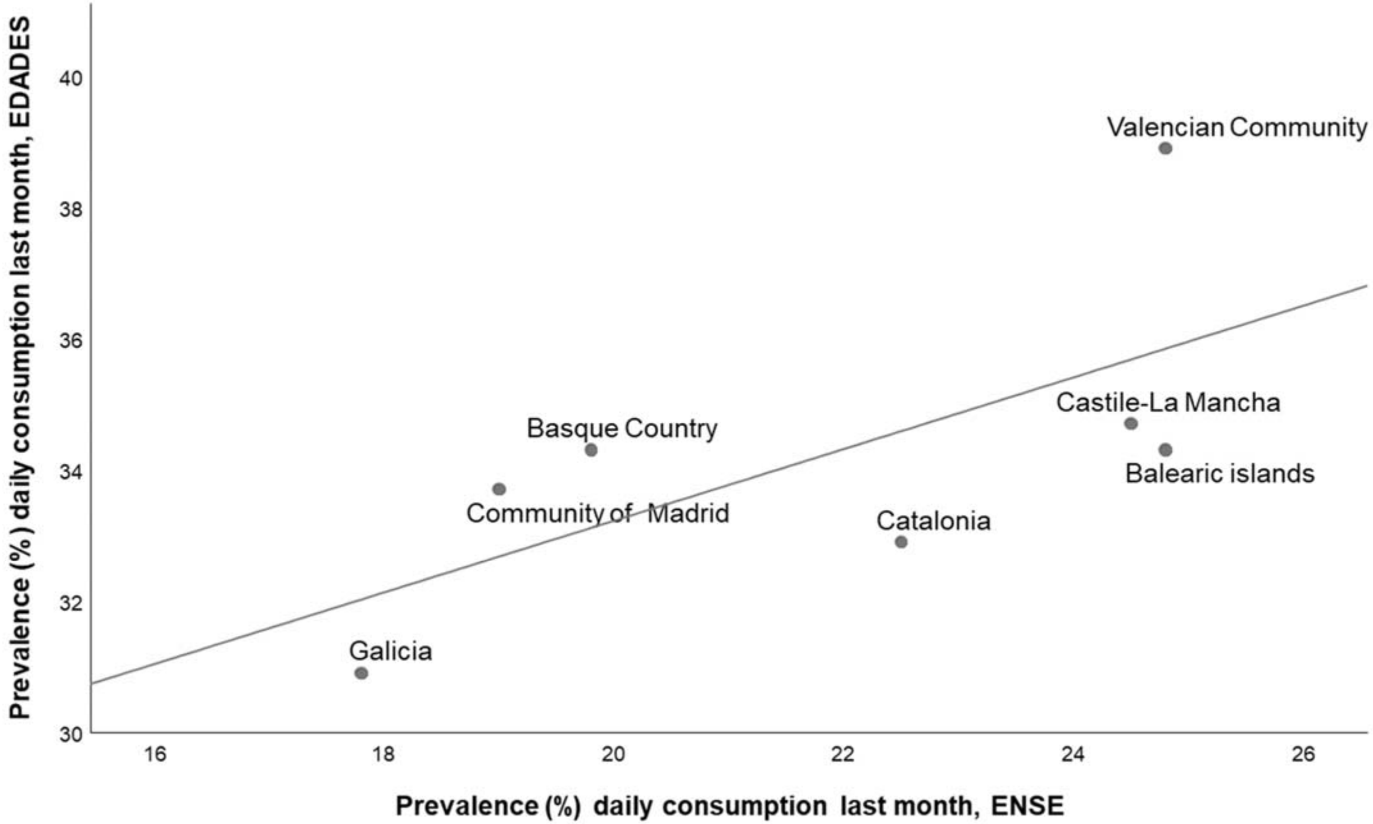

Figure S3: Representation of prevalence data from EDADES *vs* ENSE surveys ($r(5)=0.673$, $p>0.05$).

Table S1: WWTP details and sampling conditions

WWTP code	Region	Population served by the WWTP (inh.)	Locations/districts served by the WWTP [(1)]	% of main city population covered by WWTP(s) [(2)]	Method used to estimate the population served [(3)]	Location of autosampler	Autosampler refrigeration	Time of beginning of the sampling	Sampling mode [(4)]	Sampling period	Average. Flow (m^3/day)[(5)]
Santiago de Compostela	**Galicia**	136500	**Santiago de Compostela**	100%	H x 2.5	After fine screen	No	9:00	T (150 mL/10 min)	13/03/2018 - 19/03/2018	106627
Bilbao	**Basque Country**	860237	**Bilbao**, Abanto-Zierbena, Alonsotegi, Arrigorriaga, Barakaldo, Barrika, Basauri, Berango, Derio, Erandio, Etxebarri, Galdakao, Getxo, Leioa, Lezama, Loiu, Ortuella, Portugalete, Santurtzi, Sestao, Sondika, Sopelana, Trapagaran, Ugao-Miravalles, Urduliz,Zamudio, Zaratamo, Zeberio	100%	C (2016)	After pretreatment	No	8:00	T (100 mL/60 min)	17/04/2018 - 23/04/2018	263818

WWTP code	Region	Population served by the WWTP (inh.)	Locations/districts served by the WWTP (1)	% of main city population covered by WWTP(s) (2)	Method used to estimate the population served (3)	Location of autosampler	Autosampler refrigeration	Time of beginning of the sampling	Sampling mode (4)	Sampling period	Average. Flow (m^3/day)(5)
Madrid I	**Community of Madrid**	727176	**Madrid** (Districts: Chamartín, Tetuán, Moncloa-Aravaca, Chamberí, Centro, Arganzuela, Retiro, Ciudad Lineal, Salamanca, Moratalaz, Puente de Vallecas)	30%	COD	After sieving	Yes	8:00	T (400 mL/30 min)	16/05/2018-22/05/2018	108901
Madrid II		227869	**Madrid** (Districts: Chamartín, Tetuán, Moncloa-Aravaca, Fuencarral-El Pardo, Pozuelo de Alarcón, Las Rozas, Majadahonda)		BOD	After fine screen	Yes	8:00	T (100 mL/60 min)	20/06/2018-26/06/2018	43563
Móstoles		187281	**Móstoles**, Alcorcón, Fuenlabrada (all served also by other WWTPs)	90%	H x 3.5	After fine screen	Yes	8:00	T (100 mL/60 min)	17/05/2018-23/05/2018	26891

WWTP code	Region	Population served by the WWTP (inh.)	Locations/districts served by the WWTP (1)	% of main city population covered by WWTP(s) (2)	Method used to estimate the population served (3)	Location of autosampler	Autosampler refrigeration	Time of beginning of the sampling	Sampling mode (4)	Sampling period	Average. Flow (m^3/day)(5)
Guadalajara	**Castile-La Mancha**	94755	**Guadalajara**	100%	BOD	Before fine screen	No	10:00	T (200 mL/60 min)	02/05/2018-08/05/2018	29490
Toledo		79793	**Toledo**	100%	BOD	After sieving	No	8:00	T (100 mL/15 min)	17/04/2018-23/04/2018	14017
Barcelona	**Catalonia**	1163154	**Barcelona**, Cervelló, Cornellà de Llobregat, Esplugues de Llobregat, Hospitalet de Llobregat, El Prat de Llobregat, Sant Boi de Llobregat, San Joan Despí, San Just Desvern	35%	C (2017)	Mechanical bar screens	Yes	9:00	T (50 mL/10 min)	14/03/2018-20/03/2018	270672
Lleida		143612	**Lleida**, Alpicat	100%	C (2017)	Before fine screen	No	6:00	T (200 mL/60 min)	07/03/2018-13/03/2018	42264
Reus		115000	**Reus,** Castellvell, Almoster	100%	C (2017)	After fine screen	No	20:00	F	17/04/2018-23/04/2018	17217
Tarragona		142635	**Tarragona**, La Canonja, els Pallaresos	100%	C (2017)	Before fine screen	No	8:00-9:00	T (450 mL/60 min)	17/04/2018-23/04/2018	23985

WWTP code	Region	Population served by the WWTP (inh.)	Locations/districts served by the WWTP [(1)]	% of main city population covered by WWTP(s) [(2)]	Method used to estimate the population served [(3)]	Location of autosampler	Autosampler refrigeration	Time of beginning of the sampling	Sampling mode [(4)]	Sampling period	Average. Flow (m^3/day)[(5)]
Valencia I	**Valencian Community**	527222	**Valencia**	100%	COD	After fine screen	Yes	8:00	T (100 mL/60 min)	10/04/2018-16/04/2018	124587
Valencia II		788242	**Valencia**, Albal, Alcasser, Alfafar, Benetusser, Beniparrell, Burjassot, Catarroja, Llocnou de la Corona, Massanassa, Mislata, Paiporta, Paterna, Picanya, Picassent, Sedaví, Silla, Torrent		COD	After fine screen	Yes	8:00	T (100 mL/60 min)	10/04/2018-16/04/2018	204014
Valencia III		162249	**Valencia** metropolitan area: Alaquàs, Aldaia, Manises, Mislata, Quart de Poblet, Xirivella		COD	After fine screen	No	8:00	F	10/04/2018-16/04/2018	29593
Castellón		171669	**Castellón De La Plana**	100%	C (2015)	Before fine screen	No	8:30	T (100 mL/15 min)	11/04/2018-17/04/2018	34285
Palma de Mallorca I	**Balearic Islands**	406492	**Palma city**, Palma beach, Sant Jordi, El Pil·lari, Son Sant Joan airport	100%	C (2017)	After fine screen	No	10:00	T (100 mL/15 min)	10/04/2018-16/04/2018	45902
Palma de Mallorca II		47961	**Palma city**, Son Castelló, Can Valero and Son Rosinyol		C (2017)	After fine screen	No	10:00	T (100 mL/15 min)	18/04/2018-24/04/2018	49292

WWTP code	Region	Population served by the WWTP (inh.)	Locations/districts served by the WWTP [(1)]	% of main city population covered by WWTP(s) [(2)]	Method used to estimate the population served [(3)]	Location of autosampler	Autosampler refrigeration	Time of beginning of the sampling	Sampling mode [(4)]	Sampling period	Average. Flow (m^3/day)[(5)]
			Industrial States, Marratxí, Esporles and Bunyola								

[1] Name of the main city served by the WWTP marked in bold (some WWTPs receive wastewater from other towns included in the capital metropolitan area)

[2] Estimated with the total population at 1/01/2018. Source: National Institute of Statistics (INE). WWTPs serving parts of the same main city were considered together

[3] C: census (year); BOD: biochemical oxygen demand; COD: chemical oxygen demand; H: number of homes connected to the sewage system

[4] T: time proportional (volume sampled/frequency of sampling); F: flow proportional

[5] Average week flow. For back-calculations daily flows were used.

Table S2: LC-MS/MS instrumental parameters

Compound	MRM transition	Transition type	Capillary voltage (V)	Collision Energy (V)	Transitions ratio
COT	177 < 80	Quantification	60	19	3.4 ± 0.9
	177 < 98	Qualification	60	15.5	
COT-d3	180 <80	Quantification	60	19	3.4 ± 0.9
	180 <101	Qualification	60	15.5	
OH–COT	193 <80	Quantification	56	21.5	1.4 ± 0.3
	193 <134	Qualification	56	13.5	
OH–COT-d3	196 <80	Quantification	56	21.5	1.4 ± 0.3
	196 <137	Qualification	56	13.5	

Nebulizing (55 psi, 50ºC) and drying (18 psi, 200ºC) gas: N_2. Ion spray voltage: 4500 V. Collision gas: Ar

Table S3: Average measured concentration levels of COT and OH-COT by WWTP

	[COT] ng/L		[OH-COT] ng/L	
WWTP	Mean	SD	Mean	SD
Santiago de Compostela	584	154	860	262
Bilbao	3084	559	4265	587
Madrid (I)	3828	592	5814	1020
Madrid (II)	3954	844	5519	948
Móstoles	7711	1453	13283	3289
Guadalajara	2819	366	4221	664
Toledo	3149	271	4968	516
Barcelona	3675	699	5654	1099
Lleida	2110	229	3214	341
Reus	6011	584	8928	856
Tarragona	5104	1096	7288	1612
Valencia (I)	2380	546	3710	952
Valencia (II)	2985	543	4209	692
Valencia (III)	3672	634	5295	821
Castellón	4434	665	6457	1298
Palma de Mallorca	2987	591	4189	886

Table S4: Daily NIC consumption estimated in each WWTP

NIC g/(day 1000inh.)			
WWTP	Mean	SD	*HSD Tukey post-Hoc* [(1)]
Santiago de Compostela	1.4	0.3	a
Bilbao	2.8	0.4	e
Madrid (I)	1.8	0.3	a,b
Madrid (II)	1.4	0.3	a
Móstoles	3.7	0.8	f
Guadalajara	2.7	0.3	e
Toledo	1.8	0.1	a,b
Barcelona	2.6	0.2	c,d,e
Lleida	2.0	0.2	a,b,c
Reus	2.8	0.2	e
Tarragona	2.6	0.5	c,d,e
Valencia (I)	1.8	0.3	a,b
Valencia (II)	2.3	0.5	b,c,d,e
Valencia (III)	2.0	0.3	a,b,c,d
Castellón	2.7	0.3	d,e
Palma de Mallorca	2.3	0.3	b,c,d,e

[(1)] Same letter codifies homogeneous groups.

Table S5: Significance (p) of the HSD Tukey multiple (pair-wise) comparisons for NIC loads g/(day 1000 inh) in each WWTP.

	Santiago de Compostela	Bilbao	Madrid (I)	Madrid (II)	Móstoles	Guadalajara	Toledo	Barcelona	Lleida	Reus	Tarragona	Valencia (I)	Valencia (II)	Valencia (III)	Castellón	Palma de Mallorca
Santiago de Compostela		**2.9E-08**	7.9E-01	1.0E+00	**2.3E-12**	**1.8E-07**	9.0E-01	**2.0E-06**	2.7E-01	**4.0E-08**	**2.3E-06**	8.5E-01	**2.3E-03**	1.3E-01	**4.7E-07**	**2.3E-03**
Bilbao	**2.9E-08**		**2.5E-04**	**1.7E-08**	**3.3E-03**	1.0E+00	**1.1E-04**	1.0E+00	**4.8E-03**	1.0E+00	1.0E+00	**1.7E-04**	4.0E-01	**1.4E-02**	1.0E+00	4.0E-01
Madrid (I)	7.9E-01	**2.5E-04**		7.2E-01	**2.6E-12**	**1.2E-03**	1.0E+00	**7.9E-03**	1.0E+00	**3.4E-04**	**8.9E-03**	1.0E+00	5.5E-01	1.0E+00	**2.6E-03**	5.5E-01
Madrid (II)	1.0E+00	**1.7E-08**	7.2E-01		**2.3E-12**	**1.1E-07**	8.5E-01	**1.2E-06**	2.2E-01	**2.4E-08**	**1.5E-06**	7.9E-01	**1.5E-03**	1.0E-01	**2.9E-07**	**1.5E-03**
Móstoles	**2.3E-12**	**3.3E-03**	**2.6E-12**	**2.3E-12**		**7.7E-04**	**2.4E-12**	**9.2E-05**	**2.0E-11**	**2.6E-03**	**8.0E-05**	**2.5E-12**	**4.7E-08**	**8.9E-11**	**3.4E-04**	**4.7E-08**
Guadalajara	**1.8E-07**	1.0E+00	**1.2E-03**	**1.1E-07**	**7.7E-04**		**5.1E-04**	1.0E+00	**1.8E-02**	1.0E+00	1.0E+00	**7.7E-04**	6.8E-01	**4.7E-02**	1.0E+00	6.8E-01
Toledo	9.0E-01	**1.1E-04**	1.0E+00	8.5E-01	**2.4E-12**	**5.1E-04**		**3.8E-03**	1.0E+00	**1.4E-04**	**4.3E-03**	1.0E+00	4.0E-01	1.0E+00	**1.2E-03**	4.0E-01
Barcelona	**2.0E-06**	1.0E+00	**7.9E-03**	**1.2E-06**	**9.2E-05**	1.0E+00	**3.8E-03**		8.5E-02	1.0E+00	1.0E+00	**5.5E-03**	9.4E-01	1.9E-01	1.0E+00	9.4E-01
Lleida	2.7E-01	**4.8E-03**	1.0E+00	2.2E-01	**2.0E-11**	**1.8E-02**	1.0E+00	8.5E-02		**6.2E-03**	9.3E-02	1.0E+00	9.6E-01	1.0E+00	**3.5E-02**	9.6E-01
Reus	**4.0E-08**	1.0E+00	**3.4E-04**	**2.4E-08**	**2.6E-03**	1.0E+00	**1.4E-04**	1.0E+00	**6.2E-03**		1.0E+00	**2.2E-04**	4.5E-01	**1.8E-02**	1.0E+00	4.5E-01
Tarragona	**2.3E-06**	1.0E+00	**8.9E-03**	**1.5E-06**	**8.0E-05**	1.0E+00	**4.3E-03**	1.0E+00	9.3E-02	1.0E+00		**6.2E-03**	9.5E-01	2.0E-01	1.0E+00	9.5E-01
Valencia (I)	8.5E-01	**1.7E-04**	1.0E+00	7.9E-01	**2.5E-12**	**7.7E-04**	1.0E+00	**5.5E-03**	1.0E+00	**2.2E-04**	**6.2E-03**		4.7E-01	1.0E+00	**1.7E-03**	4.7E-01
Valencia (II)	**2.3E-03**	4.0E-01	5.5E-01	**1.5E-03**	**4.7E-08**	6.8E-01	4.0E-01	9.4E-01	9.6E-01	4.5E-01	9.5E-01	4.7E-01		1.0E+00	8.1E-01	1.0E+00
Valencia (III)	1.3E-01	**1.4E-02**	1.0E+00	1.0E-01	**8.9E-11**	**4.7E-02**	1.0E+00	1.9E-01	1.0E+00	**1.8E-02**	2.0E-01	1.0E+00	1.0E+00		8.5E-02	1.0E+00
Castellón	**4.7E-07**	1.0E+00	**2.6E-03**	**2.9E-07**	**3.4E-04**	1.0E+00	**1.2E-03**	1.0E+00	**3.5E-02**	1.0E+00	1.0E+00	**1.7E-03**	8.1E-01	8.5E-02		8.1E-01
Palma de Mallorca	**2.3E-03**	4.0E-01	5.5E-01	**1.5E-03**	**4.7E-08**	6.8E-01	4.0E-01	9.4E-01	9.6E-01	4.5E-01	9.5E-01	4.7E-01	1.0E+00	1.0E+00	8.1E-01	

Significant differences at 95% confidence level marked in bold

Table S5: Results of K-S normality test for data on socio-economic [15] and health condition [33].

	N	D	P[1]
Death rate(%) year 2018	7	0.166	0.200
Unemployment rate (%) year 2018	7	0.210	0.200
Education level (%) Primary or less	7	0.200	0.200
Education level (%) Secondary	7	0.189	0.200
Education level (%) University	7	0.205	0.200
Mean age (years) 2018	7	0.275	0.118
Average economic family income (€/year) 2018	7	0.282	0.098
Hospital admissions any diag."nicotine addiction" year 2017 (nº/million inh.)	7	0.166	0.200
Hospital admissions any diag."heart stroke" year 2017 (nº/million inh.)	7	0.250	0.200
Hospital admissions any diag."lung cancer" year 2017 (nº/million inh.)	7	0.233	0.200
Hospital admissions any diag."oral cancer" year 2017 (nº/million inh.)	7	0.257	0.178
Hospital admissions any diag."bronchitis" year 2017 (nº/million inh.)	7	0.352	0.009
Hospital admissions any diag."COPD" year 2017 (nº/million inh.)	7	0.181	0.200

[1]Lilliefors significance correction