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Trends in characteristics of 24-h urine samples and their relevance for human biomonitoring studies – 20 years of experience in the German Environmental Specimen Bank

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ABSTRACT

To document trends in human exposure to environmental pollutants, the German Environmental Specimen Bank (ESB) has been routinely collecting and archiving 24-h urine samples from young adults at four sampling sites in Germany on an annual basis. For the purpose of normalizing measured analyte concentrations, urinary creatinine (UC), specific gravity (SG), conductivity (CON), and total urine volume (UV_{tot}) of 24-h urine samples have also been recorded. These parameters are however susceptible to variation over time, as well as within/among participants and normalization against them can thus affect the interpretation of data regarding exposure to environmental pollutants. To evaluate the influence of normalization against these parameters, we first sought to determine variations of these parameters with regard to differences between sexes and trends over time. We analysed data from 8619 urine samples collected from 1997 to 2016. We observed an inverse relation between UV_{tot} and UC, SG, and CON. We also found differences between sexes for UC, SG and CON, but not UV_{tot}. UC, SG, and CON showed significant decreasing trends over time in both sexes. In contrast, a significant increase of over 30% in UV_{tot}, independent of participant age and BMI, was revealed. This increase in UV_{tot} and the concomitant sample dilution is likely to have an impact on measured analyte concentrations in 24-h urine samples. Hence, normalization of urinary concentrations is warranted when interpreting time trends of human exposure. Next, urinary calcium (Ca^{2+}) concentrations of ESB participants were used to demonstrate the effects of normalization against each of the four urine parameters. From 1997 to 2016, measured Ca^{2+} concentrations showed a statistically significant but scientifically implausible decrease. Normalization of Ca^{2+} concentrations against UV_{tot} (by calculating the total daily excretion), UC, or CON, but not SG, eliminated this decrease. Consistent with previous work, Ca^{2+} concentrations in urine and total daily Ca^{2+} excretion were higher for males than females. Normalization against UC, SG, or CON, however, attenuated this difference. Thus, to avoid misinterpretation in trend analysis and sex-specific excretion in 24-h urine samples, the calculation of the total daily excretion is recommended.

1. Introduction

The German Environmental Specimen Bank (ESB) was officially launched in 1985 by the German Federal Government to document trends in exposure to environmental pollutants using human and environmental samples. It is financed by the Federal Ministry for the Environment, Nature Conservation and Nuclear Safety (BMU) and administratively and scientifically coordinated by the German Environment Agency (UBA). The German ESB combined with the German Environmental Survey (GerES) builds the cornerstone of human biomonitoring (HBM) at the federal level in Germany (Kolossa-Gehring et al., 2012a, b). Regarding the HBM activities of the German ESB, sampling takes place at four different sampling sites annually. Sampling began in 1981 at the sampling site Muenster (North Rhine-Westphalia) and was expanded to the sites Greifswald (Mecklenburg West-Pomerania, since 1992), Halle/Saale (Saxony-Anhalt, since 1995), and Ulm (Baden-Württemberg, since 1997). Although a broad variety of human samples were collected within the first years (e. g., scalp and

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pubic hair, saliva, and breast milk) a set of three matrices, which are readily accessible and considered to provide sound HBM data, was set as standard. These matrices are whole blood, blood plasma, and 24-h urine, and they have been collected continuously since sampling began in 1981. The concept of the German ESB aims at collecting samples from a minimum of 120 young adults per year and sampling site, aged 20 to 29 with a balanced sex ratio. The main HBM-related goal of the German ESB is monitoring of trends in exposure over time (Wiesmüller et al., 2007; Kolossa-Gehring et al., 2012a; Lermen et al., 2014). Samples have been routinely stored under cryogenic conditions to minimize chemical, biological, and physical alterations. To be able to investigate the relevance of matrix-specific properties for the interpretation of HBM data, several clinical, chemical, and physical parameters of the different matrices have also been measured routinely.

24-h urine is considered the gold standard to investigate total daily exposures. Compared to spot or morning urine samples, 24-h urine samples can provide a more complete picture of the daily pollutant excretion (Scher et al., 2007). However, collecting 24-h urine samples is often considered to be time-consuming and cumbersome (Johansson et al., 1998; Barr et al., 2005; Suwazono et al., 2005; Ye et al., 2011). Furthermore, errors might occur due to under- or oversampling. The correct sampling of 24-h urine and defined pre-analytical processes that guarantee a complete 24-h urine sample are important for sound interpretation of analytical data and correct risk assessment. To avoid misinterpretation of analytical data due to incorrect 24-h urine samples, it is widely accepted to adjust analyte concentration using urinary creatinine (UC), and report the concentration as analyte quantity per gram creatinine. In this regard, creatinine is assumed to be excreted from the body at a fairly constant rate (Seifert et al., 2000; Becker et al., 2003; Calafat, 2005; Li et al., 2015; Wang et al., 2016; Urbancova et al., 2017). Other parameters commonly used to adjust analyte concentrations in 24-h urine samples include specific gravity (SG), conductivity (CON), osmolality and total volume of the 24-h urine sample (UV_{tot}) (Haddow et al., 1994; Berglund et al., 2011; Weaver et al., 2014). Single studies indicate that several urine parameters may change over longer periods of time, which can in turn affect observed HBM concentration trends (Koch et al., 2012). The interpretation of normalized analyte concentrations using UC, SG, CON, or UV_{tot} can also be confounded by other factors such as sex, ethnicity, diet, exercise, age, BMI, and kidney function (Barr et al., 2005; Suwazono et al., 2005). Analyte concentrations in urine of ESB participants are used to evaluate changes in human internal chemical exposure and support informed policy making. Thus, misinterpretation due to a lack of, or inappropriate normalization can lead to flawed environmental health policy decisions and ultimately to inefficient regulations.

UC, SG, CON, and UV_{tot} was measured routinely by the German ESB in 24-h urine samples for each participant. For UC, SG, and UV_{tot}, data from all four sampling sites is available from 1997. Data on CON is available from 2001. This data set spanning 20 years offers the unique opportunity to investigate the effect of normalizing against these parameters and to discuss their relevance for human biomonitoring studies.

2. Materials and methods

2.1. Ethical statement

The study protocol of the German ESB was reviewed and approved in 2011 by the ethics committee of the Medical Association Saarland, Germany. Before 2011, the study protocol has been approved by the ethics committee of the Medical Association of Westphalia-Lippe and the Medical Faculty of the University of Muenster, Germany. All study participants gave written informed consent. If requested by the participants, participants received their results after the analysis of the samples was completed.

2.2. Recruitment and sampling of 24-h urine

Recruitment of participants and sampling of 24-h urine is described in detail elsewhere (Eckard et al., 2011; Lermen et al. 2014, 2015a; Schröter-Kermani et al., 2016). Briefly, each year young adults considered healthy have been recruited by promoting the study at the medical faculties of the universities and by sending e-mails to all students of the medical faculties at all four sampling sites. 120 participants per year and site with a balanced sex ratio were set as a target, equalling 480 participants per year. In the earlier years, this target was not always met. The lowest sample size was 339 participants (~70% of the target sample size) in 1998. Over the presented time-frame, the sample sizes increased gradually and since 2011 the target sample sizes have approximately been met every year(Supplementary Table 1). After registration, students receive a parcel that contains information material, a declaration of consent, instructions on how to collect the 24-h urine, and a urine-sampling container. Urine samples are then processed for analysis using on-site cryo-preservation (Wiesmüller et al., 2007; Lermen et al., 2015a).

2.3. Measurements and analysis

From 1997 to 2016, a total of 8902 24-h urine samples from participants aged 20–29 were collected. The 24-h urine samples are deemed complete based on the participants' statement to have adhered to the German ESB's collection guidelines. However, since data on the precise collection time of the 24-h urine samples is not documented for this study, the validity of the 24-h urine samples cannot be guaranteed. Without a precise collection time, a correction according to the creatinine excretion rate is not applicable. According to current literature, mean voiding frequencies of healthy adults vary between 5.8 and 8.3 times in 24 h (Homma et al., 2000; Blanker et al., 2001; Fitzgerald et al., 2002; Latini et al., 2004; Van Haarst et al., 2004; Amundsen et al., 2007; Tissot et al., 2008). Furthermore, Tissot et al., (2008) reported a mean minimum void volume in healthy males of 113 mL. In healthy females a mean minimum void volume of 81 mL is reported by Amundsen et al., (2007). Assuming a mean voiding frequency of 7 times within 24 h based on data from literature and considering the reported minimum void volumes, 24-h urine samples of less than 700 mL may not have been collected correctly, and the fact that participants providing such samples suffer from oliguria cannot be excluded. An upper limit for the volume of a 24-h urine sample depends on the dietary liquid uptake among other factors. Unfortunately, dietary liquid uptake for the specific time period in which the 24-h urine samples were collected is not available as control. A daily urine volume above 4000 mL might however indicate renal dysfunction (De Leiva Hidalgo et al., 2009; D'angelo et al., 2010; Özkan et al., 2010). Hence, 24-h urine samples in this study with volumes above 4000 mL may not have been collected correctly, and we cannot exclude that participants providing such volumes suffer from polyuria. Thus, to avoid or at least minimize misinterpretation, samples with an UV_{tot} below 700 mL or above 4000 mL were excluded from statistical analysis. From the 8902 24-h urine samples collected between 1997 and 2016, 8619 were considered valid 24-h urine samples. 283 samples were excluded from this study due to over- or undersampling or missing physical parameters.

Physical parameters as well as UC in urine have been measured according to the respective standard operating procedures (SOPs) of the German ESB and are described in detail elsewhere (Eckard et al., 2011; Lermen et al., 2015a). In brief, UV_{tot} has been measured using an electronic balance (Mettler Toledo, 11124926), SG has been measured with an aerometer (Assisstent, 60008), and CON has been recorded since 2001 using a conductivity meter (Mettler Toledo, 51302936). From 1997 until 2010, UC was measured by UKM photometrically according to Jaffe (Boeringer Mannheim, MPR3 Creatinin testkit, 124 192). In 2010, 2011, UC was analysed by the central laboratory for clinical chemistry of UKM. Since 2012, urine samples have been

analysed for UC by Fraunhofer IBMT using a colorimetric determination based on the Jaffe reaction using a cobas c111 analyser (Roche, 04 777 433 001).

Until 2010, calcium (Ca^{2+}) concentrations in 24-h urine samples were measured by the University Hospital Muenster (Germany) using inductively coupled plasma emission spectroscopy (ICP-OES, LOQ = 6.5 µg/L). From 2010 on, Ca^{2+} in 24-h urine samples has been measured by the Institute and Outpatient Clinic of Occupational, Social and Environmental Medicine, Friedrich-Alexander-University Erlangen-Nuernberg (Germany) using inductively coupled plasma mass spectrometry (ICP-MS, LOQ = 1.0 µg/L).

Total daily Ca^{2+} excretion (TDE Ca^{2+}) in mg/24 h was calculated as the product of Ca^{2+} concentration and UV_{tot} divided by 1000. Urinary Ca^{2+} concentration normalised by urinary creatinine concentration (Ca^{2+}PerUC) in mg Ca^{2+} per mg creatinine was calculated as the ratio between Ca^{2+} and UC divided by 10. Ca^{2+} concentration normalised by the sample's specific gravity (Ca^{2+}PerSG) was calculated as $Ca^{2+} \times (SG_{ref} - 1)/(SG-1)$, SG_{ref} = 1.015 g/mL, as proposed by (Sauvé et al., 2015). Ca^{2+} concentration normalised by the sample's conductivity (Ca^{2+}PerCON) was calculated as the ratio between Ca^{2+} and CON.

All analytical methods were based on respective standard operation procedures of the German Environmental Specimen Bank (Umweltbundesamt, 1996).

Quality assurance was improved over time considering internal and external control schemes. Quality control for Ca^{2+} analysis has been conducted according to external quality assessments schemes (G-EQUAS) explained in detail elsewhere (Schaller et al. 2001, 2002; Wiesmüller et al., 2007; Göen et al., 2012; Lermen et al., 2014). Since 2014 quality control for analysis of urine parameters has been conducted according to the DIN EN ISO 9001 and DIN EN ISO/IEC 17025 standards additionally (Lermen et al. 2014, 2015b).

2.4. Statistical analysis

Statistical analysis was carried out using R Version 3.2.3. P values < 0.05 were considered statistically significant. All measured parameters were visually inspected (using histograms and QQ-plots) to follow approximately normal distributions. As the visual inspection indicated better conformity with a normal distribution after log-transformation, UC, SG, CON, and UV_{tot} parameter values were transformed with the natural logarithm. Due to lacking variance homogeneity, statistical significance of differences between sexes in UC, SG, CON, and UV_{tot} after log-transformation was evaluated using Welch-corrected t-tests. Trends in urine parameters over time were evaluated separately for male and female participants assuming a linear relationship between yearly geometric means of non-transformed parameter values and sampling year. For ease of interpretation, all figures depict non-transformed parameter values. Spearman's rank correlation coefficients were determined to evaluate relationships between individual non-transformed urine parameters. Persistence of the correlation between individual urine parameters after adjustment for sampling year was assessed using partial Spearman correlation adjusted for sampling year calculated using the function “pcor.test” from the R-package “ppcor” v1.1.

The hypothesis of a dependency of UC, SG, and CON on UV_{tot} was evaluated using three separate linear regression models. The dependency of UV_{tot} on sampling year was modelled with a fourth linear regression analysis. Preliminary analysis of the data with linear regression models revealed heteroscedastic residuals (Breusch-Pagan test). In consequence, linear regression with heteroscedasticity-consistent covariance estimator (HC3) was used (functions “coeftest” from package “lmtest” v0.9 and “vcovHC” from package “sandwich” v2.4). Potential confounding influences of participant BMI, age, and sex as well as sampling site were adjusted for by including corresponding terms into each regression model. Linearity of association between the dependent and each interval-scaled independent variable was visually evaluated using partial-regression plots (function “avPlots” from package “car” v3.0). Independent variables were transformed with the natural logarithm in case it improved the linearity of association with the dependent variable. Collinearity of explanatory variables was assessed using variance inflation factors (VIF) and determined to be uncritical in all regression models (VIF < 2). Significance of explanatory variables in the four regression models was expressed as a p-value and corrected for multiple testing using the Bonferroni correction.

In order to account for the presence of non-linear trends in the normalized Ca^{2+} data, a Mann-Kendall test was applied to geometric mean values per year separately for male and female participants. Steepness of the trends was evaluated with Sen's slope method (Sen, 1968). Trend tests and slope calculations were carried out using the functions “mk.test” and “sens.slope” from the R package “trend” version 1.0.1. Due to lacking variance homogeneity, statistical significance of differences between sexes in normalized and non-normalized Ca^{2+} concentrations was evaluated per individual sampling year using Welch-corrected t-tests applied after log-transformation of measurements.

3. Results

3.1. Sex-specific differences

Table 1 gives an overview of selected characteristics of young adults from all four sampling sites of the German ESB that participated in the sampling in 2016. Shown are median (MD), arithmetic mean (AM), geometric mean (GM), and the range for age, BMI, UC, SG, CON, UV_{tot}, and Ca^{2+} of the 24-h urine samples. With regard to all investigated participants, from 1997 to 2016, for UC, SG, and CON significant sex specific differences are revealed. Generally, male participants show higher values than female participants in these three parameters. Interestingly, regarding all investigated participants from 1997 to 2016 no sex specific differences are identified in UV_{tot}. These patterns are observed at each individual sampling site (Fig. 1).

3.2. Trends in urine parameters over time

Fig. 2 shows the sex-specific course of geometric mean values of UC, SG, and UV_{tot} of participants at all sampling sites from 1997 to 2016, for CON from 2001 to 2016. The data clearly reveal decreasing trends for UC, SG, and CON with a similar rate of decrease in both sexes. In contrast, UV_{tot} values increased from 1997 to 2016 again with a similar rate in both sexes. A sex-specific trend analysis confirmed that the increase of UV_{tot} in both sexes from 1997 to 2016 is highly significant. In males, mean UV_{tot} increased by 32% from 1532 mL/24 h in 1997–2039 mL/24 h in 2016 and in females by 36% from 1459 mL/24 h in 1997 to 1987 mL/24 h in 2016. The decreasing trends for UC, SG, and CON are also significant in female and male participants.

3.3. Dependency of urine parameters on UV_{tot}

Table 2 shows that all investigated urine parameters are strongly and significantly correlated with each other. Correcting for the present trends over sampling years did not change these strong correlations (data not shown). The most obvious explanation for these observations is a dependence of UC, SG, and CON on UV_{tot} due to dilution effects. Taken together with intrinsic differences between sexes, this dilution effect creates parallel sex-specific decreasing trends in UC, SG and CON. Consistent with this explanation, linear regression models with adjustment for participant BMI, age, and sex as well as sampling site showed a strong dependency of UC, SG, and CON on UV_{tot} (see Table 3). Furthermore, the multivariate regression analysis confirmed that the differences between sexes in UC, SG, and CON as well as the absence thereof in UV_{tot} are persistent when confounding factors are corrected

Table 1
Overview of median (MD), arithmetic mean (AM), geometric mean (GM), 5th and 95th percentiles (5^{th}PE, 95^{th}PE), min and max values for age, BMI, urinary creatinine (UC), specific gravity (SG), conductivity (CON), total urine volume (UVtot), and urinary Ca^{2+} for all participants of the German ESB sampling in 2016.

		MD (95% CI)	AM (± SD)	GM (95% CI)	5^{th} PE	95^{th} PE	Min	Max
All	Age	24 (23.8–24.2)	23.8 (± 2.4)	23.7 (23.5–23.9)	20	28	20	29
n = 494	BMI	22.2 (22.1–22.4)	22.7 (± 3.3)	22.5 (22.2–22.8)	18.7	28.7	16.1	44.1
	UC	64.8 (63.1–66.6)	72.4 (± 35.6)	65 (62.4–67.7)	31.9	141	17.9	224.2
	SG	1.013 (1.0126–1.0134)	1.0135 (± 0.005)	1.0135 (1.013–1.014)	1.007	1.024	1.001	1.031
	CON	12.3 (12–12.6)	13.4 (± 5.5)	12.4 (11.9–12.8)	6.5	24.4	2.4	30.9
	UV_{tot}	2073 (2026–2119)	2035 (± 644)	1922 (1863–1982)	973	2977	721	3875
	Ca^{2+}	76.5 (73.7–79)	90 (± 55.5)	74 (69.8–78.4)	24.4	192.7	7.6	352.3
Female	Age	23 (22.8–23.2)	23.6 (± 2.4)	23.5 (23.2–23.8)	20	28	20	29
n = 246	BMI	21.3 (21.1–21.5)	22 (± 3.6)	21.8 (21.4–22.2)	18.2	28.7	16.1	44.1
	UC	55 (52.9–57.2)	60.8 (± 27.8)	55.2 (52.3–58.4)	26.6	115.4	17.9	163
	SG	1.011 (1.0106–1.0114)	1.0119 (± 0.004)	1.0119 (1.0114–1.0125)	1.0062	1.0198	1.004	1.029
	CON	10.8 (10.4–11.2)	11.7 (± 4.6)	10.9 (10.3–11.4)	6	21	2.4	24.8
	UV_{tot}	2024 (1963–2085)	2001 (± 636)	1890 (1809–1975)	934	2943	721	3875
	Ca^{2+}	66 (62.6–69.4)	76.1 (± 47.8)	62.1 (57.1–67.6)	22	172.2	7.6	245.6
Male	Age	24 (23.8–24.2)	24 (± 2.3)	23.9 (23.6–24.2)	20	28	20	29
n = 248	BMI	23.1 (22.9–23.3)	23.4 (± 2.8)	23.3 (22.9–23.6)	19.6	28.6	17.4	36.4
	UC	75.1 (72.3–77.8)	83.9 (± 38.6)	76.3 (72.3–80.5)	39.9	167.4	23.2	224.2
	SG	1.014 (1.0135–1.0145)	1.0151 (± 0.005)	1.0151 (1.0144–1.0157)	1.008	1.026	1.001	1.031
	CON	13.9 (13.4–14.4)	15.1 (± 5.8)	14 (13.4–14.7)	7.9	26.2	4.8	30.9
	UV_{tot}	2096 (2027–2164)	2068 (± 651)	1953 (1869–2041)	1019	3033	726	3220
	Ca^{2+}	94.2 (89.8–98.5)	103.7 (± 59.1)	87.9 (81.6–94.8)	31.4	220.6	9.8	352.3

Age in years, BMI in kg/m^2, UC in mg/dL, SG in g/mL, CON in mS/cm, UV_{tot} in mL/24 h, Ca^{2+} in mg/L, CI = Confidence Interval, TDE = Total Daily Excretion.

for. The regression models adjust for significant positive association of BMI with UC, SG, and CON. UV_{tot} on the other hand is not significantly affected by BMI. The regression models also correct for small (one order of magnitude smaller than the observed difference between sexes), but significant differences between individual sampling sites for all investigated urine parameters. UC is the only one of the four investigated

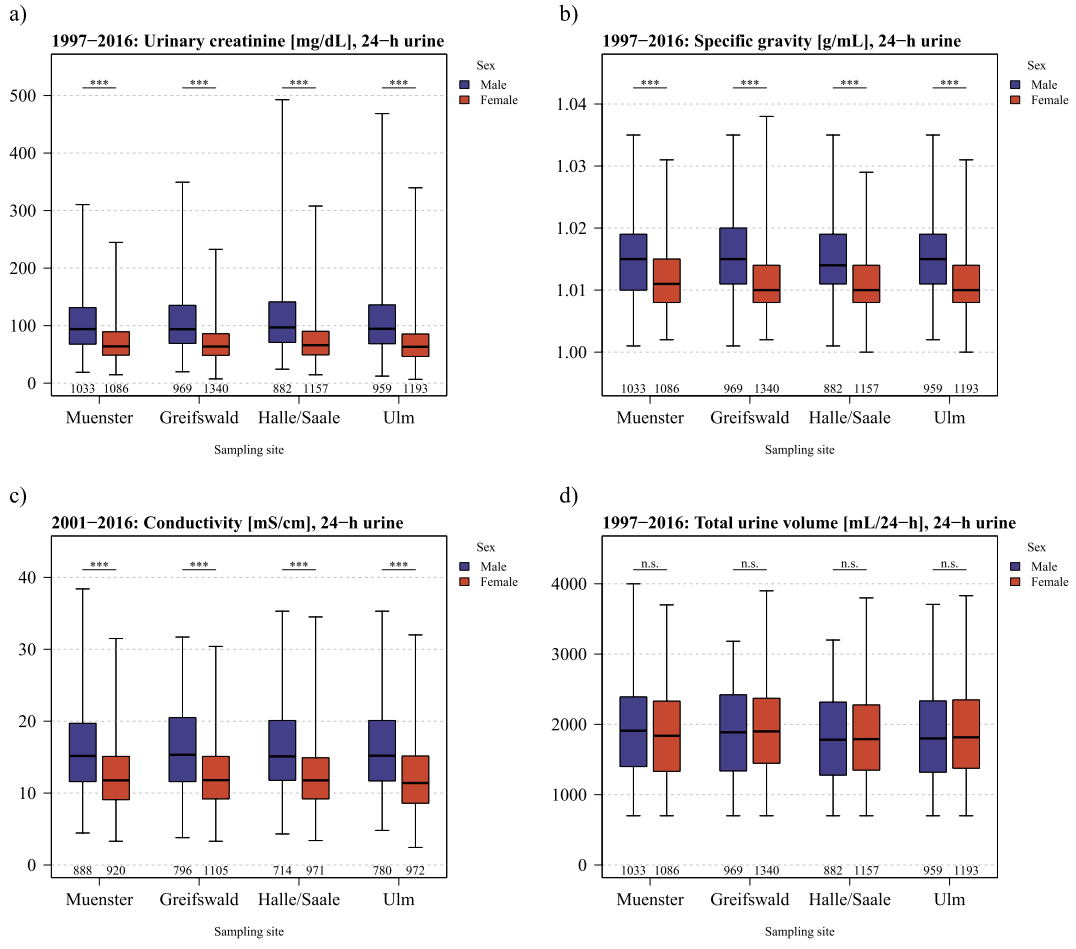

Fig. 1. Sex specific boxplot diagrams for a) urinary creatinine, b) specific gravity, c) conductivity, and d) total urine volume from all four sampling sites from 1997 to 2016. Median is indicated by the horizontal line. The 25th and 75th percentile are indicated by the lower and upper boundary line of each box. Whiskers represent range. The number of measurements represented by each box is indicated at the bottom of each panel. ***. Difference is significant at $\alpha = 0.001$; n.s. not significant.

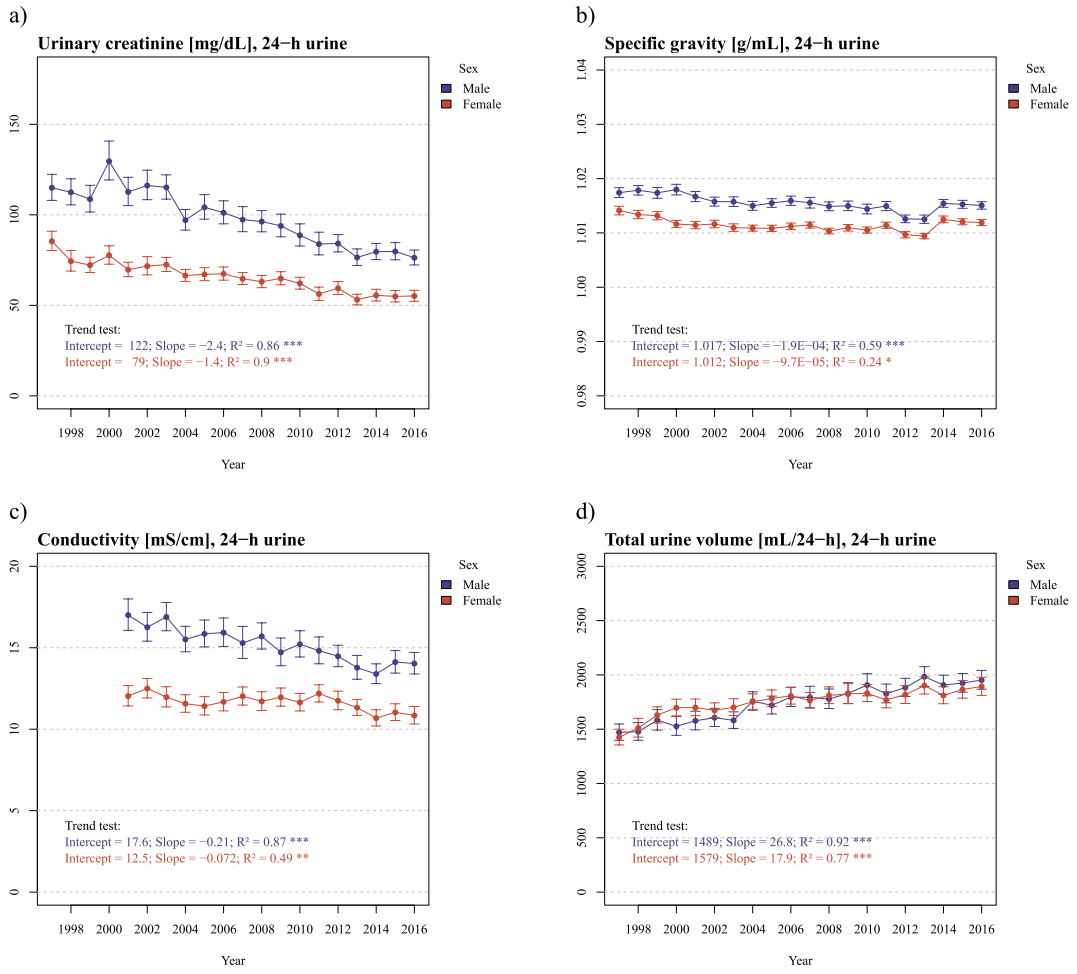

Fig. 2. Geometric mean values per sampling year of a) urinary creatinine, b) specific gravity, c) conductivity, and d) total urine volume for all participants differentiated by sex. Error bars indicate the 95% confidence interval of geometric means. Intercept, slope, model fit and significance of linear trends in GMs versus sampling year are shown in blue text for male and red text for female participants. ***. Trend is significant at $\alpha = 0.001$; **. Trend is significant at $\alpha = 0.01$; *. Trend is significant at $\alpha = 0.05$. Number of participants per sex and year: 164 to 289 (for details see Supplementary Table 1). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Pairwise associations (Spearman's rank correlation) between physiological parameters UC, SG, UV_{tot}, and CON by sex for young adults from 1997 to 2016 (CON since 2001).

Male n = 3843		UC	SG	UV_{tot}	CON
Correlation according to Spearman	UC		.81	-.81	.81
	SG			-.69	.86
	UV_{tot}				-.67
Female n = 4776		**UC**	**SG**	**UV_{tot}**	**CON**
Correlation according to Spearman	UC		.74	-.80	.74
	SG			-.68	.82
	UV_{tot}				-.65

All presented associations are significant at $\alpha = 0.001$ (two-tailed test).

parameters slightly affected by participant age, with younger participants generally having higher UC values.

3.4. Effect of normalization approaches

To investigate the effects of normalizing measured analyte concentrations against individual urine parameters, Ca^{2+} concentrations in urine of young adults were used as an example and normalized against UV_{tot}, UC, CON, and SG. Fig. 3 a) shows the Ca^{2+} concentrations in urine of ESB participants from 1997 to 2016 differentiated by sex.

A highly significant monotonically decreasing trend in non-normalized urinary Ca^{2+} concentrations is observed for female ($p = 0.0017$, Sen's slope $= -1.1$) and male ($p = 0.0001$, Sen's slope $= -2.2$) participants (Fig. 3, panel a). Panels b–e of Fig. 3, show Ca^{2+} concentrations normalized against UV_{tot} (resulting in the total daily excretion), UC, CON, and SG. Normalization against UV_{tot}, UC, or CON changes the observed trend in urinary Ca^{2+} concentrations: In contrast to the non-normalized Ca^{2+} concentrations, no significant trend is observed after normalization (females and males: $p > 0.1$). Interestingly, a less pronounced but still significant downward trend in urinary Ca^{2+} concentrations (females: $p = 0.025$, Sen's slope $= -1.1$; males: $p = 0.041$, Sen's slope $= -1.1$) remains after normalizing against SG.

Moreover, highly significant sex-specific differences exist for most years in urinary Ca^{2+} concentrations with males having higher values than females (see Supplementary Table 2). These highly significant differences can also be observed when Ca^{2+} concentrations are normalized against UV_{tot} (Fig. 3, Panel b, Supplementary Table 2). However, when normalized against UC, CON, or SG (Fig. 3, Panels c–e, Supplementary Table 2), significant sex specific differences occur only in few individual years. Sex specific differences are demonstrated for UC, CON, and SG and confirmed by results of this study (Fig. 1). With regard to urinary Ca^{2+} concentrations, normalization against one of these three parameters hence cancels out the previously observed

Table 3
Multivariate regression analysis: Dependency of UC, CON and SG on UV_{tot} – Each major column shows the result of a linear regression model, respectively with UC, CON, SG, and UV_{tot} as dependent variable. Independent (explanatory and confounder) variables are organised by rows. For each model and independent variable, the determined regression coefficient (Coeff) and its significance in the model (p-value) are shown. Variables with significant effects ($p < 0.05$) are marked in boldface.

	Log(UC)		Log(CON)		Log(SG)		Log(UV_{tot})	
	Coeff	p-value	Coeff	p-value	Coeff	p-value	Coeff	p-value
Explanatory variables								
Log(UV_{tot})	−1.04	0	−0.707	0	−0.0105	0		
Log(Sampling year)							24.7	8.8e-71
Confounders								
Sex: Female	−0.355	0	−0.236	8.33e-220	−0.00354	5.92e-310	0.0082	1
Log(BMI)	0.551	3.88e-85	0.34	1.66e-29	0.00555	1.66e-48	0.063	0.493
Age	−0.0072	3.1e-06	0.00166	1	−3.58e-05	0.412	0.00129	1
Site: Ulm	−0.0388	2.28e-04	−0.0339	0.00375	−0.0004	0.0135	−0.00911	1
Site: Halle	0.00742	1	−0.0229	0.163	−0.0007	6.05e-9	−0.032	0.0281
Site: Greifswald	0.00688	1	0.0169	0.512	−8.22e-05	1	0.0151	1
Full model: adj. R^2	0.67		0.49		0.55		0.04	

Adj. R^2: Coefficient of determination adjusted for model complexity; Coeff: Regression coefficient.

differences between sexes.

4. Discussion

The German ESB routinely has been recording data on UC, SG, CON, and UV_{tot} from 24-h urine samples of each participant. The availability of data from 20 successive years allows an unprecedented evaluation of the above-mentioned parameters' suitability for normalization and their respective effects on HBM data interpretation. Investigation of differences between sexes revealed that UC, SG, and CON measurements in 24-h urine samples were significantly higher in males than in females. These findings have been described previously and can be attributed to differences in body-build, physiological functions, and potentially diet. For instance, higher UC values in males are common already during childhood (Remer et al., 2002). Higher UC in males results from higher muscle mass and therewith higher total body creatine, the precursor of urinary creatinine (Heymsfield et al., 1983; James et al., 1988; Barr et al., 2005). Similar differences between sexes in SG and CON have equally been described previously (Carrieri et al., 2000; Junlong et al., 2007; Silva et al., 2010). Furthermore, UC concentrations decrease with increasing age and can additionally be influenced by multiple factors (e.g., BMI, ethnicity, diseases) (Bulusu et al., 1970; James et al., 1988; Barr et al., 2005; Ix et al., 2010; Waikar et al., 2010). Due to the narrow age range and since the majority of participants of the German ESB can be assumed to be of central European ethnicity, caution is warranted when generalizing results of this study to populations that are more diverse in terms of age and ethnicity. For UC, especially in the elderly, findings might differ (Bulusu et al., 1970; Rowe et al., 1976; Friedman et al., 1989).

The observed increase in total urine volume of over 30% from 1997 to 2016 in both sexes is remarkable. The German ESB has recorded data on dietary liquid uptake annually for each participant using a self-administered questionnaire. This data revealed that the reported uptake of non-alcoholic beverages increased in females from 10.6 L per week in 1997 to 14.7 L per week in 2016 (40%) and in males from 11.0 L per week in 1997 to 15.7 L per week in 2016 (43%) (data not shown). This is well in line with the observed increase in UV_{tot}. Considering water uptake only, an even stronger increase is documented. In females, reported water intake increased by about 104% from 1997 (5.2 L per week) to 2016 (10.7 L per week) and in males by about 97% from 1997 (5.7 L per week) to 2016 (11.3 L per week). This indicates that in general the uptake of water increased substantially, whereas the uptake of other non-alcoholic beverages (e. g., lemonade, juice) decreased. In conclusion, the revealed increasing trend in UV_{tot} can be attributed to a comparable increase in dietary liquid uptake. A dilution effect caused by this increase is apparent and is reflected in a concomitant decrease in the parameters UC, SG, and CON. Hence, the increase in UV_{tot} has a direct impact on these urine parameters. As described in the Material and Methods section, quality control measures have been adapted over time. However, given that UV_{tot} has been measured by a robust and simple weighing method, a potential impact of quality control or laboratory changes on the identified trend in UV_{tot} and the corresponding trends in the parameters UC, SG, and CON is unlikely.

Since trends in UC, SG, and CON are affected by changes in UV_{tot} to a varying extent, normalization against them can generate differing results. It can be reasoned that the increase in UV_{tot} would also lead to a dilution of other urinary solutes including environmental pollutants. This is especially relevant when interpreting trends in exposure to pollutants, where a lack of appropriate normalization may lead to erroneous conclusions about the prevalence of a substance of interest.

Normalization effects are demonstrated exemplarily for urinary Ca^{2+} concentrations, which show a decreasing trend from 1997 to 2016 in both sexes. Since calcium in healthy adults of similar age is commonly excreted on a fairly constant rate (Knapp, 1947; Bulusu et al., 1970), this observed decrease in Ca^{2+} concentrations is highly surprising. The Ca^{2+} concentrations show a rather high variation in particular in the years 2009–2012, which could be interpreted as a result of a laboratory change in 2011. However, while both natural variation in a sample over the years and change in laboratory may result in deviation from a monotonic trend in urinary Ca^{2+}, they do not call into question the much stronger overall decrease from 1997 to 2016. This can be indeed best explained by a dilution effect (increase of urine volumes), especially because the decreasing Ca^{2+} trend attenuates substantially with normalization.

The dilution effect and its consequences for trend analysis on Ca^{2+} concentrations is pertinent with regard to analysis of environmental pollutant concentrations, as was reported by Koch et al., (2012) for Bisphenol A in samples from the German ESB. In this study, when only the urinary Bisphenol A concentration over time was considered, a decreasing trend was identified. When urinary Bisphenol A concentrations were normalized against UC, the initially indicated decreasing trend was not observed anymore.

As mentioned before, UC, CON, and SG show significant differences between females and males caused by differing physiology, metabolism or potentially diet. Therefore, when evaluating sex-specific exposures, normalization of urinary concentrations of environmental pollutants using UC, CON, or SG may enhance or attenuate differences in concentrations between sexes. We confirmed this assumption in the example of urinary Ca^{2+} concentrations. Based on differences in body-build and physiological functions, urinary Ca^{2+} concentrations differ between sexes with females having lower urinary Ca^{2+} levels than males (Rathod et al., 2015). When UC, CON, or SG is used for

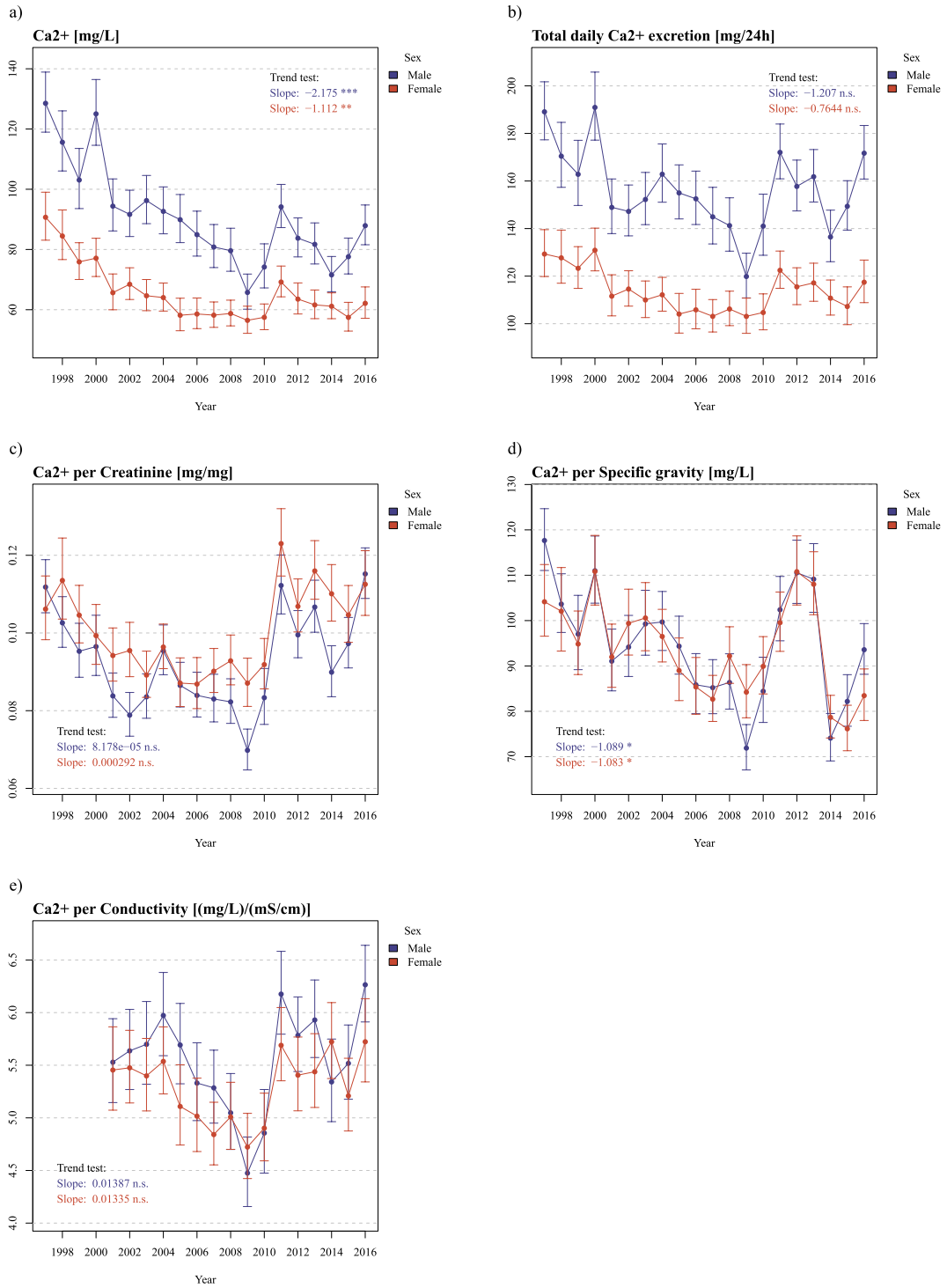

Fig. 3. Geometric mean values per sampling year of a) Ca^{2+} concentration b) total daily Ca^{2+} excretion, c) Ca^{2+} normalized by urinary creatinine, d) Ca^{2+} normalized by specific gravity and e) Ca^{2+} normalized by conductivity for all participants differentiated by sex. Error bars indicate the 95% confidence interval of geometric means. Detected trends in GMs versus sampling year are shown in blue text for male and red text for female participants. ***. Trend is significant at $\alpha = 0.001$; **. Trend is significant at $\alpha = 0.01$; *. Trend is significant at $\alpha = 0.05$. Number of participants per sex and year: 164 to 289. Details see Supplementary Table 1. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

normalization, sex specific differences between males and females are greatly reduced. Normalizing Ca^{2+} concentrations using these parameters minimizes the sex-specific differences observed for the excretion of urinary Ca^{2+} while such sex-specific excretion is evident from the total daily excretion of Ca^{2+} results.

Therefore, when examining trends and sex specific differences in urinary concentrations of environmental pollutants, we suggest calculating the total daily excretion of the pollutants. However, because the effects of normalization likely differ among individual environmental chemicals depending on the chemicals toxicokinetics, the normalization method may need to be tailored to the evaluated analyte. Currently this hypothesis is being tested with data from the German ESB on the exposure of young adults to different environmental pollutants.

While 24-h urine samples are described as the gold standard for determining daily exposure, they also have limitations, as they might conceal short term temporal or diurnal variations in excretion (Ye et al., 2011; Frederiksen et al., 2013; Lassen et al., 2013). Furthermore, in some study settings the collection of urine over 24 h may be impractical or even impossible. Depending on the research question and study design, other types of urine collections, e.g. spot samples, or morning urine samples, can also provide useful information. However, comparability of results and appropriate normalization methods for these types of urine samples remains the subject of scientific investigations (Alconcher et al., 1997; Carrieri et al., 2000; LaKind and Naiman, 2015; Weaver et al., 2015).

Among the parameters investigated in this study, UC is used most commonly for normalization of concentrations measured in 24-h and spot urine samples. UC is affected by body weight, age, and gender and can be influenced by several diseases. Therefore, it might not always be appropriate for normalization (Barr et al., 2005; Nermell et al., 2008; Pearson et al., 2008; Bulka et al., 2017). Results of this study clearly show that CON and SG, similarly to UC, depend on UV_{tot}. Therefore, either of the two parameters could be considered as a possible alternative to UC (Berlin et al., 1985; Barber and Wallis, 1986; Suwazono et al., 2005; Edmands et al., 2014; Hoffman et al., 2014). Another approach to correct for dilution of timed spot urine samples is the normalization using urine flow rate (UFR), which is calculated as the void volume divided by the time since the last void. Hays et al. (2015) calculated analyte excretion rates by multiplying measured analyte concentrations with UFR and proposed that these excretion rates can be used as exposure metrics in the assessment of exposure health outcome associations. A subsequent study by Middleton et al. (2016) found that the dependency between excreted analyte concentration and UFR varies between individual analytes and can only be used for dilution correction once that dependency has been experimentally determined. Furthermore, they concluded that adjustment with osmolality is similarly effective as UFR-based adjustment methods for spot urine samples. In summary, the choice of method to normalize urinary analyte concentrations depends on the type of urine sample, the study design and question, the investigated cohort, and the target analyte(s) (Pearson et al., 2008; Akerstrom et al., 2012; Middleton et al., 2016). Therefore, the current study findings may not directly apply to studies based on spot urine samples. Collecting spot urine samples in addition to 24-h urine samples for a subset of ESB participants could provide useful data to better understand the adequacy of the various normalization approaches.

5. Conclusion

Among young adults investigated by the German ESB, we observed a remarkable increase in UV_{tot} from 1997 to 2016 that directly affects UC, SG, and CON in 24-h urine samples. Independently of the observed increase over time, participants with high UV_{tot} consistently show low values in UC, SG, and CON and vice versa. The observed increase in total urine volume is also likely to affect trends in urinary concentrations of environmental pollutants over time. This finding can also affect the comparison of results from different studies and between individuals. Collecting 24-h urine samples and calculating total daily excretion can minimize impact when studying trends and sex-specific urinary excretion (e.g., compared to normalization with UC), as illustrated for Ca^{2+}. However, if collection of 24-h urine samples is not feasible, it is valuable to record multiple urinary parameters, since their suitability for standardization may vary related to the analyte of interest, the study design, and the type of urine sample taken.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2019.04.009.

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