Exposure of Portuguese children to the novel non-phthalate plasticizer di-(iso-nonyl)-cyclohexane-1,2-dicarboxylate (DINCH)

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A B S T R A C T

Di-(iso-nonyl)-cyclohexane-1,2-dicarboxylate (DINCH) is used as substitute for high molecular weight phthalate plasticizers such as di-(2-ethylhexyl) phthalate (DEHP) and di-(iso-nonyl) phthalate (DINP). Due to a rapid substitution process we have to assume omnipresent and increasing DINCH exposures. The aim of this study was to evaluate DINCH exposure in 112 children (4–18 years old) from Portugal, divided in two groups: 1) normal-/underweight following the usual diet; and 2) obese/overweight but under strict nutritional guidance. First morning urine samples were collected during the years 2014 and 2015. Oxidized DINCH metabolites (OH-MINCH, oxo-MINCH, cx-MINCH) were analyzed after enzymatic hydrolysis via on-line HPLC-MS/MS with isotope dilution quantification. We detected DINCH metabolites in all analyzed samples. Urinary median (95th percentile) concentrations were 2.14 μg/L (15.91) for OH-MINCH, followed by 1.10 μg/L (7.54) for oxo-MINCH and 1.08 μg/L (7.33) for cx-MINCH. We observed no significant differences between the two child-groups; only after creatinine adjustment, we found higher metabolite concentrations in the younger compared to the older children. Median (95th percentile) daily DINCH intakes were in the range of 0.37 to 0.76 (2.52 to 5.61) μg/kg body weight/day depending on calculation model and subpopulation. Body weight related daily intakes were somewhat higher in Group 1 compared to Group 2, irrespective of the calculation model. However, in terms of absolute amounts (μg/day), DINCH intakes were higher in Group 2 compared to Group 1. In regard to age, we calculated higher intakes for the younger children compared to older children, but only with the creatinine-based model. This new data for southern European, Portuguese children adds information to the scarce knowledge on DINCH, confirming omnipresent exposure and suggesting higher exposures in children than adults. Significant sources and routes of exposure have yet to be unveiled. For now, all calculated daily intakes are far below established health benchmark levels (TDI, RfD). However, rapidly increasing exposures have to be expected over the next years.

Keywords:
DINCH
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1. Introduction

Di-iso-nonyl-cyclohexane-1,2-dicarboxilate (DINCH) is used as substitute for some High Molecular Weight (HMW) phthalate plasticizers like di (2-ethylhexyl) phthalate (DEHP) and diisononyl phthalate (DINP). These two HMW phthalates have been going through intensive scrutiny worldwide, due to their endocrine disrupting and reprotoxic activity (CPSC, 2008; EU, 2005; EU, 2006). Since 1999 (EU, 1999) (Directive 1999/815/EC) the European Union (EU) has banned DEHP in toys and childcare articles in concentrations above 0.1%, and DINP, diisodecyl phthalate (DIDP) and di-n-octyl phthalate (DnOP), in toys and childcare articles which are intended for mouthing at concentrations above 0.1% (entry 51/52 of Annex XVII of the Regulation EC No. 1907/2006) (EU, 2006). Since 2001, DEHP is classified as a reproductive toxicant in the EU (Directive 2001/59/EU) (EU, 2001); since February 2015, DEHP (listed in Annex XIV of the Regulation EC No. 1907/2006) (EU, 2006) must not be placed on the EU market any more (REACH sunset date). Due to these changes in the regulatory landscape and increasing evidence for adverse health effects on humans the demand for safe non-phthalate, non-aromatic substitutes increased.

In 2002, the plasticizer DINCH was introduced into the market, intended for the use in sensitive products such as toys, food contact materials and medical devices (Bhat et al., 2014; Biedermann-Brem et al., 2008; David et al., 2015; EFSA, 2006). DINCH is produced by catalytic hydrogenation of the aromatic ring of DINP, and commercialized as

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Hexamoll® DINCH® (Koch et al., 2013a). In 2014, 200,000 tons were produced according to data from BASF SE (BASF, 2015) with a rapidly increasing production volume over the past decade. It is expected that production volumes will continue to grow in the future.

The currently available data suggest that DINCH, contrary to phthalates such as DEHP and DINP, is neither an endocrine disruptor nor a reproductive toxicant (EFSA, 2006; Furr et al., 2014). According to the data summarized by European Food Safety Authority (EFSA) (EFSA, 2006), in a sub chronic oral toxicity study in rats only at high level doses (1000 mg/kg bw/day for both genders and 300 mg/kg bw/day for males) DINCH could cause renal toxicity. The same study showed an increased incidence of thyroid hyperplasia in males at all tested doses and at high level dose for females. However, these effects were contributed to substantial differences between rats and humans. The NOAEL for thyroid hyperplasia was considered inapplicable to establish a tolerable daily intake (TDI). A TDI of 1 mg/kg bw/day with a factor of uncertainty of 100 was established by EFSA (EFSA, 2006) based on the NOAEL for renal effects. Recently, Bhat et al. (2014) derived an oral reference dose (RfD) of 0.7 mg/kg/day for thyroid hypertrophy/hyperplasia seen in F1 rats from a two-generation study.

Similar to other external plasticizers, DINCH is physically dissolved in the polymer and not chemically bound to it. In a Swiss market survey in 2005, DINCH was found to migrate from gaskets of metal closures into oily food (Fankhauser-Noti et al., 2006). DINCH seems to have emission rates similar to DINP from plastic material (Holmgren et al., 2012). EFSA assigned a specific migration limit of 1 mg/kg food (EFSA, 2006).

Once DINCH has entered the human body it is rapidly broken down to its simple monoester by ester cleavage. The alkyl side chain of this monoester is further oxidized to the cyclohexane-1,2-dicarboxylic acid-mono(hydroxyl-iso-nonyl) ester (OH-MINCH), the cyclohexane-1,2-dicarboxylic acid-mono(carboxy-iso-octyl) ester (cx-MINCH) and the cyclohexane-1,2-dicarboxylic acid-mono(oxo-iso-nonyl) ester (oxo-MINCH). These oxidized metabolites are the major DINCH metabolites excreted in urine and currently used for human-biomonitoring (HBM) purposes (Koch et al., 2013a; Schütze et al., 2015; Silva et al., 2012; Völkel et al., 2016).

The results from HBM population studies currently available (CDC, 2015; Fromme et al., 2016; Giovanoulis et al., 2016; Gomez Ramos et al., 2016; Schütze et al., 2014; Schütze et al., 2012; Silva et al., 2013) suggest that DINCH exposure is already widespread across the globe. Additionally, a steep increase in DINCH exposure has been reported (Schütze et al., 2014; Silva et al., 2013) since its introduction in the market, with children presenting higher urinary metabolite levels and daily intakes (DI) than adults (Fromme et al., 2016; Schütze et al., 2014; Silva et al., 2013).

The aim of this study was to investigate DINCH exposure in Portugal, as another country in the EU, in a group of 112 children. Moreover, according to other studies children seem to be a population of special concern regarding plasticizer exposure, as their exposure levels are higher when compared to adults (Cutanda et al., 2015; Den Hond et al., 2015; Kasper-Sonnenberg et al., 2014), which makes the results of this study of special interest. Finally, as our study population was composed by two groups, one with healthy normal-/underweight and another with obese/overweight children without other known associated diseases (with nutritional counselling) another aim was to assess possible differences in DINCH exposures among these two groups.

2. Material and methods

2.1. Subjects and urine samples

The present study is part of an ongoing study to assess possible differences between obese/overweight and normal-/underweight children regarding the exposure to several environmental compounds. The initial aim of this project was the determination of exposure to several suspected or confirmed endocrine disruptors and/or obesogens. Later, considering the regulation and the increasing and relevant substitution by novel compounds, DINCH was added to list. Children were recruited from the pediatric appointment at Hospital de S. João, and several local schools, in the years of 2014 and 2015. Children came from two Portuguese districts, Oporto and Aveiro, belonging respectively to the North and Central region of the country. In all, one hundred and twelve children (55 boys, 57 girls) participated in this study with an age range of 4 to 18 years (median 10 years).

The children were divided in two groups according to the body mass index (BMI). In the Portuguese public health system, the Direcção-Geral de Saúde adopted the World Health Organization (WHO) growth charters since 2012 (WHO, 2007). Group 1 included healthy children (without associated diseases) which were normal-/underweight. Group 2 included children diagnosed for obesity/overweight without other known associated diseases. The obese/overweight group was recruited from a pediatric nutritional appointment, thus counselled for healthy and balanced nutrition and was set on a prescribed diet (at least for three months), based on fresh food and less packaged and processed food items. The children in Group 1 continued with the usual diet.

A summary of anthropometric data for the two groups of children is given in Table 1.

The majority of the children was overweight/obese (62%; $n = 69$). While the discriminators body weight and BMI differed significantly ($p < 0.05$) between the 2 groups, age, gender, height and urinary creatinine were evenly distributed ($p > 0.05$) (Table 1).

During the course of the study, we collected a first morning urine sample from each participating child. All the specimens were kept cool during transportation and then stored at −20 °C until analyses.

The study was approved by the ethics committee of the Centro Hospitalar S. João/FMUP (Medicine Faculty of Oporto University ref. 163.13) and all the parents provided written consent.

2.2. Analysis of DINCH metabolites in urine

Oxidized DINCH metabolites were analyzed after enzymatic hydrolysis via on-line HPLC-MS/MS with isotope dilution quantification (Schütze et al., 2012). Briefly, to 300 μL urine 100 μL of 1 M ammonium acetate buffer (pH 6.0), 10 μL of internal standard solution and 6 μL of β-glucuronidase (from *E. coli* strain K-12, without arylsulfatase activity) were added. Then, the samples were gently mixed and placed in a water bath at 37 °C for 2 h. After adding 10 μL of acetic acid, the samples were stored at −18 °C overnight to precipitate proteins. The samples were then thawed at room temperature and centrifuged at 1900 × *g* for 10 min, and 25 μL supernatant were injected into an Agilent Technology LC1200 system coupled with an AB Sciex QTrap 5500 tandem mass spectrometer. We used a Capcell PAK 5u C18 MG-II column for clean-up and enrichment and, after back flush, an Atlantis dC18 (2.1 × 150 mm; 3 μm) for chromatographic separation. Based on the 1/x weighted calibration curves of the 4-methyl octyl derived standard substances the sum of all C9 alkyl chain isomers of MINCH and the alkyl chain isomers with oxidative functional groups were quantified. Mean accuracies for

Table 1
General characteristics of the studied population.

Population characteristics	Group 1 Normal-/underweight ($n = 43$)[a] usual diet	Group 2 Overweight/obese ($n = 69$)[a] special nutritional guidance
Age (years) mean	10.81	10.20
Gender (%)	44% Female 56% Male	55% Female 45% Male
Weight (kg) mean	36.50	52.48
Height (cm) mean	139.94	142.26
BMI (kg/m^2) mean	17.53	25.25
Creatinine (g/L) mean	1.08	1.08

[a] The underweight/normal weight and obese/overweight groups were defined according to the WHO charters (WHO, 2007).

the metabolites were between 90.1 and 97.9% and relative standard deviations of the laboratory control material (measured within each analytical batch) were consistently below 10%. The limits of quantification (LOQ) were 0.05 μg/L for all oxidized metabolites OH-MINCH, oxo-MINCH, and cx-MINCH.

2.3. Determination of urinary creatinine

Urinary creatinine concentration was measured through a modified Jaffé method (Jaffé, 1886) with an Olympus AU5400® (Beckman-Coulter®, Porto, Portugal) at São João Hospital, Department of Clinical Pathology.

Four out of the 112 children had creatinine values below 0.3 g/L. However, all urine samples were included in the statistical analyses, because creatinine values below this, in children do not necessarily indicate excessive dilution but can be indicative of lower muscle mass compared to adults (Koch et al., 2011).

2.4. Daily intake calculations

Based on the urinary levels of the major oxidized DINCH metabolite OH-MINCH, we calculated the DI of DINCH for each child. For this purpose two calculation models were applied: the first model was based on the creatinine-related OH-MINCH concentration (Koch et al., 2007; Wittassek et al., 2007), adjusted to the height/age dependent reference values for creatinine excretion (Remer et al., 2002). The second DI calculation model was based on the volume-related urinary OH-MINCH concentration (Fromme et al., 2016; Koch et al., 2007; Schütze et al., 2014; Wittassek et al., 2007) together with reference values for the daily body weight-related urine volume in children and adolescents until 14 years old (Geigy, 1983). For adolescents from 14 until 18 years old values were taken from elsewhere (Hays et al., 2011). For children and adolescents until age 14 as mentioned the urinary volumes are body weight related, but for the older children reference values are gender based (Hays et al., 2011). Thus we normalized the DI to body weight for these children. We calculated the DI based on the OH-MINCH concentration with a factor of urinary excretion (*fue*) of 0.107 (Koch et al., 2013a).

We also calculated the absolute amount of DINCH taken up per child per day (straight mass exposure to DINCH in μg/day) by multiplying the calculated intake per kg body weight per day (according to both calculation models) with each childs's body weight.

2.5. Statistical analysis

Statistical analysis was performed using SPSS 20.0 (IBM Corporation). Several statistical data are presented, namely descriptive such as medians and percentiles. Concentrations below the LOQ were set to ½ LOQ for all metabolites. For the present analysis age was categorized into two categories in agreement with the European guidelines for clinical studies in pediatric patients (ICH, 2000): Children from 2 to 11 years old and adolescents from 12 to 18 years old.

A non-parametric test (Mann-Whitney *U* test) was performed to assess possible differences across distribution between the study groups for the urinary values (μg/L and μg/g creatinine) and DI (for both creatinine and volume-related models). Statistical analysis for region, gender and age dependency regarding urinary values was also tested by a Mann-Whitney *U* Test. The same analysis (concerning group, gender and region) was performed for the DI, however regarding age a Spearman's rank correlation was applied to assess the possible tendency. Additionally, a Spearman's rank correlation was also used to assess the correlation between the two DI calculation models as well as between the oxidized metabolites.

3. Results and discussion

3.1. Urinary metabolites levels

In our study we could detect OH-MINCH and cx-MINCH in 100% and oxo-MINCH in 99% of all urine samples, showing the omnipresent exposure of the Portuguese children to DINCH. Only one child from Group 2 (overweight or obese/nutritional guidance) had a urinary concentration level below LOQ for oxo-MINCH. Results in μg/L and μg/g creatinine, median concentrations, 95th percentile and maximum values for both groups and all children together are shown in Table 2.

OH-MINCH was the metabolite with the highest concentration levels (for the non-normalized and the creatinine corrected values) with a median of 2.44 μg/L and 2.03 μg/g creatinine for Group 1 children and with a median of 1.83 μg/L and 2.22 μg/g creatinine for the Group 2 children. The other two metabolites cx-MINCH and oxo-MINCH were generally lower by a factor of 2. This general distribution of metabolites is in accordance with findings from previously published human metabolism studies (Koch et al., 2013a; Schütze et al., 2016).

In regard to metabolite concentrations, we found no significant differences between the two groups of children, neither for non-normalized values nor for creatinine adjusted concentrations. While non-normalized median metabolite concentrations in μg/L seemed a bit lower in Group 2 (obese or overweight children with nutritional guidance) compared to Group 1 (regular weight children following the usual diet), creatinine adjusted median values were very similar between the groups. On the one hand, these minor (non-significant differences) might be due to differences in external DINCH exposure. The Group 2 children were being followed in a nutritional appointment, thus a dietary consultation program was set up for these children. The dietary pattern of these children relied primarily on fruits and vegetables, whole grains, low-fat dairy products, beans, fish, and lean meat. Additionally, the amount of calories was set according to their nutritional needs (energy intake is appropriate for the maintenance of a normal weight for height and for the adequate intake of micronutrients). The Group 1 children continued their usual diet. As seen by other authors regarding compounds that have short half-lives and food as an important exposure source, dietary interventions can have an impact in lowering exposure (Rudel et al., 2011). On the other hand, renal clearance and urinary creatinine excretion (and therefore creatinine adjustment) might also be influenced by the physiological differences of the two groups (obese/overweight vs. normal-/underweight) and lead to some disparities between μg/L and μg/g creatinine concentrations (Hays et al., 2015).

Table 2
Urinary metabolite levels for the Portuguese children (μg/L and μg/g creatinine) for total population and the two study groups.

Metabolites	Non-normalized values (μg/L) median (95th percentile/maximum)				Creatinine adjusted values (μg/g) median (95th percentile/maximum)			
(n)	Group 1 (43)	Group 2 (69)	Total (112)	*p value*[a]	Group 1 (43)	Group 2 (69)	Total (112)	*p value*[a]
OH-MINCH	2.44 (6.85/60.80)	1.83 (20.95/114.00)	2.14 (15.91/114.00)	0.938	2.03 (9.80/125.36)	2.22 (18.06/132.56)	2.14 (17.25/132.56)	0.758
cx-MINCH	1.11 (4.13/23.60)	0.98 (10.74/47.00)	1.08 (7.33/47.00)	0.926	1.16 (6.01/48.66)	1.16 (10.34/54.65)	1.16 (8.79/54.65)	0.820
oxo-MINCH	1.39 (4.06/18.40)	0.87 (8.13/54.40)	1.10 (7.54/54.50)	0.827	1.11 (4.45/37.94)	1.07 (7.90/63.26)	1.09 (7.22/63.26)	0.858

Group 1 – Normal weight/underweight following the usual diet.
Group 2 – Obese/overweight with nutritional guidance.
[a] Significance level is 0.05.

Table 3
Urinary metabolite levels for the Portuguese children (μg/L and μg/g creatinine) according to age groups.

Metabolites	Non-normalized values (μg/L) median (95th percentile/maximum)			Creatinine corrected values (μg/g) median (95th percentile/maximum)		
	4–11 years ($n = 71$)	12–18 years ($n = 41$)	p value[a]	4–11 years ($n = 71$)	12–18 years($n = 41$)	p value[a]
OH-MINCH	1.83 (17.78/114.0)	2.33 (17.73/23.30)	0.505	2.55 (22.12/132.56)	1.72 (14.41/18.13)	0.003
cx-MINCH	1.03 (7.49/47.00)	1.11 (11.13/23.90)	0.450	1.44 (10.08/54.65)	0.74 (8.42/14.86)	0.003
oxo-MINCH	0.89 (9.18/54.40)	1.37 (6.90/7.88)	0.280	1.22 (9.92/63.26)	0.88 (5.58/7.79)	0.033

[a] Significant level is 0.05.

In a second approach, and because metabolite levels were rather comparable between the 2 original study groups, we investigated the age of the children as a possible determinant of urinary DINCH metabolite levels. We divided the whole study population into two age groups: younger children from 4 to 11 years and older children from 12 to 18 years as described previously in Section 2.5. In Table 3 we present the urinary metabolite levels (for non-normalized and creatinine corrected values) by age group.

For the main metabolite OH-MINCH the young children (4–11 years) had a median of 1.83 μg/L and 2.55 μg/g creatinine, the older children (12–18 years) had a median of 2.33 μg/L and 1.72 μg/g creatinine. Similar distributions applied to the other metabolites, at lower levels. Thus, based upon μg/L values, metabolite levels were lower for the younger children, while based upon creatinine adjusted concentrations metabolite levels were higher for the younger children. These differences, however, were significant only for the creatinine adjusted values. This considerable difference is probably due to the rapidly changing creatinine excretion with age and development in children (Barr et al., 2005; Remer et al., 2002). The median creatinine excretion determined for the children with ages between 4 and 11 years was 0.79 g/L, and 1.39 g/L for older children respectively ($p < 0.05$). Therefore, in regard to creatinine adjustment, it is important to bear in mind that creatinine in children varies more with the extent of the child development rather than with urine dilution (Koch et al., 2011; Langer et al., 2014).

We observed no statistical differences ($p < 0.05$) between genders and regions (Oporto and Aveiro), neither for the non-normalized nor the creatinine adjusted concentrations (data not shown).

We also investigated the correlation between the three DINCH metabolites and found strong correlations (see Fig. 1). The spearman correlations for all three metabolites resulted in rho-values above or equal to 0.882. Similar correlations, albeit at lower metabolite concentration levels and for fewer samples, have been reported previously by Schütze et al., 2015. These strong correlations, observed over several orders of magnitude of urinary concentration confirm the ruggedness of the metabolites to be used as biomarkers of DINCH exposure. The correlations were not influenced by the BMI-category/nutritional regime of the children.

3.2. Daily intakes

We calculated daily DINCH intakes applying two different calculation models (creatinine and volume based) based on the urinary concentrations of OH-MINCH. The calculated daily intakes for the whole population as well as for Group 1 (normal-/underweight weight following the usual diet) and 2 (overweight or obese with nutritional guidance) are shown in Table 4.

Depending on the model, we calculated median daily DINCH intakes for the whole study population between 0.44 and 0.61 μg/kg body-weight/day. Intakes at the 95th percentile ranged from 2.76 to 4.82 μg/kg body-weight/day and at the maximum from 26 to 36 μg/kg body-weight/day. Intakes calculated via the volume-based calculation model were generally higher than intakes calculated via the creatinine calculation model. A similar tendency towards higher daily intakes via the volume-based model has previously been described for phthalate intakes by Wittassek et al. (2007) and Koch et al. (2007). As one possible explanation to this, body height and gender-based reference values for daily urinary creatinine excretion used in the creatinine calculation model were more detailed (Remer et al., 2002) while corresponding data for the daily urine volume excretion in children was less detailed

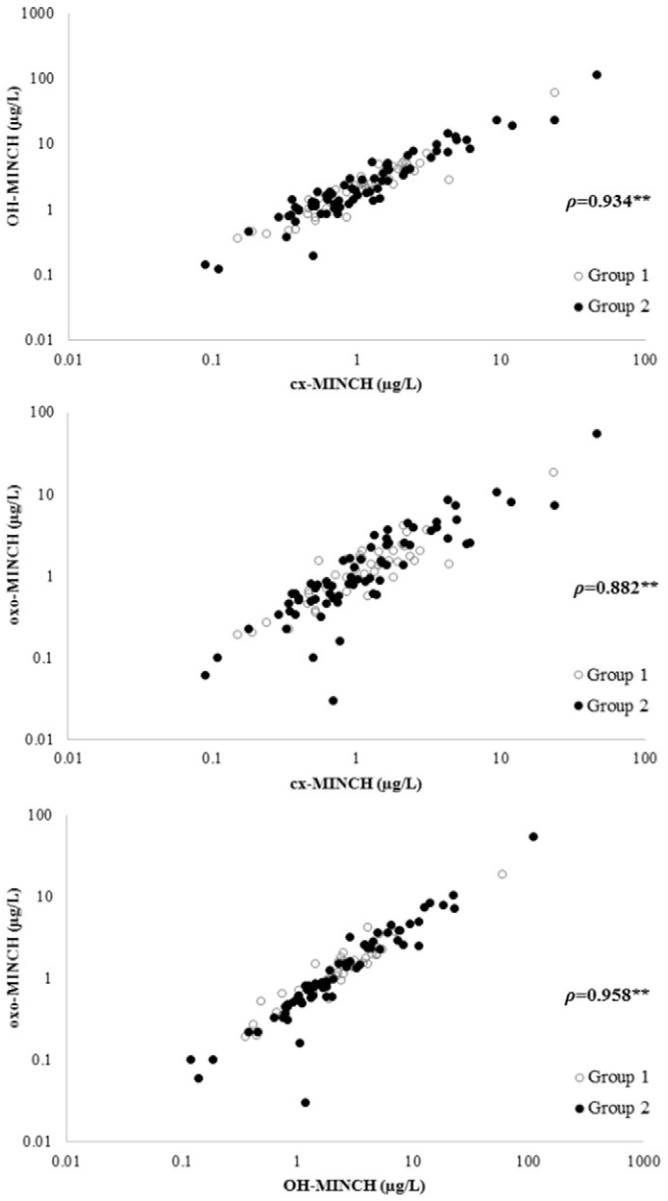

Fig. 1. Correlations between the oxidized metabolites of DINCH (in μg/L). White circles represent children of Group 1, black circles represent children of Group 2 (ρ = Spearman's rank correlation coefficient significant level is 0.01**).

Table 4
Daily intake of DINCH in Portuguese children, total, Group 1 and 2.

Calculation model	Population 4–18 years	Daily DINCH intake (μg/kg body weight/day)			p value[a]
		Median	95th percentile	Maximum	
crt	Total (112)	0.44	2.76	26.24	0.086
	Group 1 (43)	0.53	2.52	26.20	
	Group 2 (69)	0.39	3.05	26.24	
vb	Total (112)	0.61	4.82	35.98	0.693
	Group 1 (43)	0.76	4.65	22.26	
	Group 2 (69)	0.57	5.06	35.98	

crt – creatinine-based.
vb – volume-based.
Group 1 – Normal weight/underweight following the usual diet.
Group 2 – Obese/overweight with nutritional guidance.
[a] Significant level is 0.05.

and robust (Geigy, 1983). Notwithstanding, the discrepancy between all models is smaller than a factor of 2. Furthermore, we obtained strong and significant correlations between the two DI calculation models (spearman correlation of 0.802 with p value < 0.01). In regard to daily DINCH intakes, we observed no statistically significant difference between the two groups of children. However, the median DI values were always slightly higher for Group 1 children. This tendency was observed regardless of the calculation model. Again, we speculate that these differences might arise from the fact that the children in Group 2 were on special nutritional guidance (namely fresh and unprocessed food) which might have led to somewhat lower DINCH intakes. As reported by other authors dietary interventions can have an impact on lowering the exposure to food-borne environmental chemicals, such as plasticizers (Koch et al., 2013b; Rudel et al., 2011). In regard to body weight/BMI recent studies reported on positive associations with internal exposure to phthalate plasticizers like DEHP (Buser et al., 2014; Hou et al., 2015; Trasande et al., 2013). We could not observe such an association for DINCH. However, again we have to point out that it was the obese/overweight children that were on a "healthy" diet.

To further investigate DINCH exposure in Group 1 and Group 2 children, we also calculated the absolute amount of DINCH taken up per child per day (straight mass exposure to DINCH in μg/day) by multiplying the calculated intake per kg body weight per day with each childs's body weight. For the creatinine-based data we obtained median absolute DINCH intakes of 18.2 and 19.3 μg/day for Group 1 and 2 respectively. For the volume-based data we obtained absolute DINCH intakes of 24.2 and 28.8 μg/day for Group 1 and 2, respectively. Based upon absolute intakes, the obese children (under nutritional guidance) would have incorporated a slight higher amount of DINCH than the regular weight children (following their usual diet). However, for the body weight related intake data, differences between both groups were not significant. Combining these results supports the hypothesis that DINCH exposure is driven by food exposures and that the diet intervention probably had an impact in Group 2 (obese/overweight) DINCH exposures.

The daily intakes calculated separately for the young (4–11 years) and older children (12–18 years) are presented in Table 5.

Table 5
DINCH DI in Portuguese children by age groups.

Calculation model	Age	Daily DINCH intake (μg/kg body weight/day)		
		Median	95th percentile	Maximum
crt	4–11 years	0.51	3.51	26.24
	12–18 years	0.37	2.81	3.28
vb	4–11 years	0.60	5.61	35.98
	12–18 years	0.61	5.28	5.59

crt – creatinine-based.
vb – volume-based.

Regarding age, it seems to exist a trend for higher intake with decreasing age (Table 5). But this correlation was only significant for the creatinine-related model (spearman correlation of −0.314, p value < 0.01). In the volume model, however, daily intakes were very similar for both age groups. Again, as already pointed out above for the urinary metabolite levels, the rapidly changing child physiology complicates the age-dependent daily intake calculations of children. If there were age-dependent differences, these are rather small (less than a factor of 1.5). These differences are rather similar to the age-dependent daily intake differences previously reported for phthalates such as DEHP (Wittassek et al., 2007) or DnBP/BBzP (Koch et al., 2007) that were indicating to 2-times higher exposures in young children compared to older children. Furthermore, behavioral factors can make children a highly exposed group of the population for chemicals that are widespread (Ginsberg et al., 2016). Thus, one can presume that exposures especially in young children might additionally be caused by other age dependent behaviors, like mouthing or playing near to the floor (Biedermann-Brem et al., 2008; Fromme et al., 2016; Ginsberg et al., 2016; Koch and Angerer, 2012; Lee et al., 2014; Nagorka et al., 2011; Xie et al., 2016).

3.3. Risk assessment

For risk assessment we can either compare urinary metabolite levels or extrapolated daily intakes with health based guidance values. The German Human Biomonitoring Commission derived an HBM-I value for urinary metabolite levels (sum of OH-MINCH and cx-MINCH) that represents a level below which no adverse health effect should be expected (3000 μg/L for children, 4500 μg/L for adults) (Apel et al., 2016). All children of this study were considerably below the HBM-I value with a factor of almost 1000 at median metabolite levels (3.16 μg/L) and a factor of 18 at the maximum metabolite levels (161 μg/L). The European Food Safety Authority (EFSA, 2006) established a Tolerable daily intake (TDI) for DINCH of 1 mg/kg body weight/day. Bhat et al. (2014) derived an Oral Reference Dose (RfD) of 0.7 mg/kg body weight/day. Independent of the calculation model, median daily intakes of our study (0.44–0.76 μg/kg body weight/day) are a factor of 1000 below these benchmark doses, the maximum daily intake (35.95 μg/kg body weight/day) is a factor of >10 below. Daily intakes have been calculated from first morning urine samples as the only bio-specimen available in our study. First morning urine samples have been previously shown to underestimate exposures to food-borne plasticizers (such as DEHP) to some extent (possibly a factor of 2). Food related exposures during lunch or dinner (from the day before) are underrepresented in these samples due to the rapid elimination kinetics of the DINCH metabolites (Aylward et al., 2011; Lorber et al., 2011). Thus, even with this slight possibility for underestimation kept in mind, daily intake levels of DINCH can currently be considered as safe.

3.4. Comparison with international data

Until now, only few studies reported and evaluated DINCH exposure using urinary metabolite data. A comparison between the Portuguese children of this study and the available studies so far is presented in Table 6. Three of these studies were conducted in German populations (in children with 27 to 80 months and adults) (Fromme et al., 2016; Schütze et al., 2014; Schütze et al., 2012) and one in an adult US population (Silva et al., 2013). Recently, DINCH exposure was also assessed in pooled urine samples from Australia (Gomez Ramos et al., 2016), and in adults from Norway (Giovanoulis et al., 2016). Additionally, the CDC fourth report from 2015 (CDC, 2015) also presents values for OH-MINCH for the US population starting at 6 years of age.

Urinary metabolite levels of our study agree remarkably well with levels previously reported for German children (Fromme et al., 2016) and with levels in pooled urine samples from Australian children (Gomez Ramos et al., 2016). Levels in children reported for the US seem

Table 6
Concentration of DINCH metabolites (μg/L) and DI (μg/kg bw/day) in worldwide populations.

Country	Reference	Subjects	Age	Sampling years	Metabolites levels (μg/L) median (95th percentile)			Daily intakes (μg/kg bw/day) median (95th percentile)	
					OH-MINCH	cx-MINCH	oxo-MINCH		
Child population studies									
Portugal	This study	112	4–18 years	2014/2015	2.14 (15.91)	1.08 (7.33)	1.10 (7.54)	cm	0.44 (2.76)
								vm	0.61 (4.82)
Germany	(Fromme et al., 2016)	208	27–80 months	2011/2012	1.66 (9.95)	1.14 (6.11)	1.54 (7.98)	vm	0.50 (2.80)
Australia	(Gomez Ramos et al., 2016)	400	5–14 years	2012/2013	4 pooled urines: 2.70; 1.80; 3.10; 3.50	nd	nd	nd	
US	(CDC, 2015)[a]	396	6–11 years	2011/2012	<LOD (4.10)	nd	nd	nd	
		388	12–19 years		<LOD (1.30)	nd	nd	nd	
Adult population studies									
Germany	(Schütze et al., 2012)[b]	22	23–57 years	2010	0.36	0.23	0.22	nd	
	(Schütze et al., 2014)[b]	60	20–30 years	2012	0.39 (2.09)	0.17(0.86)	0.25 (1.81)	Uv24	0.14 (1.07)
US	(Silva et al., 2013)[a]	121	Adults	2012	<LOD (1.40)	<LOD (2.40)	<LOD (1.00)	nd	
	(CDC, 2015)[a]	1705	≥20 years	2011/2012	<LOD (0.80)	nd	nd	nd	
Norway	(Giovanoulis et al., 2016)	61	20–66	2013/2014	GM = 0.25	nd	GM = 0.23	cm	0.23 (4.00)

nd – not determined.
cm – creatinine-based DI calculation model.
vm – volume-based DI calculation model.
Uv24 – urinary volume for 24 h.
GM – geometric mean.
[a] LOD (Limit of Detection) equal to 0.4 μg/L.
[b] LOQ (Limit of Quantification) equal to 0.05 μg/L.

to be somewhat lower (CDC, 2015). However, when comparing these datasets one has to keep in mind not only the spatial differences in sampling location but also differences in the study population (age of children) and the year of sampling. As pointed out earlier we have to assume that younger children are higher exposed than older children and that DINCH exposure has been consistently increasing over the last few years.

Comparing the urinary DINCH metabolite levels of the children with adult populations, it is rather apparent that levels in children are higher than in adults. The median levels in both the Portuguese and German children are about 5 times higher than median or geometric mean levels in adults from Germany and Norway. The same seems to be true for the US, albeit at lower levels. These findings support previous assumptions that children are additionally exposed to plasticizers (and DINCH) due to a higher food intake per kg body weight and due to child specific exposure routes such as mouthing of toys (Koch and Angerer, 2012). Furthermore, DINCH was introduced in the market especially for sensitive applications, namely in food contact, childcare products and toys (EFSA, 2006). Thus it is reasonable to assume that children are indeed additionally exposed to DINCH compared to adults.

The DINCH intake calculations from our study and all previous intake calculations (Fromme et al., 2016; Giovanoulis et al., 2016; Schütze et al., 2012) consistently indicate to current median intakes around or below 0.5 μg/kg bw/day and to 95th percentile intakes below 5 μg/kg bw/day. These levels are still considerably below health based limit values such as the TDI of 1 mg/kg/day (EFSA, 2006) or the RfD of 0.7 mg/kg/day (Bhat et al., 2014).

4. Conclusions

With this study we for the first time report the omnipresent DINCH exposure in a population of Portuguese children, confirming recent findings on widespread DINCH exposure from other countries. DINCH, having been introduced in the world market as a substitute for phthalate plasticizers only in 2002 seems to have established itself as an alternative to critical phthalate plasticizers.

Generally, children seem to be exposed to DINCH at approximately 5-times higher levels than adults. We observed tendencies to lower DINCH exposures in the children with nutritional guidance (healthy diet) and to somewhat higher DINCH exposures in younger children than in older children. However, contrary to previous reports on (phthalate) plasticizer exposure, these observations did not reach significance, except for age and DINCH DI based on creatinine-related model. Future studies will have to investigate general and child specific sources and routes of DINCH exposure in more detail.

Current DINCH exposures in the Portuguese children (and all other populations reported by now) are far from any level of concern. However, because rapidly increasing DINCH exposures have to be expected in the coming years, the ongoing surveillance of DINCH exposure seems warranted, preferably in high-exposure subpopulations such as infants and children. With this data we provide valuable information for the risk assessment and risk management of the alternative plasticizer DINCH. We can also advise exposure reduction measures if exposure trends indicate to reaching critical levels e.g. when exposure would approach TDI or RfD values, or if new toxicological findings lead to a re-evaluation of health based limit values.

Conflict of interest

The authors claim, that they do not have any conflict of interest.

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