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Urinary deoxynivalenol as a biomarker of exposure in different age, life stage and dietary practice population groups

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ABSTRACT

The *Fusarium* mycotoxin deoxynivalenol (DON) and its modified forms are present in most samples of grain and grain-based products. Due to the widespread presence of DON in these highly consumed food commodities, nearly all individuals are exposed to DON. Previous estimates of the dietary DON intake in Norway indicated that children's dietary intake is close to or exceed the TDI of 1 μg/kg bw/day for the sum of DON and three modified forms. One aim of the current study was to determine whether the concentrations of DON in morning urine differ between population groups like men, women, children, vegetarians, and pregnant women. An additional aim was to compare a set of models for estimating the dietary intake of DON based on urinary DON concentrations and also compare these models with DON-intakes estimated using food consumption data. DON and metabolites were detected in the morning urine from 256 out of 257 individuals and with concentrations in similar range as reported from other countries. Children have higher urinary DON-concentration than adults and elderly. The urinary DON-concentration in pregnant women and vegetarians did not differ from other adults. The estimated intake of DON was higher for children than for other age groups on a body weight basis. The correlations between different models for estimating DON-intake based on urinary concentration as well as based on individual food consumption were good (0.79–0.99), but with some outliers. We conclude that Norwegians are exposed to DON in the same range as reported from other countries and that children have a higher exposure than adults. Furthermore, we conclude that intake estimates based on urinary DON concentration is a useful tool for evaluation of the exposure at population level, but due to outliers, the estimates for individuals are uncertain. There are also uncertainties in intake estimates both from food consumption and from urinary DON concentration, and we could not conclude on which approach provides the most accurate exposure estimate.

1. Introduction

Fusarium fungi, well-known producers of a range of mycotoxins, are widespread pathogens in cereals worldwide. Deoxynivalenol (DON), belonging to the group of mycotoxins termed trichothecenes, is probably the most widespread Fusarium toxin and is commonly found in cereals and cereal-derived food products (EFSA, 2017). DON was present in most samples of cereal-based food products including bread and breakfast cereals (EFSA, 2017; Sirot et al., 2013). In accordance with findings from other countries, earlier Norwegian studies demonstrated that DON was present in practically all samples of flour collected at mills producing for the Norwegian marked (VKM, 2013, Sundheim et al., 2017). In other countries, DON has generally been analysed in final food products rather than flour. DON is stable, does not decompose during normal food processing procedures such as milling or baking (Bergamini et al., 2010; Ivanova et al., 2017; Voss and Snook, 2010) and is therefore present in flour-containing food products.

DON is rapidly excreted in urine (Eriksen et al., 2003; EFSA, 2017), and urinary DON concentrations have been developed as a biomarker of human exposure (Turner et al., 2008). Toxicity of DON has been the subject of national and international risk assessments, including risk assessments by JECFA (2001) and EFSA (2017), and both JECFA and EFSA derived a Tolerable Daily Intake (TDI) of 1 μg/kg bw/day. The EFSA TDI applies to the sum of DON, 3- and 15-acetyl DON and DON-3-glucoside, while the JECFA TDI does not include the latter.

EFSA estimated the chronic dietary intake of DON including acetylated DON and the conjugate DON-3-glucoside in 111 dietary surveys from the European Union (EU) countries (EFSA, 2017). In the worst-case

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estimates, assuming that all samples with levels below the LOQ had concentrations equal to the LOQ (upper bound estimations), the estimated mean intakes exceeded the TDI for all dietary surveys in children and in several dietary surveys conducted among adolescents and adults. Assuming that the DON content in all samples below the LOQ were zero (lower bound estimations), only the high (95-percentile) intake estimates for infants and toddlers exceeded the TDI. These exposure estimates were based on mean DON concentrations in food sampled in Europe combined with consumption data from the different surveys. Flour used for food production in Norway is produced from nationally grown cereals mixed with a highly variable proportion of cereals imported from the world market (VKM, 2013). The occurrence of DON in Norwegian cereal products may therefore vary from the mean concentrations calculated by EFSA. Furthermore, the Norwegian diet is characterized by a relatively high consumption of whole grain products, and as DON is normally found in higher concentrations in the outer part of the grain, this may also contribute to a higher intake of DON in Norway. This may be particularly relevant for the growing group of vegetarians, assuming they have a higher grain consumption. Vegetarians is a population group for which there is limited information on DON exposure both in Norway and internationally (Leblanc et al., 2005; Wells et al., 2017).

Currently, there are two different analytical approaches for measuring DON in urine. The first method developed was based on enzymatic deconjugation of DON glucuronide (Meky et al., 2003). Later, an approach where the DON conjugates are measured separately in the urine was developed (Warth et al., 2011). The latter approach is currently becoming more widespread as the analytical standards for DON-glucuronides have become available, the approach is less work-intensive, and allows simultaneous detection of several mycotoxins.

The estimated dietary intake of DON in humans is associated with uncertainty, both in food consumption data and in the occurrence levels in food. An alternative way to obtain exposure estimates is to calculate the daily dietary DON intake based on measured concentrations of DON in spot urine samples. Several publications describe such calculations using varying models. One main approach is to combine the urinary DON concentrations with a daily urine volume. Most studies using this approach have applied a default volume of either 1.5 or 2 L for 24-hour urine excretion in adults, but a few studies estimated the 24 h urinary excretion based on body weight (De Santis et al., 2019; Papageorgiou et al., 2018a). Another main approach is to normalize the urinary DON concentrations to creatinine (ng DON/mg creatinine), estimate the 24 h-creatinine excretion and then use this estimate to calculate the amount of DON excreted. Several models for estimation of daily creatinine excretion have been published and at least one of them has previously been used to estimate the daily DON intake (Turner et al., 2010). The main metabolites of DON in humans are 3- and 15-DON-glucuronides and the de- epoxide DON (DOM-1) and its conjugated form DOM-1 glucuronide (EFSA, 2017). The DON-glucuronides and DOM-1 are considered as much less toxic than DON and were virtually not cytotoxic in the tested concentration range (Eriksen et al., 2004; Wu et al., 2007).

A main aim of the current study was to determine the urinary concentrations of DON and its main metabolites in individuals and to assess whether the urinary DON concentrations differ between men, women, children, vegetarians, and pregnant women. A second aim was to compare a set of models estimating the dietary intake of DON based on the urinary DON concentrations and compare these estimates with DON intake calculations based on reported food consumption data.

2. Material and methods

This study was a part of an international study including data from Italy, Norway, and the UK. The results were reported by Brera et al. (2015) and national results have been published in scientific papers from Italy (De Santis et al., 2019) and the UK (Papageorgiou et al., 2018a, b; Wells et al., 2016, 2017). The current study reports more in-depth results from Norway. The study population includes some additional participants that were not included in the joint report. All methods for recruitments, food registration, sampling, sample handling and analytical methods were strictly harmonized, and analytical procedures adapted to the at the time best validated method and the facilities available at the participating laboratories. However, the food record used in Norway was more detailed than in the other countries.

2.1. Ethics

The study was conducted according to the guidelines laid down in the Declaration of Helsinki. All adult participants and parents of participants younger than 16 gave an informed written consent to participate. The study was approved by the Regional Committee for Medical and Health Research Ethics (REK 2014/207).

2.2. Recruitment

The study was designed as a cross-sectional study. A part of this study was reported in a report from an international study of dietary intake of DON (Brera et al., 2015), but the present paper includes additional participants. In addition, here we have used the urinary DON concentrations to estimate the dietary exposure. We recruited participants among employees at the Norwegian Institute of Public Health and the Norwegian Veterinary Institute from April to December 2014. In order to recruit participants from a wider range of age and population groups, we also recruited children and extended family member from the employees. The study included 257 participants representing men, women, children, adolescents, adults, elderly, pregnant, and vegetarians. Information about sex, age, weight, height, dietary restriction, vegetarian dietary practice, smoking and other lifestyle habits were recorded during a short interview. Together with a project worker, they chose two days to record all food, drink and dietary supplements consumed in an open diary and to provide first morning void spot urine samples on the two mornings after completing the food records. Most participants (90%) collected data on two consecutive days.

2.3. Food intake data

Participants received an open food diary and information on how to register all food and drink items for two whole days (2 × 24-h food record for each participant). The participants were instructed to record all items to the nearest gram (or mL). When a kitchen scale was not available, we asked participants to report consumed amounts in household measures and to include a description of the portion size (small, medium, large). For mixed dishes, we asked for the name of the dish and a list of single ingredients in addition to the total weight or household measure.

2.4. Urine sampling

All participants collected fasting spot urine samples the two mornings following food recording. The participants received two 0.5 L plastic bottles with a wide opening and a double lid for collecting urine samples. The urine samples were kept cold until collection by a project worker on the day of the last sample, distributed into vials and stored at −20 °C until analysis.

2.5. Analytical methods

2.5.1. Deoxynivalenol

All urine samples were analysed for DON, DON-3 and DON-15 glucuronides and DOM-1 using the method described by Turner et al. (2008). Briefly, frozen urine samples were thawed, centrifuged at 2000 g for 15 min at 4 °C. Two aliquots of 1 mL were mixed with ^{13}C labelled DON (Sigma, St Louis, MI, USA) to a final concentration of 20 ng/mL.

β-glucuronidase (Type IX-A, Sigma (St Louis, MI, USA)) was added to one aliquot and left for 18 h at 37 °C. Both aliquots were then diluted to 4 mL with phosphate buffered saline (PBS, pH 7.4) and passed through an immune affinity column (VICAM G1066, Milford, MA, USA). DON was eluted by adding 4 mL HPLC-grade methanol. The aliquots were then evaporated at 50 ºC under N2 using an XCV-5400 XcelVap™ Automated Evaporation System (Horizon technology, Salem, USA), reconstituted in 250 μL of 10% ethanol in water and injected to the LC-HRMS.

The amount of glucuronic acid-conjugated DON was determined by subtracting the free DON concentration from the total DON concentration that was obtained following treatment with β-glucuronidase. DOM–1 and DOM-1- glucuronide were determined in the same aliquots. Urinary DON and DON-metabolites were analysed by liquid chromatography interfaced to high-resolution mass spectrometry (LC–HRMS). The instrument used was a Thermo Fisher Scientific (Waltham, MA, USA) Vanquish Horizon UHPLC interfaced to a Q-Exactive™ Hybrid Quadrupole-Orbitrap mass spectrometer equipped with a heated electrospray ion source (HESI-II). Chromatographic separation was performed using a 2.1 × 100 mm Acquity HSS T3 column (1.8 μm particles; Waters, Milford, MA, USA). The compounds were eluted using a mobile phase consisting of 5 mM ammonium acetate and 0.1 % formic acid in water (A), and 98% acetonitrile containing 5 mM ammonium acetate and 0.1% acetic acid (B). Elution proceeded isocratically with a flow rate of 0.4 mL/min for 1 min using 0% B, followed by a linear gradient to 15% B over 15 min, and then to 95% in 1 min. After flushing the column with 95% B for 3 min, the mobile phase composition was returned to the starting conditions over 1 min, and the column equilibrated for 3 min. The column was maintained at 30 °C. The injected sample volume was 1 μL and the autosampler tray temperature was maintained at 17 °C. The HESI-II interface was operated at 300 °C, automatically switching between positive and negative mode during the same run, and the source parameters were: spray voltage 4 kV, transfer capillary temperature 250 °C, sheath gas flow rate 35 units, auxiliary gas flow rate 10 units, and S-lens RF level 55%. In screening and quantification experiments, full scan (FS) mass spectra were recorded in the *m/z* range 200–710 in both ionization modes. The automatic gain control (AGC) target was set to 5×10^5 and the maximum injection time (IT) was set to 250 ms. Data were acquired with a mass resolution of 70,000 full width half maximum (FWHM) at *m/z* 200. The Xcalibur™ version 2.3 software (Thermo Fisher Scientific) was used for instrument control and calculation of mass errors and elemental compositions. Target compounds were quantified based on extracted ion chromatograms of acetate adducts (±5 ppm, *m/z* 355.1387 for $[DON + acetate]^-$ and *m/z* 339.1438 for $[DOM\text{-}1 + acetate]^-$). DON was quantified using internal calibration with reference to U-^{13}C-labelled DON, while DOM-1 was quantified using matrix-matched calibration.

The calibration curve was set by the injection of DON and ^{13}C-DON standard solution (prepared in 10% ethanol) covering the range 2–250 ng/mL, corresponding to 0.50–50 ng/mL urine. Limits of detection (3 × S/N ratio) were 0.005 ng/mL urine for DON and 0.009 ng/mL for DOM-1, while the limits of quantification (9 × S/N ratio) were 0.015 ng/mL and 0.27 ng/mL for DON and DOM-1 respectively. The method was validated in a comparison study between three laboratories as described by Brera et al. (2015).

2.5.2. Creatinine

Urinary creatinine concentrations were analysed at the Department of Drug Analysis, Norwegian Institute of Public Health by an accredited method using a colorimetric assay (modified kinetic Jaffe method) on a Beckman Coulter AU680 analyser (Beckman Coulter Inc., Brea, CA, USA). The method is based on a reaction with alkaline picrate forming a red–orange complex. The colour intensity is directly proportional to the creatinine concentration and measured spectrophotometrically.

2.6. Exposure estimates

2.6.1. Exposure estimates based on urinary volumes

We calculated the dietary intake of DON from urinary concentrations by combining urinary DON concentrations with either standard volumes of 24-hour urine excretion or by estimated 24-hour urinary secretion volume. Previous studies of urinary DON as a biomarker have used default values of either 1.5 or 2 L of urine as 24-hour volume for adults. To enable comparison with other studies, we have used 1.5 L for adults, 1.25 L for adolescents and 1.0 L for children. These are the same as used by Heyndrickx et al. (2015) and Turner et al. (2010), while EFSA (2017) and Warth et al. (2012) used 2 L for adults. According to Turner et al. (2010), 72% of the ingested DON was excreted in the urine within 24-hours. Since then, this carry-over value has been used in most intake estimates. Using these parameters, we calculated the daily intake to DON according to Equation Eq. (1a):

$$\text{Estimated daily intake } (\text{ng/kg bw/day}) = c \times (V/bw) \times (100/E) \quad (1a)$$

where:

c = total DON concentration in the analysed urine samples (ng/mL urine);
V = urine volume of 1.5 L/day for adults, 1.25 L/day for adolescents and 1.0 L/day for children;
bw = body weight (kg) reported in the questionnaire;
E = urinary excretion rate of DON in 24 h, 72% (Turner et al., 2010).

A mean 24-hour urinary production rate of 1.0 mL/kg bw per hour for adults and 2.0 mL/kg bw per hour for children was used to estimated DON intake in Italy (De Santis et al., 2019) and UK (Papageorgiou et al., 2018a). Urinary volumes were estimated based on Klingensmith et al. (2008) and Kliegman and Geme (2015) for adults and children respectively. Using these estimates for urine secretion, a daily DON exposure was estimated using the same equation (Eq. (1a)), but where the 24 h urine volume (V) in L was estimated as follows:

$$\begin{aligned} V(\text{L per day}) = {} & 0.001 \text{ L/kg bw/day} \times 24 \text{ h} \\ & \times \text{bw (kg) for adolescents and adults; } 0.002 \text{ L/kg bw/day} \\ & \times 24 \text{ h} \times \text{bw(kg) for children;} \end{aligned} \quad (1b)$$

2.6.2. Exposure estimates based on creatinine adjusted DON concentrations

To adjust for the variability in urine volume, excretions are calculated based on daily creatinine. We estimated the daily creatinine excretion using three different models and then we calculated the total DON excretion based on the estimated daily creatinine excretion. Several models for estimating daily creatinine excretion are available. Input parameters in the models are for example age, sex and either body weight or BMI. We have estimated the individual daily creatinine excretion using an online creatinine calculator (http://www.clinicalculator.com/english/nephrology/excrea/excrea.htm) previously used by Turner et al. (2010) to estimate DON excretion, as well as by other more recent models for creatinine excretion developed for clinical use (Daugirdas and Depner, 2017; Forni Ogna et al., 2015; Ix et al., 2011).

We used the following equations according to the online clinical calculator:

$$Creatinine\ Excretion\ (mg/day)\ (women) = \left(22 - \left(\frac{age}{9}\right)\right) \times BM \quad (2)$$

$$Creatinine\ Excretion\ (mg/day)\ (men) = (28 - \left(\frac{age}{6}\right)) \times BM \quad (3)$$

where BM is body mass (kg) and age is expressed in years. The equation is based on the Cockcroft-Gault equations for estimating creatinine excretion originally developed for control of kidney function in patients

with kidney disease (Cockcroft and Gault, 1976).

We also used a second model for estimating daily creatinine excretion developed in the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI), based on a *meta*-study of 6 cohorts from several countries and involving 3453 patients (Ix et al., 2011):

$$\begin{aligned} &Creatinine\ excretion\ (ng/kg\ bw/day): \\ &= 879.89 + 15.51 \times BM - 6.19 \\ &\times age\ (years) + (34.51\ if\ black) - (379.42\ if\ female) \end{aligned} \quad (4)$$

Finally, we used a more recently developed model based on two cohorts in Switzerland, one designed for estimating the dietary intake of salt and hypertension and one longitudinal study focusing on the associations between genes, kidney hemodynamics and environment on hypertension, with a total 2131 participants from European descent (Forni Ogna et al., 2015):

$$\begin{aligned} &\text{Creatinine excretion}(\mu mol/day): \\ &= 266.16 - 47.71(\text{if woman}) - 2.33 \\ &\times \text{BMI} + 0.66 \times \text{age} - 0.017 \times \text{age}^2 \end{aligned} \quad (5)$$

2.6.3. Exposure estimates based on consumption data

Two trained research assistants coded all food and drink items in the food records manually. Food items were assigned a food code number used in the 2015 version of the Norwegian Food Composition Table. Food intakes in grams per day were multiplied with DON occurrence in foods using two deterministic approaches. First, we used the lowest mean DON concentration measured in a total of 111 samples of Norwegian milled wheat flour, sieved wheat flour, wheat bran and oat flakes during the years 2008 to 2011 to estimate DON concentrations (μg/kg) in bread, rolls, bakery wares and cereal products (VKM, 2013; Sundheim et al., 2017). Modified forms of DON were not included in these data as occurrence data were not available for these forms. For other breakfast cereals, rye bread and pasta, we use mean DON values from the European occurrence data reported in the EFSA risk assessment of DON (EFSA, 2017). Secondly, we used mean European occurrence values for all the above food items. For both approaches, we only included DON values for foods with <10% of samples having values below the limit of detection. The mean DON occurrence data were then combined with the individual food consumption data for all cereal-containing food items.

2.7. Statistics

We present participant age and weight as mean ± SD. However, the urinary DON and creatinine concentrations were not normally distributed and are described by median and quartiles (25 and 75 percentiles). We used non-parametric statistics to compare differences in the urinary concentrations between groups and the Kruskall-Wallis 1-way ANOVA to check for factors affecting the urinary concentrations. Wilcoxon rank sum pairwise comparison was used to compare urinary concentrations for more than two groups, and Spearman rank correlation to evaluate agreement between the different models for estimating dietary intake based on urinary concentrations and the agreement between estimated DON intakes from urinary analysis and food intake data. Statistical differences were considered as significant when *p*-values were <0.05. Bonferroni corrections for multiple comparisons were used.

3. Results

3.1. Participant characteristics

A total of 257 persons were recruited, 155 (60%) females and 102 males. The study included 30 vegetarians, of which two where adolescents and 28 adults. The vegetarian group included all persons from all age groups with no intake of meat and fish, but some of them consumed eggs and/or milk. Pregnant women and vegetarians were not included in the age groups. Table 1 shows participant characteristics according to age, life-stage and vegetarian diet.

3.2. Urinary DON concentrations

DON and metabolites were detected in all samples apart from both samples from one individual. This individual had not registered any consumption of cereal or cereal products. There were no significant differences between days 1 and 2 (Overall *p*-value 0.51, *p*-values 0.09–0.95 in different population groups, Supplementary data, S1). We therefore used the mean concentration of the two samples from each individual in further analyses as this is thought to better reflect the exposure over time. DON concentrations were expressed as ng total DON (sum of DON, DON-Glc-A and DOM glucuronide/mL as well as ng total DON/mg creatinine (Fig. 1, Supplementary Table S2). Acetylated DON was not detected in any sample. The urinary concentration of total DON was slightly, but statistically significantly higher in males than in females (overall median values of 6.9 vs 5.1 ng/mL, p = 0.0005), but there was no significant difference when the concentration was expressed as ng DON/mg creatinine (median 7.2 vs 6.1 ng/mg creatinine, p = 0.095).

The urinary concentration of total DON differed significantly between some age groups (p < 0.0001, Kruskall-Wallis) and was significantly different between age groups when stratified by sex (Fig. 1, Table S2).

DON was mainly present in urine as DON-glucuronide (Table 2). Overall, 81 % of the total DON excreted in urine was present as DON-glucuronides, with a range from 40 to 100% (P25: 77% and P75: 85%). In addition to having higher urine concentrations of DON, the children and female adolescents also had significantly higher proportion of unconjugated DON compared to adults (Table 2).

In addition, the conjugated de-epoxide metabolite DOM-1-glucuronide was detected in the morning urine sample of 12% of the participants. In contrast to several other papers (summarized in Ali et al., 2016), we included this metabolite in the total DON. However, DOM-1 glucuronide constituted a low percentage of total DON in almost all individuals and the 95-percentile for DOM-1 did not exceed 5% of total DON. There were no differences in the prevalence or concentration of the de-epoxide metabolite between population or age groups.

3.3. Estimated intake of DON based on urinary DON concentrations

Using the urinary DON concentrations, we estimated the mean dietary intake to 0.05–0.73 μg/kg bw/day depending on population group and models for estimating the intake (see methods Sections 2.6.1 and 2.6.2) (Table 3). Three out of the five models used creatinine adjusted DON concentrations (urinary DON adjusted for creatinine is reported in Supplementary Table S2) and two models used either estimated or assumed volume of urine secretion. All three models using creatinine adjusted DON concentrations resulted in similar estimated DON intakes.

Table 1
Participant characteristics by subgroups. Total n = 257[1].

Subgroup	n	Age (years)	Sex Male n (%)	Body weight (kg)
Children 3–9 years	47	6.3 ± 1.9	30 (64)	23.3 ± 5.6
Adolescents, 10–17 years	46	13.4 ± 2.3	17 (37)	49.7 ± 12.7
Adults 18–64 years	71	42.1 ± 11.4	34 (48)	73.2 ± 12.7
Elderly 65 + years	23	70.7 ± 6.7	11 (48)	72.1 ± 11.3
Pregnant women	40	33.8 ± 4.2		66.9 ± 8.1
Vegetarians	30	36.0 ± 12.6	10 (33)	67.8 ± 14.9

[1] Pregnant women and vegetarians are not included in the statistics for age groups.

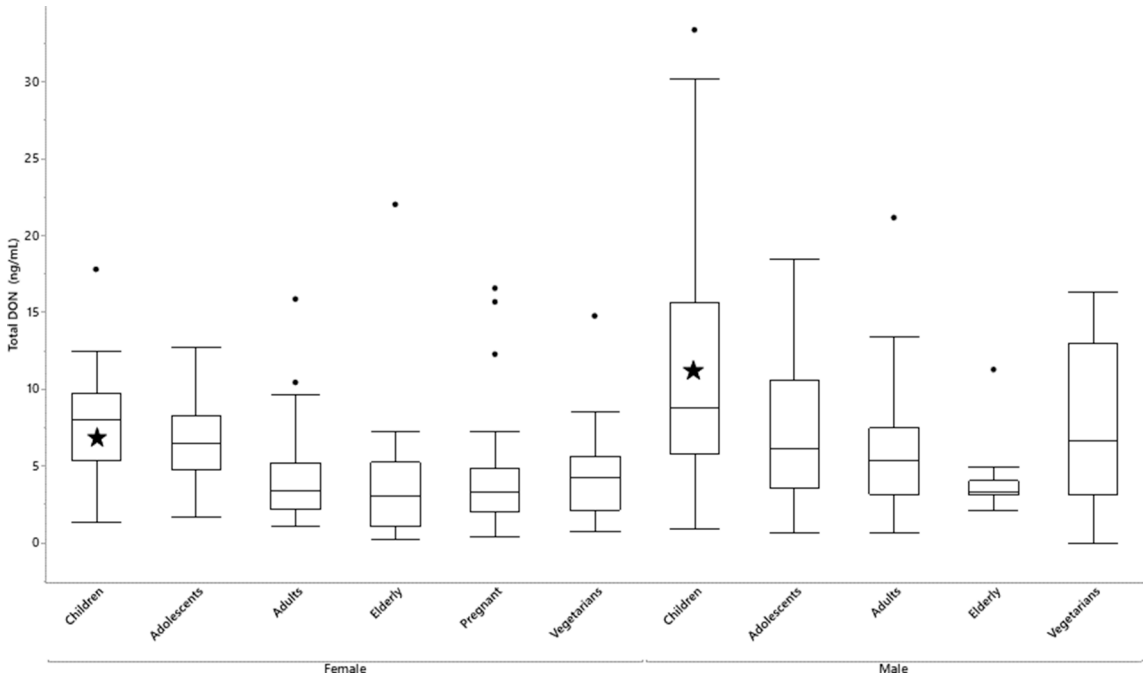

Fig. 1. Concentrations of sum of DON-metabolites (DON, DON-glucuronides and DOM-1 glucuronide) in morning urine in different age and population groups. Unconjugated DOM-1 was not detected in any sample. The boxes show median value and 25 and 75 percentiles for each group. Whispers indicate 5 and 95 percentiles. The concentrations of DON metabolites in morning urine for each age group were compared to adults of the same gender. *: statistical different from adults of same gender.

Table 2
Proportion of unconjugated DON in morning urine in different age groups. All comparisons were made using Wilcoxon pair-wise comparisons. P values were from comparisons with adults of the same sex. Vegetarians were included as vegetarian independent of age. Differences regarded as statistically significant when $p < 0.0056$ according to Bonferroni correction for multiple comparisons using $p < 0.05$ and 9 comparisons.

Subgroup	Median unconjugated DON (%) (25th and 75th percentile)			
	male	*p*-value	*female*	*p*-value
Children 3–9 years (n = 47)	21 (18, 24)	*0.001**	25 (20, 28)	*0.003**
Adolescents, 10–17 years (n = 46)	21 (19, 22)	*0.259*	20 (19, 24)	*0.002**
Adults 18–64 years (n = 71)	16 (13, 19)	–	15 (12, 21)	–
Elderly 65 + years (n = 23)	17 (15, 28)	*0.136*	12 (5, 17)	*0.436*
Pregnant women (n = 40)			16 (11, 20)	*0.585*
Vegetarians (n = 30)	23 (17, 35)	*0.008*	17 (14, 24)	*0.634*

* Statistical difference by comparisons with adults of the same sex using Wilcoxon rank sum comparisons.

The estimated intakes were 3 to 5-fold higher in children than in adults and slightly higher in males than in females. The creatinine adjusted estimates were however lower than those based on urine volumes. This was particularly evident for the calculations based on estimated urine excretion. This model also resulted in higher mean intakes in females than in males in most population groups. Pregnant women had estimated intakes similar to other women. Even though the group "Vegetarians" included all age groups and are thus not directly comparable to other age groups, the median values were in the same range as for adults, probably reflecting the majority of individuals belonging to this age group. Hence, depending on the model used, the estimated daily intake based on creatinine-adjusted concentrations exceeded the TDI for 0–3 male children. In addition, the model estimating the urinary excretion volume resulted in 3 female and 7 male children also having an intake above the TDI. The estimated intake was below the TDI for all other participants.

The correlations between the different models were high (0.79–0.99, Supplementary Table S3).

The pairwise comparisons between the different models estimating exposure did not reveal any significant differences between the exposure models based on creatinine-adjusted DON concentrations (Eqs. (2)–(5), *p*-values 0.521–0.997), while the models based on urine volumes differed from each other ($p = 0.0005$) as well as from the exposures estimated on the creatinine-adjusted DON concentrations (*p*-values in Supplementary Table S4).

3.4. Estimated intake of DON based on food consumption data

As for urinary DON, there were no significant differences between DON intake calculated from food for day 1 and day 2 in the food record. Therefore, the mean of the two days is presented. In all participants combined, the mean (95th percentile) DON intake was 18.1 (34.5) μg/day using Norwegian occurrence data and 15.6 (29.5) μg/day using European occurrence data. The estimated dietary intake using Norwegian occurrence data from the years 2008–2011 resulted in significantly higher exposure than using the occurrence data from the EFSA database ($p < 0.001$). Bread, rolls, and bakery wares contributed on average 74% of total DON intake, while breakfast cereals contributed 15% and pasta 11% using the Norwegian occurrence data.

Calculated DON intakes expressed per kg body weight and evaluated by age, life stage or dietary practice, were significantly higher in children and adolescents than in the other groups (Table 4). The calculations indicated that 12 children and 1 adolescent had a dietary intake exceeding the TDI when the Norwegian occurrence data were used, while 5 children exceeded the TDI when the EFSA occurrence data were used in the calculations.

Table 3
Exposure estimates (Mean (P25, P75)) for different population groups based on urinary concentrations of DON, DON-glucuronides, DOM-1, and DOM-1 glucuronide.

Group	DON exposure estimates based on creatinine-adjusted concentrations μg/kg bw per day						DON exposure estimates based on urine volumes μg/kg bw per day			
	Calculated using model from creat calc[1]		Calculated by model from Ix et al., 2011[2]		Calculated by model from Ogna et al., 2015[3]		Calculated by estimated urine excretion[4]		Calculated using standard urine volume[5]	
	Male	*Female*	*male*	*Female*	*Male*	*female*	*male*	*female*	*Male*	*Female*
Children 3–9 years (n = 30 m, 17f)	0.34 (0.22, 0.50)	0.18 (0.14, 0.39)	0.67 (0.47, 0,94)	0.24 (0.21, 0.65)	0.34 (0.22, 0.47)	0,18 (0.14, 0.39)	0.59 (0.39, 1.05)	0.73 (0.51, 0.92)	0.55 (0.38, 0.97)	0.38 (0.25, 0.62)
Adolescents, 10–17 years (n = 17 m; 29f)	0.14 (0.11, 0.21)	0.13 (0.10, 0.18)	0.17 (0.13, 0.26)	0.14 (0.096, 0.19)	0.14 (0.11, 0.21)	0.13 (0.10, 0.18)	0.21 (0.12, 0.35)	0.31 (0.22, 0.39)	0.25 (0.13, 0.35)	0.26 (0.16, 0.36)
Adults, 18–64 years (n = 34 m; 37f)	0.11 (0.061, 0.16)	0.074, (0.060, 0.12)	0.11 (0.060, 0.14)	0.070 (0.055, 0.11)	0.13 (0.067, 0.17)	0.081 (0.06, 0.13)	0.18 (0.11, 0.25)	0.17 (0.11, 0.26)	0.13 (0.089, 0.18)	0.11 (0.07, 0.19)
Elderly, 65 + years (n = 11f, 12 m)	0.081 (0.060, 0.12)	0.081 (0.046, 0.16)	0.090 (0.07, 0.13)	0.078 (0.045, 0.15)	0.092 (0.067, 0.14)	0.081 (0.49, 0.14)	0.11 (0.11, 0.14)	0.15 (0.057, 0.25)	0.084 (0.080, 0.12)	0.11 (0.040, 0.16)
Pregnant women (n = 40)		0.092 (0.074, 0.15)		0.084 (0.071, 0.14)		0.095 (0.077, 0.15)		0.16 (0.10, 0.23)		0.097 (0.072, 0.16)
Vegetarians (n = 10 m; 20f)	0.18 (0.080, 0.27)	0.14 (0.087, 0.17)	0.18 (0.077, 0.23)	0.13 (0.085, 0.16)	0.20 (0.090, 0.26)	0.14 (0.095, 0.18)	0.22 (0.11, 0.43)	0.20 (0.10, 0.26)	0.19 (0.08, 0.33)	0.14 (0.072, 0.21)
n > TDI	3 (all children)						7 (all children)	3 (all children)	7 (all children)	

M = male, f = female).

Table 4
DON intake in the different groups calculated from food by two sets of occurrence data.

		DON intake[1], μg/kg bw/day			DON intake[2], μg/kg bw/day		
Age group	n	Mean (SD)	Median (25th and 75th percentile)	n > TDI	Mean (SD)	Median (25th and 75th percentile)	n > TDI
Children, 3–9 years	47	0.73 (0.34)	0.72 (0.51, 1.05)	12	0.62 (0.30)	0.58 (0.39, 0.84)	5
Adolescents, 10–17 years	46	0.43 (0.23)	0.37 (0.25, 0.53)	1	0.37 (0.18)	0.33 (0.25, 0.44)	0
Adults 18–64, years	71	0.25 (0.12)	0.23 (0.15, 0.32)	0	0.22 (0.11)	0.19 (0.15, 0.30)	0
Elderly, 65 + years	23	0.22 (0.09)	0.21 (0.16, 0.28)	0	0.18 (0.08)	0.19 (0.13, 0.23)	0
Pregnant	40	0.28 (0.14)	0.24 (0.18, 0.34)	0	0.24 (0.12)	0.20 (0.16, 0.30)	0
Vegetarians	30	0.27 (0.17)	0.25 (0.16, 0.36)	0	0.23 (0.15)	0.21 (0.13, 0.33)	0

[1] DON intake calculated using Norwegian occurrence data based on the lowest mean DON concentrations measure in Norwegian flour during years 20008–2011 for wheat-based breads and rolls, bakery wares, and oat dishes, complemented with mean European occurrence values for pasta, rye bread and breakfast cereals other than oat. The Norwegian data did not include the modified forms of DON.

[2] DON intake was calculated using mean European occurrence data of DON excluding modified forms) for all grain-based products.

3.5. Comparisons of exposure estimates based on urine concentrations and on food consumption data

Calculated DON intake correlated moderately with urinary total DON, both unadjusted and adjusted for creatinine, independent of which occurrence data we used for the exposure estimates (r = 0.43–0.47, p < 0.0001).

The dietary intake of DON calculated from food consumption was generally higher than the DON exposures estimated from urinary DON concentrations (Tables 3, 4).

The dietary DON intakes based on estimation of creatinine excretion according to the model by Ix et al. (2011, Eq. (4)) showed the best correlations with the intake estimates based on food consumption (Table 5). The estimates based on standard excreted urine volumes had a better correlation with estimated food intake than using the urine excretion estimated based on body weight. The intake estimates based on either Norwegian or European *occurrence data correla*ted equally well with the exposure estimates based on urinary concentrations.

Table 5
Spearman rank correlation coefficients and 95% confidence intervals between estimated 24-hour dietary intake of DON from Norwegian or EFSA occurrence data and intake estimates based on DON concentrations in morning urine according to five different models. All correlations were significant with p < 0.0001 (n = 257).

	Ix et al., 2011 (Eq. (4))	Ogna et al., 2015 (Eq. (5))	Creatinine calculator (Eqs. (2), (3))	Std urine volume* (Eq. (1a))	Estimated urinary volume (Eq. (1b))
Norwegian data	0.62 (0.54, 0.62)	0.55 (0.46, 0.63)	0.55 (0.46, 0.63)	0.61 (0.52, 0.68)	0.53 (0.44, 0.61)
EFSA data	0.62 (0.54, 0.69)	0.55 (0.45, 0.63)	0.55 (0.46, 0.63)	0.60 (0.52, 0.67)	0.52 (0.42, 0.61)

* Standard amount of urine excreted = 1.0 L/24 h for children, 1.25 L/24 h for adolescents and 1.5 L/24 h for adults.

4. Discussion

4.1. Urinary concentrations

DON and/or its metabolites were present in morning urine samples from virtually all participants in the present study, confirming the widespread exposure to DON from food. The urinary concentrations of DON from pregnant and vegetarians, and their calculated intakes based on urinary concentrations or food consumption, did not differ from other adults. This seems to contradict the assumption that vegetarians have higher grain consumption than other populations groups, but is in accordance with the knowledge that women's dietary patterns change little from before to during pregnancy and pregnant women have a diet not very different from other adult women (Crozier et al., 2009).

Children in the age group 3–9 years had higher concentrations of DON in their morning urine than other age groups (Fig. 1). A higher DON concentration in morning urine from children than from adults has also been reported in studies from the UK and Italy (De Santis et al., 2019; Papageorgiou et al., 2018a; Wells et al., 2017). In contrast, 10–12-year-olds in Belgium had a significantly higher urinary DON level compared to 3–6-year-olds (Heyndrickx et al., 2015). In addition to the higher energy requirements in children, these differences in DON concentrations between age groups may also reflect age-specific dietary habits.

Despite the high intake of whole grain products in Norway (Kyrø et al., 2012), the urinary concentrations of DON and metabolites in Norwegians were in a similar range as previously reported from several other European countries but lower than in some European countries (Chen et al., 2017; Ali et al., 2016). The reason for this somewhat unexpected finding might be that the levels of DON in food in the sampling year 2014 were lower than the concentration used for exposure assessments. Unfortunately, no samples of food grain or grain-containing food items were available for analysis of DON from this year in which we collected urine samples. As a surrogate, we used levels from the years 2008 to 2011 in order to estimate the dietary DON intake. These were the data available closest in time to the year of data collection.

According to the review by Chen et al. (2017), population studies have found mean urinary DON concentrations of 1.1–59 ng DON/mL with the highest mean concentration of 59.0 ng/mL reported from Belgium (Heyndrickx et al., 2015). A study in Swedish adults indicated that the analytical results using a single toxin method with enzymatic deconjugation are slightly higher than when applying a multi-toxin method on the same samples, but the difference was small (median of 2.5 vs 0.8 ng/mL) (Turner et al., 2017). This is in contrast to the findings reported by Vidal et al. (2020) who reported that the mean urinary concentration of total DON was 27.69 nmol/mL using enzymatic deconjugation and 37.95 nmol/mL using a direct analysis of each metabolite. They did not report how values below the LOQ was dealt with when calculating total DON and with more metabolites determined in the direct determination method, this may affect the total DON differently in the two methods. In our study we used a single toxin method (analysed for DON and DOM-1) with deconjugation based on a single mycotoxin method previously used in Sweden, UK, and other countries. A mean upper bond (samples < LOQ assumed to be at LOQ) urinary DON concentration of 3.4 ng/mL was reported from Sweden using a multi-toxin method (Wallin et al., 2015). In that study, 63% of the samples were positive for DON, but the LOQ was not given. In a recent study by the same group from Sweden, the proportion of samples positive for DON was considerably lower and only 4.8% and 9.0% of the urinary samples from adolescents were positive for DON and DON-15-glucuronide respectively (Warensjo Lemming et al., 2019). The latter study used a multi-toxin method with a higher LOD/LOQ compared to the first, which according to the authors may explain the lower incidence of positive samples. Overall, the results indicate that the urinary DON concentrations from the Norwegian population is in the same range as in other countries, despite the high intake of whole-grain products.

4.2. DON metabolites in urine

As reported from other studies, DON-glucuronide conjugates constitute approximately 80% of the total DON in urine in the present study. We found a statistically significant higher proportion of unconjugated DON in children compared to the other age groups (Table 2), but the sample size in each age group is limited, and verification of this is needed. As the conjugated DON is considered to be less toxic than DON, an increased proportion of unconjugated DON may render children more vulnerable to the harmful effects of DON than older age groups. This may be further aggravated by the higher total DON intake relative to body weight in children. The reason for a higher proportion of unconjugated DON in children remains unknown. Maul et al. (2015) reported that two out of 10 recombinant human uridine-diphosphoglucuronyltransferases (UGTs) were able to conjugate DON. UGT2B4 predominantly formed DON-15-glucuronide while UGT2B7 predominantly formed DON-3-glucuronide. Previously DON-15-glucuronide has been found to be the main DON glucuronide in human urine even though also DON-3-glucuronide is present (Heyndrickx et al., 2015; Warth et al., 2013). In this study we used enzymatic deconjugation prior to analysis, and we could not separate the conjugated forms of DON. There are conflicting reports on the enzymatic capacity of UGT2B4 in children compared to adults. A capacity similar to adults was found in the first month after birth in one study (Badée et al., 2019), while another study reported that UGT2B4 still had a reduced capacity in two-year-olds compared to adults (Strassburg et al., 2002). However, UGT2B7 was already fully active during the first six months in both of these studies. It is therefore unclear if a reduced capacity of UGT2B4 in children may explain the difference in percent of unconjugated DON in urine between adults and children. Additionally, there is individual variation in percentage of unconjugated DON in urine within each age group, with an overall range from 40 to 100%. This may contribute to individual sensitivity to DON exposure. However, the proportion of the population with a low DON conjugation capacity seems to be rather low.

The DON-metabolite DOM-1, lacking the epoxide group considered essential for toxicity (Eriksen et al., 2004), was present in 12% of the Norwegian morning urine samples. DOM-1 was only detected as glucuronide conjugate and generally in low proportions of the total DON (maximum 37%, 97.5 percentile 7.5 %). DOM-1 and/or its conjugate has also previously been detected in human urine samples (Gratz et al., 2014; Föllmann et al., 2016; De Santis et al., 2019; Papageorgiou et al., 2018a; b). The de-epoxidation appears to be carried out by gut microbiota prior to absorption (EFSA, 2017). *In vitro* faecal incubations indicate individual variations in the ability of the human faecal microbiota to transform DON to DOM-1 (Eriksen and Pettersson, 2003; Gratz et al., 2013; Heyndrickx et al., 2015). Overall, the proportion of DON transformed to DOM-1 is low in both the present and in previous studies, indicating that this metabolite is of minor importance as a biomarker of exposure in a population. However, of interest, for some individuals DOM-1 may constitute a substantial proportion of total DON, and this may contribute to variation in individual sensitivity to DON.

4.3. Comparisons of different models for estimating DON exposure

The urinary DON concentration has frequently been used to estimate the intake of DON from the diet (see e.g. Turner et al., 2008; Heyndrickx et al., 2015; Chen et al., 2017; de Santis et al., 2019). In contrast to many other studies (see Ali et al., 2016), we included urinary concentrations of DOM-1 glucuronide in the total DON. This is not likely to have any significant impact on the outcome as DOM-1 was <5% of total DON in >95% of the participants. Collection of complete 24 h urine samples is not convenient in large population studies and is normally only carried out in studies with a limited number of participants. As an alternative, the dietary intake is therefore frequently estimated based on DON concentrations in the morning urine combined with estimates of urine

excretion. The different models for estimating the dietary intake of DON based on urinary DON concentrations resulted in estimated intakes in a similar range (Table 3), and the correlations between the different models were high (0.79–0.99, Table 5). This indicates that all five models have similar ability to predict the exposure. However, the available data do not allow us to decide which of the models best predicts the dietary DON intake in the population. This can only be established by comparison of estimates based on morning urine with measured DON intake, and this has to our knowledge not been reported so far. Our findings indicate that the calculations based on DON concentration in morning urine can be useful tools for estimating the dietary intake on a population level. In our study, we found that the dietary intake of DON calculated from food consumption was generally higher than the DON exposures estimated from urinary DON concentrations (Tables 3, 4).

For children there was also a significant difference ($p < 0.05$) in the estimated DON exposure between boys and girls in four of five models. The exception was the model using standard urine volumes of 1 L/day. This sex difference in estimated DON exposure was not reflected in a significant difference in urinary concentrations of total DON, even though it was a tendency towards higher DON concentration in morning urine from boys than from girls (Fig. 1, Supplementary Table S3). This discrepancy may be explained by differences in creatinine excretion. All models for creatinine excretion are validated for adults only (>17 years or > 150 cm), and the application in children is therefore associated with uncertainty and should be interpreted with care. Warth et al. (2013) used a correction factor of 2 for morning urine being more concentrated than other urine. According to the authors, this factor was, based on "observations" and no data supporting the factor was given in the paper. We therefore decided not to use this factor in our estimations. In all models, there are outliers indicating that there are individuals with a deviating excretion pattern and estimating the dietary intake on an individual level could be misleading. Despite this limitation, we calculated that some children have dietary exposure above the group TDI of 1 μg/kg bw per day for the sum of DON, 3-Ac-DON, 15-Ac-DON, and DON-3-glucoside set by EFSA. This is in line with the dietary intakes also calculated by EFSA based on dietary surveys in children in Europe (EFSA, 2017).

The proportion of ingested DON excreted in the urine within 24 h is also an important factor in the calculations. Most estimates of DON intake based on urinary excretion, including ours, have used 72% excretion as established by Turner et al. (2010). A similar carry-over of 68% was also reported from one individual consuming naturally contaminated food (Warth et al., 2013). In the study by Turner et al. (2010), the contribution from acetylated and glucosidated DON to the dietary intake of total DON was not taken into account. Therefore, the total intake of DON analogues could have been higher than the estimated intake. EFSA estimated the relative ratios of acetylated DON to 25% of DON and of DON-3- glucoside to 20% of DON indicating that the dietary intake of total DON could have been 45% higher than estimated in the present study, which would mean that the actual proportion excreted in urine is lower than the reported 72%. In a more recent study, a mean excretion rate of 64.0 ± 22.8% was reported from twenty human volunteers given a bolus with a mixture of DON and DON-3-glucoside (Vidal et al., 2018), a finding which is still close to 72% which is frequently used (Turner et al., 2010). However, there is also uncertainty related to this excretion rate, as the bioavailability in a bolus may differ from DON in food. The individual variation in the proportion of DON excreted in urine within 24 h was substantial, and the 95% confidence interval in the study by Turner et al. (2010) with 35 participants was 59–86%.

Children had a higher estimated intake of DON compared to adolescents, adults, and elderly (Table 3). The higher intake in children is as expected, based on their higher energy requirements relative to body weight, and is in accordance with previous estimates of dietary exposure in European surveys (EFSA, 2017, Chen et al., 2017). Nevertheless, few individuals had an intake above the TDI, this being the case for only 0–21% of the children aged 3–9 years, depending on model for estimating the intake (Table 3). This was seen also for DON estimated from food intake, with 5–12% of children aged 3–9 years having a DON intake > TDI depending on the origin of the samples for occurrence levels in the grain-based food. In a previous risk assessment, the chronic dietary intake of DON in Norway, excluding the modified forms, was estimated based on food consumption and occurrence of DON in food calculated by use of occurrence levels in flour and oatmeal (VKM, 2013; Sundheim et al., 2017). DON was detected above the LOQ in 98% of the food samples. The median estimated DON intake was highest for young children on a body weight basis, and it was close to or exceeding the TDI for 1–9-year-old children. The corresponding estimated 95 percentile exposures were up to 3.5 higher than the TDI. The estimated intakes for teenagers and adults were all below the TDI and not of concern. The estimated intake for adults was also in the same range as previously reported from several European countries including Norway (De Ruyck et al., 2020). The dietary intake of DON in Norway in potential vulnerable groups such as pregnant women and elderly has not previously been estimated.

4.4. Comparison of exposure estimates based on food consumption vs estimates based on urinary DON concentrations

The DON intakes based on reported food consumption during the 24 h prior to the morning urine sampling was higher than DON intakes based on urinary DON. The correlations between dietary and urinary DON were in the range of 0.43–0.47. In the context of biomarkers of dietary intake, such correlations are frequently considered as moderate (Fraser et al., 2016). Given the inherent uncertainties in self-reported food intake and the uncertainties related to the use of either a limited number of flour samples collected from the Norwegian market or samples from all EU countries with a variable origin, the correlations were higher than expected.

Biomonitoring of DON in urine can be a valuable tool for surveillance of population exposure to DON. DON levels in urine can for example reveal regional differences in exposure due to weather and/or climate-related variations in DON concentrations in food, since it will also reflect each country or region's degree of consumption of locally produced grains or in annual variations in DON content (Gratz et al., 2014; Chen et al., 2017; Ali et al., 2016). A major uncertainty when evaluating DON exposure based on food consumption is the degree of representativity of grain or food items analysed for DON for the food actually consumed (EFSA, 2017). On the other hand, urinary DON concentrations only reflect short-term intake, whereas food surveys are meant to capture habitual (chronic) intake. However, as grain and grain-based foods (staple foods) are consumed on a regular daily basis without large variation, this can be considered a small uncertainty compared to regional variations in food contamination.

5. Conclusions

DON and metabolites were present in virtually all morning urine samples collected in Norway. The concentrations were in a similar range as reported from elsewhere in Europe. Children had higher urinary concentrations of DON than adults and elderly. The concentrations of DON in urine samples from pregnant and vegetarians did not differ from other adults. Five different models for estimating the dietary intake of DON based on urinary DON concentrations resulted in highly correlating exposure estimates. The estimated intakes also correlated well with exposure estimates based on food consumption combined with concentrations in food, but the latter estimates tended to be slightly higher. The exposure estimates based on urinary concentrations may be a useful tool for evaluations of risk related to DON exposure on a population level.

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CRediT authorship contribution statement

Gunnar Sundstøl Eriksen: Conceptualization, Methodology, Writing – original draft. **Helle K. Knutsen:** Conceptualization, Methodology, Writing – review & editing. **Morten Sandvik:** Methodology, Writing – review & editing. **Anne-Lise Brantsæter:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2021.106804.

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