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Combined exposures to bisphenols, polychlorinated dioxins, paracetamol, and phthalates as drivers of deteriorating semen quality

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ABSTRACT

Background: Semen quality in men continues to decline in Western countries, but the contours of the issue remain obscure, in relation to contributing chemicals.
Objectives: To obtain more clarity about the chemicals that drive the deterioration of semen quality, we conducted a mixture risk assessment based on European exposures.
Methods: We included chemicals capable of affecting semen quality after prenatal exposures, among them androgen receptor antagonists, substances that disrupt prostaglandin signalling, suppress testosterone synthesis, inhibit steroidogenic enzymes or activate the aryl hydrocarbon receptor. We employed the Hazard Index approach (HI), based on risk quotients of exposures in Europe and reference doses for reductions in semen quality. By summing up the risk quotients of the 29 chemicals included in the assessment we examined fold-exceedances of "acceptable" mixture exposures relative to an index value of 1. For bisphenols A, F, S, phthalates DEHP, DnBP, BBzP, DiNP, n-butyl paraben and paracetamol we relied on biomonitoring studies in which these 9 chemicals were measured together in the same subjects. This allowed us to construct personalised Hazard Indices.
Results: Highly exposed subjects experienced combined exposures to the 9 chemicals that exceeded the index value of 1 by more than 100-fold; the median was a 17-fold exceedance. Accounting for median background exposures to the remaining 20 chemicals added a Hazard Index of 1.39. Bisphenol A made the largest contribution to the HI, followed by polychlorinated dioxins, bisphenols S and F and DEHP. Eliminating bisphenol A alone would still leave unacceptably high mixture risks. Paracetamol is also a driver of mixture risks among subjects using the drug.
Conclusions: Tolerable exposures to substances associated with deteriorations of semen quality are exceeded by a large margin. Bisphenols, polychlorinated dioxins, phthalates and analgesics drive these risks. Dedicated efforts towards lowering exposures to these substances are necessary to mitigate risks.

Abbreviations: AF, Assessment factor; AGD, Anogenital distance; AhR, Arylhydrocarbon receptor; AR, Androgen receptor; AUC, Area under the curve; BBzP, Butylbenzyl phthalate; BMD, Benchmark dose; BMDL, Benchmark dose (lower bound); BPA, Bisphenol A; BPF, Bisphenol F; BPS, Bisphenol S; DnBP, Di-n-butyl phthalate; DEHP, Di(ethylhexyl) phthalate; DEP, Diethyl phthalate; DI, Daily intake; DiBP, Diisobutyl phthalate; DiNP, Diisononyl phthalate; EFSA, European Food Safety Authority; EHDI, Estimated human daily intake; EPA, Environmental Protection Agency; HBGV, Health-based guidance value; HED, Human equivalent dose; HEDF, Human equivalent dose factor; HI, Hazard Index; InsL3, Insulin-like factor 3; LB, Lower bound; LC-MS/MS, Liquid chromatography – mass spectrometry,; LOAEL, Lowest observed adverse effect level; LOD, Limit of detection; MCR, Maximal cumulative ratio; MoE, Margin of exposure; MRA, Mixture risk assessment; NOAEL, No observed adverse effect level; PBDE, Polybrominated diphenyl ether; PCB, Polychlorinated biphenyl; PCDD/F, Polychlorinated dibenzo-dioxins and -furans; POD, Point of departure; RQ, Risk quotient; RfD, Reference dose; SCCS, Scientific committee on consumer safety (European Commission); TDS, Testicular dysgenesis syndrome; TEQ, TCDD equivalents; US EPA, US Environmental Protection Agency.

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1. Introduction

Semen quality, assessed in terms of sperm concentration and total sperm counts, continues to decline in Western countries (Levine et al., 2017). Fewer studies are available from non-Western countries, but recently, reports of alarming declines among healthy semen donors in China have appeared (Yuan et al. 2018). The proportion of men with normal total motile sperm count, a measure highly relevant to fertilisation success, was found to have declined by approximately 10% between 2002 and 2017 in a population of men presenting to fertility centres in Spain and the USA (Tiegs et al. 2019). Similar observations were made among sperm donors (Chang et al. 2018). Poor semen quality is part of a wider trend of deteriorating male reproductive health which has begun to escalate into a general fertility crisis (Skakkebæk et al., 2022).

The causes of these worrying developments are not fully elucidated but are likely to be complex. Lifestyle factors such as illicit drug use and maternal smoking during pregnancy play a role, as do poor nutrition, stress and genetic factors (Skakkebaek et al., 2016, 2022). It has been hypothesised that these problems are also linked to chemical exposures that originate from the use of fossil fuels in electricity generation, transport, petrochemistry, and most importantly plastic production (Skakkebaek et al., 2022). Many of the implicated chemicals - plasticisers and a host of other substances needed in plastic production - are capable of interfering with male sexual development. Attention has focused on a limited set of chemicals such as phthalates (Swan et al., 2015), (Jensen et al., 2016), dioxins (Mínguez-Alarcón et al., 2017), parabens (Boberg et al., 2016) or azole pesticides (Draskau et al., 2019). However, the general contours of contributing chemical exposures have not yet come into view. As a result, evidence of the full extent to which chemicals play a role in the alarming decline of male reproductive health is missing.

Addressing this issue requires an approach that emphatically embraces the reality of simultaneous exposures to large numbers of chemicals. This is only possible by conducting mixture risk assessments. There has been modest progress in this field. Quite a few studies have considered combined exposures to phthalates (most recently Frederiksen et al., 2022; Apel et al., 2020; see also the review by Kortenkamp and Koch, 2020). However, the contribution of other chemicals that also play a role in disrupting male sexual development has been neglected. To a large extent, this was due to a lack of clarity about other chemicals that should also be included in a mixture risk assessment of male reproductive health. This gap has now been closed. By summarising an adverse outcome pathway network for male reproductive health, Kortenkamp (2020) pinpointed multiple molecular initiating events and associated effector chains that converge on nodal points and trigger common down-stream pathways to adverse outcomes. This suggested that a more comprehensive mixture risk assessment beyond phthalates should include androgen receptor (AR) antagonists, arylhydrocarbon receptor (AhR) agonists and chemicals capable of interfering with androgen synthesis, steroid hormone conversions, InsL3 production, and prostaglandin synthesis. In addition to phthalates, a minimum list of chemicals to be considered in such an analysis should include azole pesticides, polybrominated diphenyl ethers, polychlorinated biphenyls, bisphenols, parabens, polychlorinated dibenzodioxins and analgesics such as paracetamol (Kortenkamp, 2020).

In this paper, we present a mixture risk assessment of male reproductive health comprising 29 chemicals. It was conducted as part of the European Commission-funded HBM4EU project. We employed the Hazard Index (HI) approach (Teuschler and Hertzberg, 1995) which utilises risk quotients (RQs) of daily intakes (DI) and reference doses (RfD) for relevant health endpoints. By summing up the risk quotients of all chemicals included in the mixture risk assessment, the HI examines fold-exceedances of "acceptable" mixture exposures relative to an index value of 1. To achieve consistency of the assessment, we built the RQs with RfDs for similar, if not the same toxicity endpoints.

Poor male reproductive health does not only manifest as declining semen quality but presents itself as a constellation of effects also involving non-descending testes, hypospadias, reduced anogenital distance and testis cancer, summarised as the Testicular Dysgenesis Syndrome, TDS (Skakkebaek et al., 2016). However, to be able to interpret the declining trends in semen quality in a mixture risk assessment framework, and in the interest of making the assessment as consistent as possible, we focused on semen quality as the adverse outcome. Accordingly, we searched for quantitative data that allowed us to estimate RfDs for deteriorations in semen quality. We included chemicals that met both of the following criteria:

- Evidence of in semen quality reductions (sperm numbers, concentration, morphology, motility) either from human epidemiological studies or experimental studies in mammals
- Evidence of a hormonal mode of action (MoA), including AR antagonism, suppression of testosterone synthesis, inhibition of enzymes involved in androgen synthesis, suppression of InsL3 production, disruption of prostaglandin synthesis

In deriving RfDs for the HI method, we gave preference to studies that demonstrated negative effects on semen quality after gestational exposures, either in epidemiological studies or in animal studies. Where suitable data on impaired semen quality was not available from animal studies, we used evidence from human studies. For phthalates and antiandrogenic pesticides, we relied on reductions in fetal testosterone synthesis and other TDS effects (Kortenkamp and Koch, 2020, Kortenkamp and Faust, 2010). Where data from gestational exposures in animal experiments were missing, but data of impaired semen quality was available from studies in adult animals, we only included substances that had a clear hormonal MoA (acrylamide).

Accordingly, we selected the following 29 chemicals or chemical groups for a mixture risk assessment relevant to male reproductive health with a focus on deterioration of semen quality:

- **AR antagonists**: Bisphenols A, F, S; n-butyl paraben; polybrominated diphenyl ethers BDE 99, 100, 183, 209; PCB 118, 126; chlorpyrifos, vinclozolin, procymidone, fenitrothione
- **Disruption of prostaglandin signaling and InsL3 production**: Paracetamol
- **Suppression of testosterone synthesis**: Phthalates DEHP, DnBP, BBzP, DiNP; acrylamide
- **Inhibition of steroidogenic enzymes**: linuron
- **AhR activation**: polychlorinated dibenzodioxins and -furans (PCDD/F, 17 congeners), PCB 118, 126, 169

To achieve consistency in terms of the exposure data for our mixture risk assessment, we had to take account of shifting exposure patterns over time. Between 1988 and 2015, exposures to the phthalates DEP, DnBP, DiBP, BBzP, and DEHP in Germany declined, while exposures to DiNP increased (Koch et al., 2017). Similar patterns have been observed in Sweden and Italy (Gyllenhammar et al., 2017), (Shu et al., 2018), (Tranfo et al., 2018). Frederiksen et al. (2020) observed decreases in exposures to several phthalates and phenolic substances between 2009 and 2017. Changing exposures have also been noted for polychlorinated dibenzodioxins (EFSA 2018) and other chemicals. Fortuitously, data of the levels of multiple phthalate metabolites and bisphenols are available in spot urine samples from young Danish men taken in 2009 (Frederiksen et al. 2020). The characteristics of the study population and sampling strategy are described in detail (Jørgensen et al., 2012, Frederiksen et al., 2020). The data were supplemented by additional analyses for paracetamol (reported here). Accordingly, we chose 2009–2010 as the period for which we collected European exposure data for all the other chemicals.

2. Material and methods

2.1. Provenance of urine samples

We based our mixture risk assessment on measurements of metabolites of bisphenol A, S and F (BPA, BPS, BPF), the phthalates DEHP, DnBP, DiBP, BBzP, DiNP and paracetamol in spot urine samples of Danish male volunteers of mean age of 20 years (range 18–30 years). For the present assessment, we utilized the results of analyses in samples taken in 2009 (n = 98). The details of sampling strategy, sample handling and storage and the features of study participants are described in detail by Jørgensen et al. (2012) and Priskorn et al. (2018) and the details regarding selection of samples for this specific sub-study are described in Frederiksen et al. (2020).

2.2. Quantification of substance metabolites

The urine samples were analysed for metabolites of BPA, BPS, BPF, DEHP, DnBP, DIBP, BBzP and DINP by using two recently developed and validated isotope dilution liquid chromatography mass spectrometry (LC-MS/MS) methods as described by Frederiksen et al. (2020). A new method was developed for paracetamol. Urine samples were analyzed for the total (free and conjugated) content of N-acetyl-4-aminophenol (paracetamol), CAS No. 103–90-2. The method was developed and validated based on previously published methods (Dierkes et al., 2014), (Modick et al., 2013) using isotope diluted online-TurboFlow LC-MS/MS equipped with a probe for heated electrospray ionization (HESI) running in positive mode and with prior enzymatic deconjugation. The preceding enzymatic de-conjugation was carried out by a mixture of ß-glucuronidase (Escherichia coli K12) and sulfatase from Aerobacter Aerogenes. All analytical equipment, the use of chemicals and other materials has been described previously (Frederiksen et al., 2020). Method validation and limit of detection (LOD) were determined by using the approach described by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines (ICH, 2005). The limit of detection was 0.48 µg/L. All samples were analyzed in three batches, also including standards for calibration curves, three blanks and three times three urine pool controls, with one of them spiked with a mixture of native standards, yielding three urine control pools with paracetamol in three different concentration levels. The relative standard deviation (RSD) in all three control levels ranged from 4.5 to 4.9%. The analyses were performed at the Dept. of Growth and Reproduction, Copenhagen University Hospital - Rigshospitalet.

2.3. Adjustment for urine dilution

To account for urinary dilution of analytes, all measured urinary concentrations above the limit of detection (LOD) were corrected for individual urinary osmolality. Osmolality was measured by using the freezing point depression method, as described by Frederiksen et al. (2020).

Analyte concentrations below LOD were substituted by $LOD/2^{0.5}$.

2.4. Estimation of daily intakes from urinary concentrations

Assuming a steady-state between intake and metabolic clearance and taking account of the metabolic pathways, the daily intake (DI) was calculated from single urine samples for two scenarios, as follows:

a) When only the parent compound c_p was measured in the urine sample, we used:

$$DI\left[\frac{\mu g}{kg\ x\ day}\right] = \frac{c_p[\mu g/l]*V_{24}[l/day]}{F_{UE}*BW[kg]} \quad (1)$$

b) When N metabolites $c_m^1, \cdots, c_m^N$ were measured in the urine sample, we applied:

$$DI\left[\frac{\mu g}{kg\ x\ day}\right] = \left(\frac{c_m^1[\mu g/l]}{F_{UE}^1 * MW_m^1[\mu g/\mu mol]} + \cdots + \frac{c_m^N[\mu g/l]}{F_{UE}^N * MW_m^N[\mu g/\mu mol]}\right) * \frac{MW_p[\mu g/\mu mol]*V_{24}[l/day]}{BW[kg]} \quad (2)$$

where MW_p and MW_m are the molecular weights of the parent compound and metabolite(s), respectively, BW is the body weight of the subject, V_{24} is an estimate of the median 24 h urine volume for which we assumed 1.33 L, based on a subset of study participants, and F_{UE} is the mass urinary excretion fraction for the analyte (mass of analyte excreted in urine/mass of parent compound ingested). F_{UE} values used here were 0.73 for mono-benzyl phthalate (derived from butylbenzyl phthalate, (Anderson et al., 2011), 0.707 for mono-*iso*-butyl phthalate and 0.193 for mono-(2-hydroxy-*iso*-butyl) phthalate (both from di-*iso*-butyl phthalate, (Koch et al., 2012), 0.84 for mono-n-butyl phthalate and 0.069 for mono-(3-hydroxybutyl) phthalate (di-n-butyl phthalate, Koch et al., 2012), 0.063 for mono-(2-ethyl-hexyl) phthalate, 0.156 for mono-(2-ethyl-5-hydroxyhexyl) phthalate, 0.113 for mono-(2-ethyl-5-oxo-hexyl)phthalate and 0.139 for 5cx-MEPP (all metabolites of di-(2-ethyl-hexyl)phthalate, Anderson et al., 2011), 0.123 for mono-hydroxy-*iso*-nonyl phthalate, 0.066 for mono-oxo-*iso*-nonyl phthalate and 0.109 for mono-carboxy-*iso*-octyl phthalate (from di-*iso*-nonyl phthalate, Anderson et al 2011), 1 for BPA (Koch et al., 2012) and 0.056 for butylparaben (Moos et al., 2017). For N-acetyl-4-aminophenol (paracetamol) we assumed a F_{UE} = 0.86 which reflects the upper range of individual excretion quantities measured in humans (David et al., 2021). For BPS and BPF we assumed the same value 1 as for BPA.

Summary statistics of estimated daily intakes for the jointly measured chemicals can be found in Table 1.

2.5. Exposure data for the chemicals not monitored jointly in urine samples

For the remainder of the substances considered here, we collated single chemical exposure data from different sources, making the tacit assumption that the general population is exposed to all these chemicals simultaneously, most of the time. We further assumed that basing the assessment on median exposure levels for multiple substances gives a reasonable approximation of the cumulative exposure patterns at a population level. To construct a worst-case scenario, we also investigated cumulative exposures with all substances at their 95th percentile.

As much as possible we relied on exposure data from human biomonitoring studies. Where this was not possible, we retrieved exposure data derived from pathway analyses and food consumption and prevalence data. To make the mixture risk assessment (MRA) consistent, we focused on a common time period as much as possible and selected the years 2009 / 2010.

An overview of exposure estimates used in this study is shown in Table 1. We used lower bound (LB) exposures which convert occurrence data of substances below their limit of detection to zero.

2.6. Reference doses for reductions in semen quality

For all the substances included in the cumulative assessment group, we collated quantitative dose estimates for declines in semen quality, termed Reference Doses for male reproductive health, from here on referred to as RfD. Declines in semen quality do not always equate with the critical toxicity used to derive Health-based Guidance Values (HBGV) for single chemical risk assessments (i.e. the toxicity that appears at the lowest doses greater than 0). In many cases, declines in semen quality become evident at doses higher than those associated

Table 1
Estimated exposures (daily intakes) of 9 chemicals measured together in urine samples and 20 chemicals not jointly monitored. Taken from Frederiksen et al., (2020), EFSA (2011a), EFSA (2011b), EFSA, (2014), EFSA (2015), EFSA (2018), EFSA (2021b), Moos et al., (2016). P95: 95th percentile.

Chemical	Daily intake (median)	Unit	Daily intake (P 95)	Unit	Comments and explanations
BPA	0.0407	μg/kg/d	0.144	μg/kg/d	Jointly monitored in urine samples. Estimated based on the data in Frederiksen et al. (2020). Paracetamol data are in Supplementary Material Table S2.
BPS	0.0019	μg/kg/d	0.011	μg/kg/d	
BPF	0.0052	μg/kg/d	0.037	μg/kg/d	
DnBP	1.12	μg/kg/d	3.75	μg/kg/d	
DiBP	1.30	μg/kg/d	5.30	μg/kg/d	
BBzP	0.30	μg/kg/d	1.15	μg/kg/d	
DEHP	3.17	μg/kg/d	12.0	μg/kg/d	
DiNP	1.24	μg/kg/d	4.66	μg/kg/d	
Paracetamol	0.9	μg/kg/d	5810	μg/kg/d	
PCDD/F	0.25	pg/kg d	0.57	pg/kg d	Median and 95th percentile lower bound (LB) dietary exposures for adults (men and women) from European dietary surveys conducted 2005–2012. EFSA (2018), Table 43, p 185
PCB 118	0.576	ng/kg/d	1.7	ng/kg/d	Mean and 95th percentile LB dietary intake for adults (men and women), calculated from % contribution of PCB congeners to exposure to 29 PCDD/F and DL-PCB congeners, EFSA (2018), Fig 19, p 187, and Table 42, p 184
PCB 126	0.0035	ng/kg/d	0.01	ng/kg/d	
PCB 169	0.00079	ng/kg/d	0.0024	ng/kg/d	
BDE 28	0.02	ng/kg/d	0.08	ng/kg/d	Mean LB dietary intake for adults (men and women), EFSA (2011) Table 17, p 63; and dietary exposure for 95th percentile adults, median LB estimates, EFSA (2011a) Table 18, p 64
BDE 47	0.58	ng/kg/d	1.8	ng/kg/d	
BDE 99	0.18	ng/kg/d	0.38	ng/kg/d	
BDE 100	0.15	ng/kg/d	0.46	ng/kg/d	
BDE 153	0.04	ng/kg/d	0.08	ng/kg/d	
BDE 154	0.05	ng/kg/d	0.17	ng/kg/d	
BDE 183	0.02	ng/kg/d	0.03	ng/kg/d	
BDE 209	0.61	ng/kg/d	1.06	ng/kg/d	
n-butylparaben	0.6	μg/kg/d	8.7	μg/kg/d	Median and 95th percentile daily intake for women in 2009, Moos et al (2016), Table 3
Acrylamide	0.5	μg/kg/d	1	μg/kg/d	Median and 95th percentile LB exposures for adults (men and women) from European surveys between 2005 and 2012, EFSA (2015), Table 8, p 61
Chlorpyrifos	0.07	μg/kg/d	0.07	μg/kg/d	Highest international estimated daily intake according to EFSA PRIMo is 7% of ADI (1 μg/kg/d), EFSA (2014), p 30; median estimates not accessible
Linuron	0.069	μg/kg/d	0.069	μg/kg/d	The 2009 EU report on pesticide residues in food, EFSA (2011b), Table 5–10, p 208; median estimates not accessible
Prochloraz	0.34	μg/kg/d	0.34	μg/kg/d	
Procymidone	0.25	μg/kg/d	0.25	μg/kg/d	
Vinclozolin	0.35	μg/kg/d	0.35	μg/kg/d	
Fenitrothione	0.06	μg/kg/d	0.06	μg/kg/d	The 2019 EU report on pesticide residues in food, EFSA (2021b), Table 3, p 52; median estimates not accessible

with the critical toxicity. Unless semen quality decline represents the critical toxicity of a substance, as is the case e.g. for PCDD/F, ***the RfDs used for this study are not applicable to single chemical risk assessments*** as they would be insufficiently conservative.

As much as possible, we retrieved relevant values from existing evaluations of competent authorities. In almost all cases, however, it was necessary to conduct separate reviews to derive the respective RfDs *de novo*. Not only did this require quantitative analyses of published data by systematic review (BPA, PBDEs, PCBs), it also necessitated examinations of the strength of evidence and study quality.

The RfD values we derived *de novo* did not rely on the use of assessment factors larger than those normally used for lowest-observed-adverse-effect-level (LOAEL) to no-observed-adverse-effect-level (NOAEL) extrapolations (AF = 3) and for inter-species extrapolations. For substances that accumulate in adipose tissue we adopted body burden considerations and estimated equivalent human daily intakes (EHDI) associated with the body burden at the point of departure in animal experiments. In line with established practice (EFSA 2011a), we used an inter-species assessment factor of 2.5 in such cases.

An overview of the RfDs used in this study, together with assessment factors is shown in Table 2.

2.6.1. Bisphenol A

We conducted a systematic review (Kortenkamp et al., 2022) to retrieve relevant data on reductions in semen quality and adopted 0.003 μg/kg/d as RfD for BPA. This was based on a weighted estimate of a NOAEL of 0.5 μg/kg/d for reductions in sperm numbers taken from studies with rats and mice. To extrapolate this to a human equivalent dose (HED) we used a human equivalent dose factor (HEDF) of 0.165 (EFSA 2021a), equivalent to an assessment factor (AF) of 6.06. This factor accounts for differences in the toxicokinetics in rodents and humans. Following the procedures adopted by EFSA (2021a), we have combined this with an additional assessment factor (AF) of 25 to allow for differences in sensitivity between humans and derived a RfD of 0.003 μg/kg/d (overall AF 6.06 × 25 = 151) (Kortenkamp et al., 2022).

2.6.2. Bisphenol S

For bisphenol S, the study by (Ullah et al., 2019) produced the same NOAEL of 0.5 μg/kg/d as for bisphenol A. BPS was administered *via* drinking water to female Sprague-Dawley rats; at 0.5 μg/L effects on semen quality in their offspring were no longer observed. Assuming the weight of a female Sprague-Dawley rat as 300 g and a daily water consumption of 30 ml (Laaksonen et al., 2013), this translates into a NOAEL of 0.5 μg/kg/d.

(Shi et al., 2019) found a LOAEL of 0.5 μg/kg/d in mice which with an AF of 3 extrapolates to a NOAEL of 0.167 μg/kg/d. The toxicokinetics of BPS has been investigated (Gayrard et al., 2020), (Karrer et al., 2018) but to our knowledge there are no data available that would allow

comparative estimations of the area under the curve (AUC) from time 0 to infinity. We therefore made the simplifying assumption that BPS kinetics are similar to BPA, with correspondingly similar HEDF (the ratio of human and test species AUC) and AF. Accordingly, these points of departure (0.5 μg/kg/d and 0.167 μg/kg/d) translate into 0.003 μg/kg/d (derived from Ullah et al., 2019) and 0.0001 μg/kg/d (derived from Shi et al., 2019). In view of indications that the toxicokinetics of BPS are more favourable than of BPA (Gayrard et al., 2020; Karrer et al., 2018) we chose the larger value of 0.003 μg/kg/d as RfD for bisphenol S.

2.6.3. Bisphenol F

The only study of BPF effects on spermatogenesis after gestational exposure is by Ullah et al. (2019) who observed alterations of spermatogenesis and decreases in sperm motility in the male offspring of female Sprague-Dawley rats at drinking water concentrations of 25 μg/L. At a concentration of 0.5 μg/L these effects were no longer observed. Assuming the weight of a female Sprague-Dawley rat as 300 g and a daily water consumption of 30 ml (Laaksonen et al., 2013), this translates into a NOAEL of 0.5 μg/kg/d.

To our knowledge, information from human or animal studies that would allow a toxicokinetic analysis of serum concentration–time profiles of BPF to estimate the (AUC) from time zero to infinity is not available. However, *in vitro* data are accessible, and we therefore used the generic PBPK model "pbtk" within the R software HTTK (v2.0.4) developed by USEPA researchers (Pearce et al., 2017). The model uses *in vitro* data to make predictions about the fate of chemicals in humans, rats, mice, dogs, and rabbits. *In silico* simulations for steady-state conditions of the parent compound resulted in an oral AUC of 1.7 nM*h for humans and 0.8 nM*h for rats, respectively. The ratio of these AUC gives an HEDF of 0.48. Multiplication of the BPF NOAEL of 0.5 μg/kg/d with 0.48 produces an HED of 0.24 μg/kg/d. With an AF of 25 we obtained 0.0096 ~ 0.01 μg/kg/d as the RfD for bisphenol F (overall AF = 50).

2.6.4. Phthalates

We adopted the values proposed by Kortenkamp and Koch (2020) which were derived based on suppressions of testosterone synthesis and other effects related to the phthalate syndrome in rats. There is evidence to associate exposure to phthalates in adult life with reductions in semen quality (Radke et al., 2018). In this MRA, we used the following RfD values: DEHP: 10 μg/kg/d; DnBP: 6.7 μg/kg/d; BBzP: 10 μg/kg/d; DiNP: 59 μg/kg/d; DiBP: 100 μg/kg/d.

2.6.5. Paracetamol

Paracetamol interferes with COX signalling and suppresses InsL3, a factor required for the first phase of testis descent. It also impairs semen quality. We were unable to locate dose–response studies of reductions in semen quality and paracetamol in laboratory animals. The only studies that administered paracetamol and examined parameters of semen quality are of Axelstad et al., (2018) in rats and Rossitto et al., (2019) in mice. Rossitto et al. (2019) dosed paracetamol twice daily in mice during gestation and saw reductions in sperm number at 30 mg/kg/d. Axelstad et al. (2018) administered 360 mg/kg/d through-out gestation and lactation and observed declines in semen quality. We used the latter study to derive a RfD of 1.2 ~ 1 mg/kg/d, by applying an AF of 3 for LOAEL to NOAEL extrapolation, and an additional 100 for inter- and intra-species extrapolation.

2.6.6. PCDD/F

We adopted the HBGV of 0.28 pg/kg/d (equivalent to the tolerable weekly intake value of 2 pg/kg d) established by EFSA (2018) for PCDD/F. This is based on the associations between PCDD/F exposures in early childhood and later declines in semen quality observed in the Russian Children's Cohort. Since these associations were only observed for PCDD/F congeners, and not for dioxin-like PCBs, we only applied this value to TCDD equivalents for 17 PCDD/F congeners (assessment group B in EFSA 2018).

2.6.7. PCBs

To establish congener-specific RfDs for PCBs we conducted a systematic review of human epidemiological and animal studies of PCB exposures and declines in semen quality (Ermler and Kortenkamp 2022a). Our keyword searches identified 6897 records, of which 182

Table 2
Reference doses (RfD) for declines in semen quality and related effects for 29 chemicals AF: Assessment factor, AGD: anogenital distance, LOAEL: Lowest observed adverse effect level, POD: Point-of-Departure, RfD: Reference dose.

Chemical	POD	Dose unit	Endpoint	Species	AF	RfD	Dose unit	Reference
BPA	0.5	μg/kg/d	Decrease in sperm number	Rat, mouse	151	0.003	μg/kg/d	Kortenkamp et al. (2022)
BPS	0.5	μg/kg/d		Rat	151	0.003	μg/kg/d	Ullah et al. (2019)
BPF	0.5	μg/kg/d		Rat	50	0.01	μg/kg/d	Ullah et al. (2019)
DnBP	2 (LOAEL)	mg/kg/d	Spermatocyte development	Rat	300	6.7	μg/kg/d	Kortenkamp and Koch (2020)
DiBP	10	mg/kg/d	Suppression testosterone synthesis	Rat	100	100	μg/kg/d	
BBzP	1	mg/kg/d		Rat	100	10	μg/kg/d	
DEHP	3 (LOAEL)	mg/kg/d	Dysgenesis genitalia	Rat	300	10	μg/kg/d	
DiNP	5.9	mg/kg/d	Suppression testosterone synthesis	Rat	100	59	μg/kg/d	
Paracetamol	360 (LOAEL)	mg/kg/d	Decrease in sperm number	Rat	300	1	mg/kg/d	Axelstad et al. (2018)
PCDD/F	not applicable		Decrease in sperm number	Human		0.28	pg/kg/d	EFSA (2018)
PCB 118			Semen quality			2.9	ng/kg/d	Ermler and Kortenkamp (2022b)
PCB 126						0.073	ng/kg/d	
PCB 169						5.3	ng/kg/d	
BDE 28			read-across from BDE 47			150	ng/kg/d	Ermler and Kortenkamp (2022 a)
BDE 47	30	μg/kg/d	Decrease in sperm number	Rat	200	150	ng/kg/d	
BDE 99	20	μg/kg/d	Decrease in sperm number	Rat	6,666	3	ng/kg/d	
BDE 100			read-across from BDE 99			3	ng/kg/d	
BDE 153						3	ng/kg/d	
BDE 154						3	ng/kg/d	
BDE 183			read-across from BDE 209			1,000,000	ng/kg/d	
BDE 209	100,000	μg/kg/d	Decrease in sperm number	Mouse	100	1,000,000	ng/kg/d	
n-butylparaben	10 LOAEL)	mg/kg/d	Decrease in sperm number	Rat	333	30	μg/kg/d	Boberg et al. (2016)
Acrylamide	2.5 (LOAEL)	mg/kg/d		Rat	300	8	μg/kg/d	Ivanski et al. (2020)
Chlorpyrifos	3 (LOAEL)	mg/kg/d		Rat	300	10	μg/kg/d	Li et al. (2019)
Linuron	50 (LOAEL)	mg/kg/d	Retained nipples	Rat	500	100	μg/kg/d	Kortenkamp and Faust (2009)
Prochloraz	5	mg/kg/d		Rat	100	50	μg/kg/d	
Procymidone	10	mg/kg/d		Rat	100	100	μg/kg/d	
Vinclozolin	5	mg/kg/d		Rat	100	50	μg/kg/d	
Fenitrothione	20	mg/kg/d	AGD changes	Rat	100	200	μg/kg/d	

qualified for full-text analysis. Of these, 56 papers were subjected to data extraction and risk-of bias assessment. Key elements of assessment included exposure characterisation (purity and stability of test compounds, absence of contaminations), outcome assessment (blinding of the outcome assessors) and power of detecting effects (sufficient number of animals per dose group). Based on the studies we rated as being of sufficient quality we estimated the following RfDs: PCB 118: 2.9 ng/kg/d; PCB 126: 0.073 ng/kg/d; PCB 169: 5.3 ng/kg/d (Ermler and Kortenkamp 2022a).

2.6.8. Poly-brominated diphenyl ethers BDE 99, 100, 183 and 209

Based on the systematic review of male reproductive effects of PBDEs by Zhang et al., (2020a, 2020b), we conducted a systematic review for the time period after publication of this paper and located an additional 10 records which, together with the 10 studies from Zhang et al. (2020a, 2020b) gave a total of 20 studies on semen quality in animals considered for full-text analysis and 11 of those were eligible for data extraction. We evaluated the reliability of the retrieved studies in terms of the purity of the dosed BDE congeners (to exclude issues of contamination with PCDD/F). We also considered blinding, appropriate numbers of animals and successful demonstration of effects with positive control substances (Ermler and Kortenkamp 2022b).

Based on the studies we rated as being of sufficient quality, we estimated the following RfDs: BDE 47: 150 ng/kg/d; BDE 99: 3 ng/kg/d and BDE 209: 1 mg/kg/d (Ermler and Kortenkamp 2022b).

Other relevant BDE congeners: Data about the ability of other abundant BDE congeners, such as BDE 28, 100, 153, 154 and 183 to produce declines in semen quality are missing. Exclusion of these congeners from consideration in the MRA will lead to underestimations of risk. We therefore adopted the read-across approach used by Martin et al. (2017) to assign RfDs to these untested congeners. In this read-across approach we assumed that the toxicity of untested congeners is driven by their bromine content and is therefore similar to that of the nearest tested congeners. Accordingly, we chose the RfD of BDE 47 for BDE 28 (150 ng/kg/d). The RfD of BDE 99 (3 ng/kg/d) was selected to also cover BDE 100, 153 and 154. Finally, we assumed that the untested BDE 183 is as potent as BDE 209.

There is epidemiological evidence for associations of PBDE exposure with poor semen quality, but these studies do not allow congener-specific evaluations, or if they do, have monitored PBDEs in tissues that make it difficult to relate the data to daily intakes (e.g. PBDEs in hair).

2.6.9. n-Butylparaben

Kang et al. (2002) observed decreased sperm numbers and motility in the offspring of rats dosed during gestation with 100 and 200 mg/kg/d n-butylparaben. Decreased sperm counts were also reported by Zhang et al. (2016) who administered 400 and 1000 mg/kg d during gestation. NOAELs cannot be established from these studies.

Boberg et al. (2016) administered a wider dose range to pregnant rats and observed reduced sperm counts at all tested doses (greater than10 mg/kg/d). We estimated that 10 mg/kg/d is a LOAEL and extrapolated a NOAEL of 3.33 mg/kg/d by application of an assessment factor of 3. With the standard assessment factor of 100 for animal to human extrapolation, this yields a RfD of 33 μg/kg/d, here rounded down to 30 μg/kg/d.

This value is slightly larger than the 20 μg/kg/d derived by SCCS (2005), based on a NOAEL for estrogenic effects of n-butylparaben in rats.

2.6.10. Acrylamide

Acrylamide produces reductions in sperm production in rodents dosed in adulthood (Ivanski et al., 2020; Kalaivani et al., 2018; Kermani-Alghoraishi et al., 2010) through a mechanism involving disturbance of testosterone levels. The lowest reported LOAEL of 2.5 mg/kg/d is by Ivanski et al. (2020). Kermani-Alghoraishi et al. 2010 and Kalaivani et al. 2018 observed LOAELs of 5 and 6.5 mg/kg/d, respectively. By application of a LOAEL to NOAEL extrapolation factor of 3, and the default factor of 100, we obtained a RfD of 8.3 rounded down to 8 μg/kg/d from the study by Ivanski et al. (2020).

2.6.11. Chlorpyrifos

Chlorpyrifos acts as an AR antagonist and produces declines in semen quality in laboratory animals (Ubaid ur Rahman et al., 2021). In the studies we retrieved, chlorpyrifos was administered at only one dose level; studies that permit a dose–response analysis could not be located. Babazadeh and Najafi (2017) observed declines in semen quality in rats that received 37 mg/kg/d, Alaa-Eldin, et al. (2017) and Hassan et al., (2021) saw similar effects at 6.5 mg/kg/d. The lowest reported dose for declines in semen quality was 3 mg/kg/d, given to adult rats over 20 weeks (Li et al., 2019). We deemed this dose as a LOAEL and estimated a RfD of 10 μg/kg/d (assessment factor of 3 for LOAEL to NOAEL extrapolation, plus a default factor of 100).

2.6.12. Vinclozolin, procymidone, linuron, prochloraz and fenitrothion

We adopted the values for vinclozolin, procymidone, linuron and fenitrothione used by Kortenkamp and Faust, (2010). The value for prochloraz is taken from Laier et al., (2006). All values are based on alterations of landmarks of male sexual development in the rat (retained nipples in male offspring, changes in anogenital distance, AGD). The estimates were used as proxies to bridge the absence of data on declines in semen quality. The following RfD were used: Vinclozolin: 50 μg/kg/d; procymidone: 100 μg/kg/d; linuron: 100 μg/kg/d; prochloraz: 50 μg/kg/d; fenitrothione: 200 μg/kg/d.

2.7. Mixture risk assessment approach

The Hazard Index (HI) approach (Teuschler and Hertzberg, 1995) utilises risk quotients (RQs) of daily intakes (DI) and reference doses (RfD) for relevant health endpoints. The HI is the sum of RQ of all chemicals included in the mixture risk assessment. It examines fold-exceedances of "acceptable" mixture exposures relative to an index value of 1.

The RfDs chosen as input for the HI method rely on the use of AFs of varying magnitude. If the differences in these AFs are too large, there are concerns that the outcome of the analysis might be driven by AFs rather than genuine potency differences. In such cases, the Point-of-Departure-Index (PODI, Wilkinson et al. 2000) might be more appropriate. However, the AFs used here (Table 2) were around 100. Higher AFs were necessary to extrapolate from LOAELs to NOAELs, but these did not exceed 500. One exception is BDE 99 with an AF of 6,666 which arose from the body burden approach used to convert doses in animal studies to human equivalent exposures (Ermler and Kortenkamp 2022b). We therefore adopted the HI approach.

2.7.1. Personalised risk quotients for substances monitored in urine samples

The availability of urinary levels for multiple chemicals in each study participant and year, allowed us to build personalised risk quotients RQ_P for DnBP, DiBP, BBzP, DEHP, DiNP, BPA, BPS, BPF and paracetamol by division of estimated daily intakes by the RfD values shown in Table 2:

$$RQ_p = \frac{DI_p\left[\frac{\mu g}{kg x day}\right]}{RfD\left[\frac{\mu g}{kg x day}\right]} \quad (3)$$

For each study subject, we then calculated the personalised HI_p by summing the RQ_p derived for each single substance:

$$HI_p = RQ_{DnBP} + RQ_{DiBP} + RQ_{BBzP} + RQ_{DEHP} + RQ_{DiNP} + RQ_{BPA} + RQ_{BPS} + RQ_{BPF} + RQ_{paracetamol} \quad (4).$$

2.7.2. Taking account of background exposures to 20 additional chemicals

To account for background exposures to the remaining 20 chemicals which were not monitored in the study subjects, we built RQs (Table 3) based on median exposures in Europe from around 2010 (Table 1). This added an HI of 1.39 to each HI_P.

2.7.3. Analysing the complexity of combined exposures

We investigated the complexity of combined personalised exposures by analysing the contribution of single substances to the sum of RQs. One global measure of this complexity is the Maximum Cumulative Ratio (MCR). The MCR_p is the ratio of the HI_p and the RQ_p with the highest numerical value among all RQs, termed RQ_P max.

$$MCRp = HIp/RQp\,max$$

We used the MCR_p to create scatter plots of MCR_p versus HI_p which yielded data points representing each study participant (Fig. 1c). In these plots, several groupings can be distinguished which delineate risk management options. We have labelled these options following (Price et al., 2012). For further details, see text in Results and legend to Fig. 1.

3. Results

3.1. Exposure data and reference doses (RfD)

We based our analysis on the measurements of urinary metabolite levels of BPA, BPS, BPF, DEHP, DnBP, DiBP, BBzP and DiNP in 98 male volunteers conducted by Frederiksen et al. (2020). We used these data to estimate the corresponding daily intakes for the 8 chemicals. In addition, we determined the urinary paracetamol levels in these samples (Supplementary Material, Table S1) and approximated the resulting daily intakes.

We conducted mixture risk assessments using the HI approach by combining the estimated daily intakes (summary statistics shown in Table 1) with RfD for declines in semen quality (Table 2) and similar effects to obtain personalised RQs (RQ_P) for each of the 9 monitored chemicals in all subjects (see also Supplementary Material, Table S3). These RQ_P allowed us to calculate personalized HI for each study participant, termed HI_P.

We structured the mixture risk assessments as follows: We first carried out an analysis based on the 9 jointly monitored chemicals. In a second step, we additionally considered combined exposures to the remaining 20 chemicals not monitored by Frederiksen et al. (2020). To this end, we retrieved median daily intakes from European data (Table 1) which we combined with the RfDs in Table 2 to derive RQs and HI. For each study subject, we added the resulting HI for the 20 additional chemicals (Table 3) to the HI_P for the 9 monitored chemicals. Finally, we analysed the special case of subjects with high urinary paracetamol levels.

3.2. Mixture risk assessment for 9 jointly monitored chemicals

Based on the RQ of the 9 chemicals monitored jointly in urine samples, all study participants exceeded an HI of 1, by a very large margin. Fig. 1a shows the HI_P arranged in ascending order. The values varied 37-fold and ranged from a minimum of 2.7 to a maximum of 103, with a median and 95th percentile of 17 and 57, respectively.

To identify the nature of the chemical(s) that contributed strongly to the HI_P, we used the median RQ_P for each of the 9 chemicals among individuals (Supplementary Material, Table S3) to define a person of average exposure. This yielded a median HI_P of 15.3. Next, we arranged the median RQ_P from all 98 study participants for each chemical in ascending order, to produce a "waterspout" graph (Fig. 1b). It can be seen that the RQs of paracetamol, DIBP, DINP, BBzP, DnBP and DEHP were relatively small. Together, they summed up to a HI = 0.55. However, added to this, the RQs of BPF, BPS and BPA raised the HI well above 1, to reach a value of 15.3. The RQ_P of BPA alone represented 90% of the HI_P. BPA, BPS and BPF can be considered as drivers of the HI. Their elimination as contributors to the HI would mean that the remaining 6 chemicals combined would not exceed HI = 1.

The same rank order of the main contributing chemicals to the HI became apparent both in more highly exposed subjects with HI_P above the 95th percentile and in participants with HI_P in the lowest quartile. Among the 5 subjects with the largest HI, BPA alone contributed more than 90% of the HI_P. This proportion decreased to 80% among study participants with HI_P in the lowest quartile (Supplementary Material, Fig. S1).

In visualizing risk management options according to the degree of exceedances of HI = 1, plots of MCR_P versus HI_P (Fig. 1c) can reveal additional relationships not obvious from the previous analyses. The MCR_P is the ratio between the HI_P and the largest contributing RQ_P. It yields a global measure of the contribution of individual chemicals in the mixture to the HI, as follows: If only one chemical explains the HI in its entirety, the MCR is 1 ($HI = RQ_{max}$); there is no mixture issue. If the HI is in excess of 1 at the same time, this signifies a single chemical

Table 3
Risk quotients and Hazard Index for the 20 chemicals not jointly monitored in urine samples. Table 3 is a compilation of relevant data from Tables 1 and 2. RfD: Reference dose; RQ: Risk quotient. P 95: 95th percentile. The sum of RQ is the Hazard Index HI.

Chemical	RfD	Unit	Daily intake (median)	Unit	RQ (median intakes)	Daily intake (P 95)	Unit	RQ (P 95)
PCDD/F	0.28	pg/kg/d	0.25	pg/kg d	0.892857	0.57	pg/kg d	2.035714
PCB 118	2.9	ng/kg/d	0.576	ng/kg/d	0.198621	1.7	ng/kg/d	0.586207
PCB 126	0.073	ng/kg/d	0.0035	ng/kg/d	0.047945	0.01	ng/kg/d	0.136986
PCB 169	5.3	ng/kg/d	0.00079	ng/kg/d	0.000149	0.0024	ng/kg/d	0.000453
BDE 28	150	ng/kg/d	0.02	ng/kg/d	0.000133	0.08	ng/kg/d	0.000533
BDE 47	150	ng/kg/d	0.58	ng/kg/d	0.003867	1.8	ng/kg/d	0.012000
BDE 99	3	ng/kg/d	0.18	ng/kg/d	0.060000	0.38	ng/kg/d	0.126667
BDE 100	3	ng/kg/d	0.15	ng/kg/d	0.050000	0.46	ng/kg/d	0.153333
BDE 153	3	ng/kg/d	0.04	ng/kg/d	0.013333	0.08	ng/kg/d	0.026667
BDE 154	3	ng/kg/d	0.05	ng/kg/d	0.016667	0.17	ng/kg/d	0.056667
BDE 183	1,000,000	ng/kg/d	0.02	ng/kg/d	0.000000	0.03	ng/kg/d	0.000000
BDE 209	1,000,000	ng/kg/d	0.61	ng/kg/d	0.000001	1.06	ng/kg/d	0.000001
n-butylparaben	30	µg/kg/d	0.6	µg/kg/d	0.020000	8.7	µg/kg/d	0.290000
Acrylamide	8	µg/kg/d	0.5	µg/kg/d	0.060241	1	µg/kg/d	0.120482
Chlorpyrifos	10	µg/kg/d	0.07	µg/kg/d	0.007000	0.07	µg/kg/d	0.007000
Linuron	100	µg/kg/d	0.069	µg/kg/d	0.000690	0.069	µg/kg/d	0.000690
Prochloraz	50	µg/kg/d	0.34	µg/kg/d	0.006800	0.34	µg/kg/d	0.006800
Procymidone	100	µg/kg/d	0.25	µg/kg/d	0.002500	0.25	µg/kg/d	0.002500
Vinclozolin	50	µg/kg/d	0.35	µg/kg/d	0.007000	0.35	µg/kg/d	0.007000
Fenitrothione	200	µg/kg/d	0.06	µg/kg/d	0.000300	0.06	µg/kg/d	0.000300
				Sum	**1.39**		Sum	**3.57**

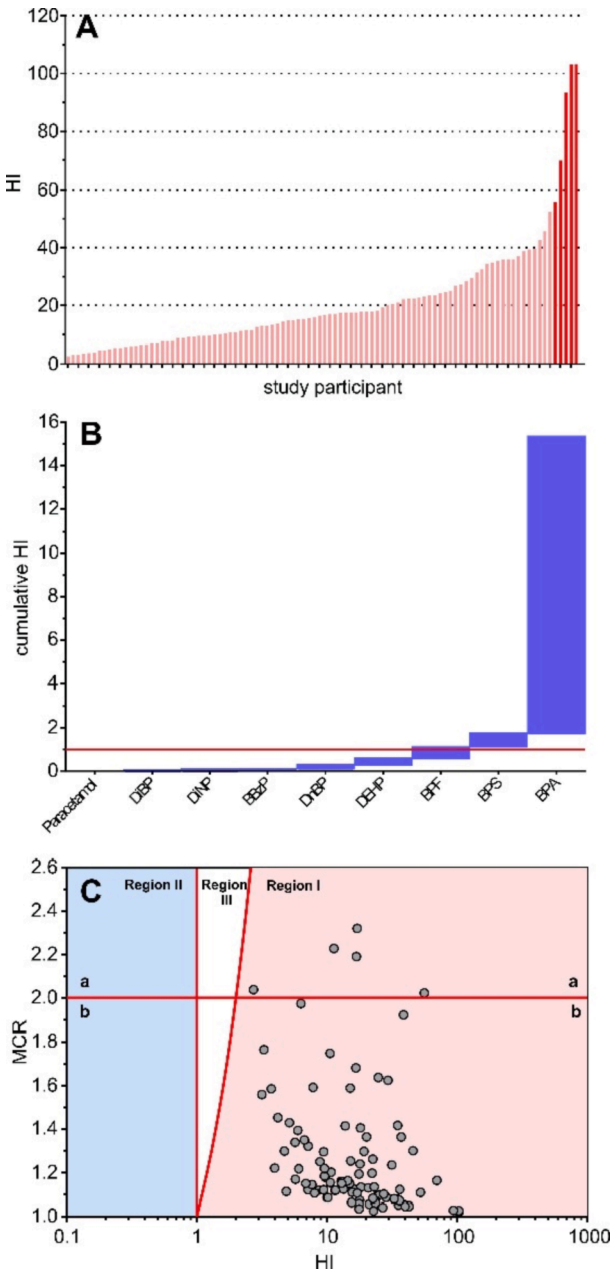

Fig. 1. Mixture risk assessment for 9 chemicals monitored jointly in urine samples from 98 Danish young men. A: Personalised Hazard Indices HI_P for each study participants, arranged in ascending order. Dark red bars show subjects above the 95th percentile. B: Median Risk Quotients RQ_P of all 98 subjects, arranged in ascending order. The red horizontal line shows HI = 1; cumulative RQ below this line stay within acceptable combined exposures. Cumulative RQ above this line contribute to exceedances of acceptable HI, here BPF, BPS and BPA. C: Scatter plot of Maximum Cumulative Ratio (MCR_P) versus Hazard Index (HI_P) with categories for risk management. Each study participant is depicted by a data point. The blue segment to the left of the vertical line marking an acceptable HI = 1 marks combined exposures that do not present concerns (Region II). The white segment defined by the vertical line for acceptable HIs and the curved line depicting MCR = HI shows subjects with combined exposures above HI = 1, but without exceeding RQ_P = 1 for any single chemical (Region III). The red segment to the right of the MCR = HI line is for subjects exceeding acceptable combined exposures and with exposures producing RQ_P greater than 1 for at least one chemical (Region I). Data points below the horizontal line corresponding to MCR = 2 show subjects in whom one chemical contributed 50% or more to the HI_P (Regions Ia, IIa and IIIa). Above this line are study participants who experienced combined exposures where multiple chemicals contributed to the HI_P (Regions Ib, IIb and IIIb).

management issue. At the other extreme, when all chemicals contribute to the same extent, the MCR equals the number of chemicals considered together, in our case 9 (each RQ is 1/9th of the HI; accordingly, the HI/RQ is 9). With 9 chemicals in the mixture, the MCR can therefore only assume values from 1 to 9. Of particular interest in the MCR_P/HI_P plot is the horizontal line MCR = 2 that defines Regions Ib, IIb or IIIb in Fig. 1c. Situated in these Regions are study subjects where the largest single RQ contributes 50% or less to the HI, indicating a more complex mixture exposure pattern which becomes especially challenging with HI_P larger than 1.

The MCR_P/HI_P plot in Fig. 1c shows a cluster of subjects where the MCR_P diminishes with increasing HI_P, along a line of data points that descends from left to right. This suggests that among most subjects with large HI_P the complexity of the mixture diminishes as almost the entire HI is explained by the RQ of one substance. We have seen earlier that this substance is BPA. But there are exceptions suggestive of more complex patterns. Five study participants exhibited MCR_P above 2, signifying a distribution of RQ_P where exposure to one single chemical only contributed a maximum of 50% or less to the HI_P. As can be seen in Fig. 1c, a small number of these individuals also showed high HI_P of 10 or more.

Of further significance is the curved line that defines the points where the MCR assumes the same value as the HI. If the condition MCR = HI is met, no single contributing RQ can exceed 1. Accordingly, positioned to the right of this line are subjects where the HI_P is composed of one or more RQ_P larger than 1. To risk managers, RQ exceeding 1 indicate non-compliance with single RfD and signal the need for exposure reduction measures to achieve HI < 1. Strikingly, all study subjects exceeded HI_P = 1 and at the same time fell in Region I of the MCR_P/HI_P plot, to the right of the MCR = HI line, with one or several RQ_P above 1.

3.3. *Mixture risk assessment for jointly monitored chemicals, excluding BPA*

Our analysis shows that BPA exerts a dominating influence on combined exposures that affect semen quality, to an extent as to potentially obscure the contours of a more complex underlying situation. To assess this issue, we removed BPA from consideration. As was to be expected, the HI_P decreased, now with a maximum of 30.1 and a minimum value of 0.5 (Fig. 2a). For a person with an "average" exposure derived by using the median RQ_P's from the study participants for each of the remaining 8 chemicals we calculated an HI of 1.7. The rank order of the RQ (Fig. 2b) remained unchanged compared with the scenario that included BPA (see Fig. 1b). This was also true for study subjects with HI_P above the 95th percentile and in the lower quartile (Supplementary Material Fig. S2).

However, the MCR_P/HI_P plot (Fig. 2c) revealed rather striking new patterns. The removal of BPA from consideration led to a shift of the cloud of data points upwards and to the left, relative to the plot in Fig. 1c. But this shift still left 85.7% of the subjects with HI_P above 1. Of note is an increase in the MCR_P to values of up to 3.6, compared with a maximum of 2.2 when BPA was included. This means that many participants (37.7%) now come to be positioned in Region III of the MCR_P/HI_P plot, with exceedance of HI = 1 but no single RQ_P above 1, the most challenging situation for risk management. Thus, elimination of BPA from the scenario would not be sufficient to mitigate mixture risks, despite its dominance.

3.4. *Mixture risk assessment incorporating a further 20 chemicals*

Next, we analysed how inclusion of the other 20 selected chemicals not monitored in the study subjects might change the outcome of the assessment. RQ based on median exposures added 1.39 to each HI_P (see Table 3) and produced correspondingly elevated minimum and maximum HI_P of 4.1 and 105, respectively. As shown in Fig. 3a, there was also an impact on the nature of the chemicals seen to drive the

mixture risks. Considering a person of median RQ_P with an HI_P of 16.7, BPA still made the strongest contribution, but the second-most important drivers of mixture risks were PCDD/F, followed by BPS, BPF and DEHP. This rank order was also seen among subjects with HI_P above the 95th percentile and in the lower quartile (Supplementary Material, Fig. S3).

Inclusion of the 20 additional chemicals shifted the cloud of data points in the MCR_P/HI_P plot to the right and upwards, with a maximum MCR_P of 3.1 (Fig. 3b), increased from 2.3based on the 9 jointly monitored chemicals considered before (see Fig. 1c). Accordingly, 9.2% of participants now came to be positioned in Region I a of the MCR_P/HI_P plot, above the MCR = 2 line, indicating more complex mixed exposures.

3.5. Exposure scenario-based analysis: The special case of paracetamol users

While most study participants showed relatively low urinary paracetamol levels, with negligible RQ_P around 0.001 (see Fig. 1b and Fig. 3a), we noticed 5 subjects with considerably higher levels, presumably due to concurrent therapeutic paracetamol use. This group exhibited a median HI_P of 28, well above the median value of 15.2 for the entire study population. The waterspout graph in Fig. 4 a shows that the RQ of paracetamol made a substantial contribution to the HI of this group, in second place after BPA. Other drivers were PCDD/F, BPS, BPF and DEHP.

We highlighted the 5 therapeutic paracetamol users in the MCR_P/HI_P plot in Fig. 4b. The individual with the highest HI_P of 71 showed a relatively low MCR_P, with the BPA RQ contributing most. In contrast, in the subjects with HI_P around 18, the RQ_P of BPA and paracetamol were similar, resulting in a correspondingly higher MCR_P. For all these 5 study participants, elimination of BPA and paracetamol would still not reduce

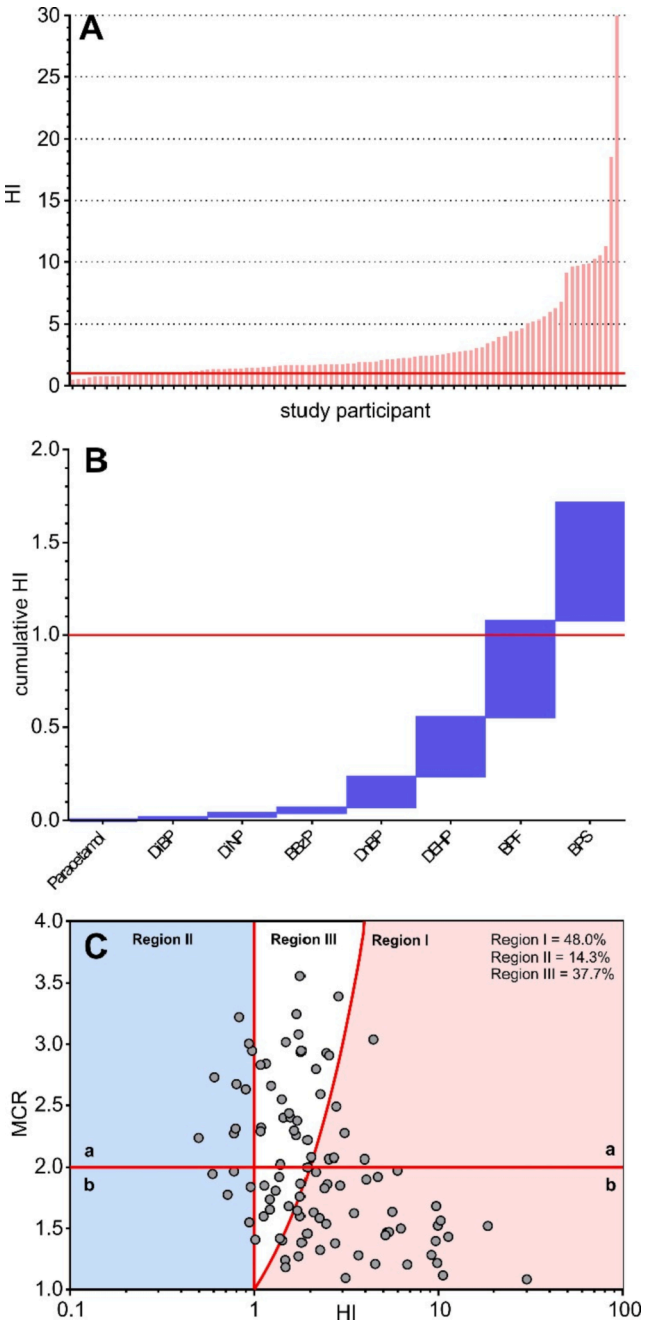

Fig. 2. Mixture risk assessment for chemicals monitored jointly in urine samples from 98 Danish young men, omitting bisphenol A from consideration. A: Personalised Hazard Indices HI_P for each study participants, arranged in ascending order. B: Median Risk Quotients RQ_P of all 98 subjects, arranged in ascending order, legend as in Fig. 1b. C: Scatter plot of Maximum Cumulative Ratio (MCR_P) versus Hazard Index (HI_P) with categories for risk management, as in Fig. 1c.

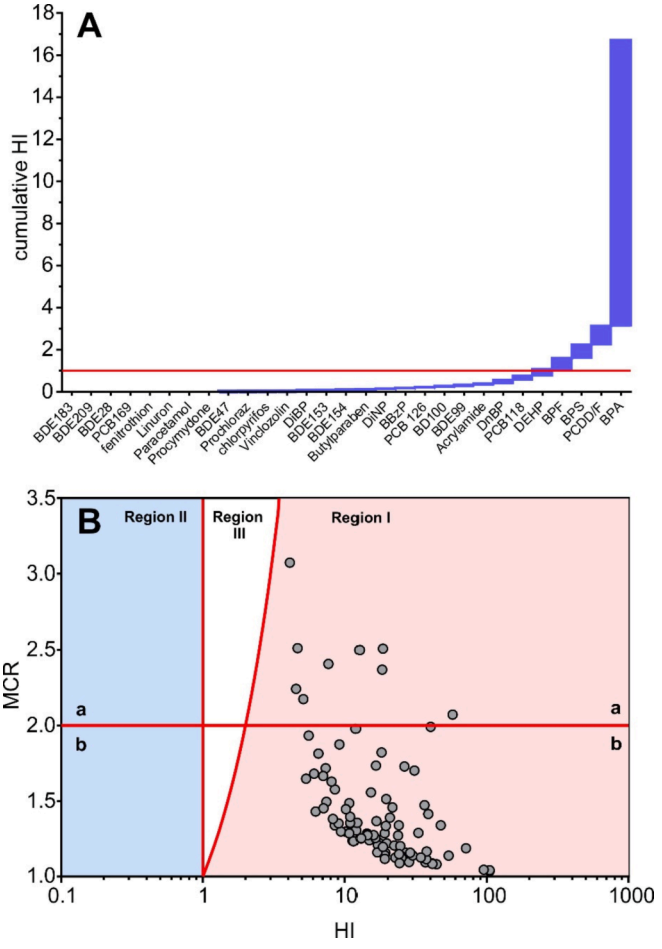

Fig. 3. Mixture risk assessment for chemicals monitored jointly in urine samples from 98 Danish young men, with exposure to additional 20 chemicals. Risk Quotients for the additional 20 chemicals not monitored in urine samples are based on lower bound median exposures in Europe, see Table 1. A: Median Risk Quotients arranged in ascending order, legend as in Fig. 1b. The RQ for the additional 20 chemicals are as shown in Table 3. B: Scatter plot of Maximum Cumulative Ratio (MCR_P) versus Hazard Index (HI_P) with categories for risk management, legend as in Fig. 1c. An HI of 1.39 representing lower bound median exposures to the additional 20 chemicals was added to each MCR_P/HI_P data point shown in Fig. 1c.

the HI_P to below 1.

4. Discussion

To our knowledge, this is the first mixture risk assessment of male reproductive health that includes a more diverse set of chemicals than previously considered, beyond phthalates. Our selection is based on experimental evidence of combination effects of chemicals on deterioration of semen quality (Axelstad et al., 2018) and is further supported by an adverse outcome pathway-driven grouping approach and a review of the relevant mixture literature (Kortenkamp 2020).

Due to the large number of chemicals incorporated in our analysis, we expected the HI to be higher than those reported for phthalates (HI = 0.39 for the year 2009, Apel et al., 2020). However, we were astonished by the magnitude of the HI_P we obtained – more than 100 for the highest exposed subjects, with a median of around 20 for the entire study population (Table 3).

Although it is difficult to obtain estimates of the extent of diminished semen quality associated with such large HI – the HI only reports fold-exceedances of acceptable exposures (abscissa of a dose–response plot), not the effect magnitude (ordinate of a dose–response plot) – it is possible to glean the scale of the problem indirectly. For this purpose, it is necessary to project fold-exceedances of exposures onto a dose–response curve and estimate the corresponding effect sizes. The principle is similar to the way PCDD/F exposures are evaluated by adding them and expressing the sum in terms of 2,3,7,8 TCDD equivalents. This principle is derived from the dose addition concept which assumes that one chemical can be replaced by an equi-effective fraction of another, without loss of combination effect. Accordingly, if all chemicals considered here are expressed as BPA equivalents, the extrapolations that produced the BPA RfD allow us to gain some estimates of effect strength, provided similar assessment factors were used for the other chemicals in the mixture. This condition is met (see Table 2). Thus, the BPA RfD was set 25 times lower than projected human exposures equivalent to doses in rats associated with extrapolated NOAELs (Kortenkamp et al., 2022). This 25-fold margin is customarily introduced to accommodate differences between highly sensitive and more resilient human subjects and to protect the more vulnerable individuals. An HI_P around 25, which we observed for many study subjects, exhausts this safety margin. An HI of 75 or more comes uncomfortably close to dose ranges where effects in rodents were observed, bearing in mind that presumed LOAELs were estimated to be 3-fold higher than NOAELs. Some subjects even exceeded an HI_P of 75. It must be borne in mind that the BPA RfD we used here is not the most conservative value. In deriving this RfD, we struck a balance between caution suggested by studies with lower LOAELs and the weight of evidence from studies with higher NOAELs. The data by Shi et al. (2019) and (Salian et al., 2009) would have supported an even lower BPA RfD. Comparable assessment margins were used to derive the RfD of the other chemicals in our analysis.

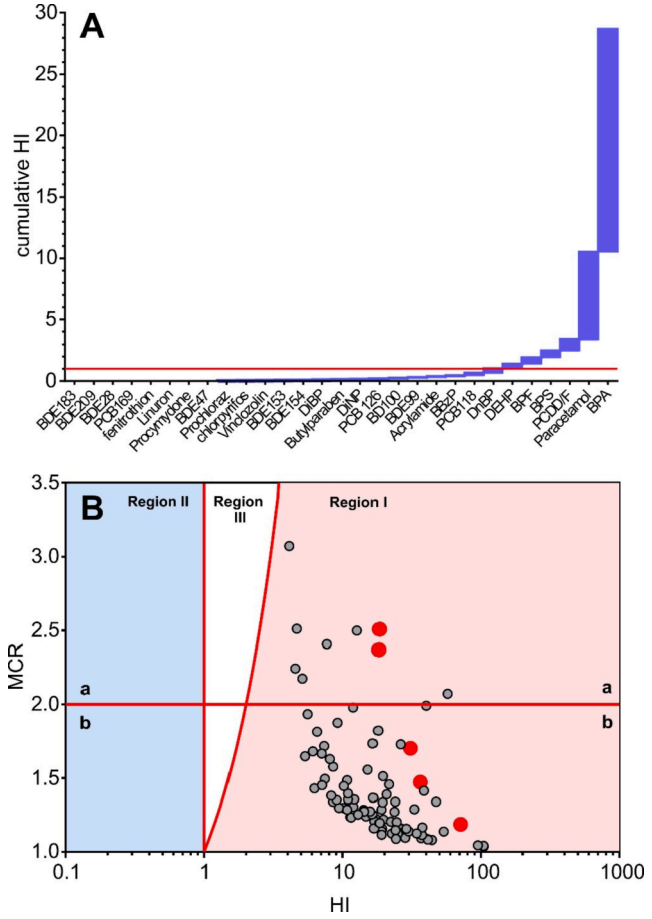

Fig. 4. Mixture risk assessment for 5 individuals with high urinary paracetamol levels, presumably due to therapeutic use. A: Personalised Risk Quotients RQ_P for 5 individuals with high RQ_P for paracetamol. RQ for the additional 20 chemicals not monitored in urine samples arranged in ascending order, legend as in Fig. 1b. The RQ for the additional 20 chemicals are as shown in Table 3. B: Scatter plot of Maximum Cumulative Ratio (MCR_P) versus Hazard Index (HI_P) with categories for risk management, as shown in Fig. 3b, but with the MCR_P/HI_P data points corresponding to the 5 therapeutic paracetamol users highlighted in red. Legend as in Fig. 1c.

The large HI_P obtained in our study are most strongly influenced by BPA. The daily BPA intakes we estimated based on spot urine samples (0.041 and 0.144 μg/kg/d, median and 95th percentile, respectively, see Table 1) agree well with the estimates from 24-hour urine samples from Germany (0.037 and 0.171 μg/kg/d, median and 95th percentile, respectively, Koch et al., 2012). However, the strong influence of BPA should not distract from the extent of the problem which came into view when we excluded BPA from the analysis. This revealed still unacceptably high HI (median HI = 3.2, 95th percentile HI = 11.1, Supplementary Material Table S2) shaped by BPF, BPS, and PCDD/F (see Figs. 3 and 4). Thus, elimination of BPA alone from the exposure scenario, although improving the situation significantly, would not be sufficient to mitigate mixture risks by reducing the HI to below 1, as BPF and BPS will still drive the HI to values in excess of 1 (see Figs. 2, 3 and 4). Our analysis supports steps to also impose restrictions on BPS and BPF, substances used to replace BPA in what has been referred to as regretful substitutions. This appears to be even more urgent, as urinary concentrations of BPS and BPF increased between 2009 and 2017, while BPA decreased (Frederiksen et al., 2020).

Our focus on paracetamol users uncovered the analgesic as an additional driver of HI. This is of relevance as pulsed exposures to paracetamol during specific time windows in pregnancy lead to an increased risk of cryptorchidism (reviewed in Bauer et al., 2021). Repeated doses during gestation precipitated declines in semen quality in rodent offspring (Axelstad et al. 2018, Rossito et al. 2019). Our observation therefore supports recent recommendations to caution expectant mothers at the beginning of pregnancy to forego paracetamol unless specifically advised by a physician (Bauer et al. 2021).

The paracetamol RQ_P for most other study participants were rather small and their contribution to the HI_P was negligible. However, the daily intakes we estimated for this group must be taken with caution, as paracetamol levels found in these urine samples are very likely due to background exposures to aniline which can be metabolised to paracetamol by N-acetylation and aromatic ring hydroxylation in para position. It is estimated that 75–86% of aniline is excreted as paracetamol (Modick et al., 2014).

In the discussion about chemical exposures and male reproductive health, phthalates have received a great deal of attention. It was therefore surprising to see that phthalates were not the most prominent drivers of mixture risks to poor male reproductive health, although they

made a fair contribution to the HI. BPA, BPF, BPS and PCDD/F exerted a much stronger influence on the HI, in every scenario we analysed. Among paracetamol users, but not in subjects exposed to background levels of paracetamol, the analgesic is also a driver of mixture risks.

Our observations might be useful for conceiving future human biomonitoring studies. It will be necessary to monitor BPA, BPS, BPF, PCDD/F and phthalates in samples from the same individual. Such data are at present not available. Similarly, future epidemiological studies of semen quality might benefit from focusing on this set of chemicals.

Despite the unprecedently high HI reported here, our analysis does not fully reveal the scale of the problem. The HI approach requires exposure and toxicity data, and accordingly, the number of chemicals we were able to incorporate in our analysis was restricted by data gaps. For some relevant chemicals, exposure estimates were missing or data on associations with semen quality declines did not permit derivation of RfDs. For example, the number of PCB congeners we could include is incomplete. There are indications that perfluorinated chemicals affect semen quality, but the available studies lack consistency or suggest only weak associations (Petersen et al., 2020). Air pollution presents a more complex assessment problem. While there is evidence that air pollution is associated with poor semen quality (Zhang et al., 2020a, 2020b), it is difficult to delineate the contribution of gaseous and particulate components which complicates estimation of RfDs. A very recent paper shows that particulate matter is associated with reductions in semen motility, but not other parameters (Zhao et al., 2022). Continued efforts to study these pollutants are needed before they can be included in a mixture risk assessment of male reproductive health. Inclusion of further pollutants in the assessment will not only increase the HI, but also drive up the MCR. Thus, the scope of our analysis is constrained by the availability of exposure and toxicity data. In view of the multitude of chemicals humans are exposed to, these constraints almost certainly mean that we have underestimated mixture risks.

There is also uncertainty about the contribution of the 20 chemicals which were not monitored together with the bisphenols, phthalates and paracetamol in our study subjects. Here, we had to estimate daily intakes by compiling data from multiple sources without direct evidence of simultaneous exposures to these substances. We had to infer this mainly from the widespread occurrence of these chemicals as food contaminants. More specifically, we utilized median daily intakes which added 1.39 to the HI for the 9 chemicals monitored together in our study subjects. Making the unrealistic assumption of exposures to all these substances at the 95th percentile of daily intakes would have contributed an additional HI of 3.6 (Table 3), with correspondingly higher risk estimates. A realistic estimate of the true contribution of these 20 chemicals lies between these extremes.

The daily intakes that we estimated for this Danish population of young men agree well with the exposure estimates for other European populations (Koch et al. 2017, Karrer et al., 2020). However, the RfD employed in our mixture risk assessment are geared towards prenatal exposures which are better judged based on urinary concentrations found in expectant mothers. The available evidence from Europe suggests that there are negligible gender differences in the exposures to BPA, BPF and BPS (Koch et al., 2012, Husøy et al. 2019) or phthalates (Koch et al., 2017). It can therefore be assumed that expectant mothers in European countries will experience BPA, BPF, BPA and phthalate exposures similar to those found in men. However, in the USA higher urinary BPA, BPF and BPS levels were measured in men (Lehmler et al., 2018). Such gender differences were also seen in other countries such as Canada, Australia or China (discussed in Lehmler et al., 2018), although they did not always reach statistical significance. The cause of these differences is not entirely clear, but they could be due to different exposures and/or sex-specific toxicokinetics. Men and women metabolise BPA differently, with a higher proportion of glucuronides found in men's urine than in women who have a higher proportion of sulfate conjugates, but these differences did not have an impact on the total urinary levels of BPA conjugates (Kim et al., 2003) and therefore would not have altered our daily intake estimates. If gender differences in BPA, BPF and BPS urinary levels also exist in Europe, the corresponding RQ_P will be slightly lower than those estimated here but would not alter the general picture of significant exceedances of HI.

Our daily intake estimates were made based on a 24-hour urine volume of 1.33 L, as measured in a sample of our study population. The use of the larger recommended volume of 1.7 L (Aylward et al., 2017) would have led to correspondingly higher exposure estimates. Repeated sampling or the collection of 24-hour urine samples will reveal the extent to which the high BPA exposures we observed are episodical or represent a chronic situation.

The reliability of the potency estimates (RfDs) for the substances we identified as drivers of mixtures risks requires scrutiny. While the experimental data supporting the RfDs for BPA and BPS are robust (Kortenkamp et al., 2022, Beausoleil et al., 2022), there are fewer studies of BPF. Another source of uncertainty are the factors used to account for toxicokinetic differences during the estimation of human equivalent doses. The toxicokinetic data available for BPS and BPF are incomplete and forced us to make simplifying assumptions when converting animal doses to HED. Consequently, we have limited confidence in the RfDs for BPS and BPF. Better information of the toxicokinetics may lead to adjustments of these RfDs, however, it is at present impossible to anticipate the likely direction of this change. We have little confidence in the RfD for paracetamol. Our estimation is based on studies which investigated the effects of only one dose level (Axelstad et al., 2018, Rossito et al., 2019). Dose-response studies of paracetamol on semen quality in experimental animals are missing altogether and are urgently needed. It is likely that such studies will reveal effects at even lower doses than employed by Rossito et al. (2019). For paracetamol, BPS and BPF, we assumed relatively high excretion factors and thus we might have underestimated their daily intake doses from the kinetic back-calculation of the urinary concentrations. For PBDEs, our use of a read-across approach to extrapolate the toxicity of untested congeners may have introduced errors, but the impact on our assessment is small, considering the small RQs.

Finally, the summing up of RQs to derive the HI is analogous to the addition of PCDD/F congener exposures as 2,3,7,8 TCDD equivalents (TEQ). Both approaches are based on the mixture effect assessment concept of dose addition. Our approach makes the tacit assumption of additive mixture effects and disregards the possibility of synergisms which would increase the risk estimates. Our recent systematic review and quantitative appraisal of ten years of mixture studies generally supports this assumption (Martin et al., 2021). The more targeted review of relevant literature of mixture studies relevant to male reproductive health also did not uncover strong evidence of synergisms and showed that the available experimental studies agree well with the principle of dose addition (Kortenkamp, 2020).

Due to the use of biomonitoring data of multiple chemicals in the same subjects, and to the choice of RfDs for similar toxicities, the analysis presented here is a high tier mixture risk assessment. There is limited scope for refinement, other than by including monitoring values for more chemicals, by considering additional exposures, or by extending toxicity studies for specific chemicals, as discussed above.

Our findings have relevance to investigations in other mammalian species. Temporal trends in declining semen quality similar to those in humans have also been observed in dogs, a species that closely shares the human environment (Sumner et al. 2020). Whether these trends are due to similar chemical exposures remains an open question, but the available studies have related effects in dogs to PBDEs, PCBs and DEHP (Sumner et al. 2021). There may be merit in also considering the contribution of bisphenols and PCDD/F, all drivers of declines in semen quality in our analysis. Similar considerations apply to the reproductive crisis in cetaceans which thus far has been investigated largely in terms of PCB exposures (Williams et al. 2021).

In conclusion, our mixture risk assessment of chemicals which affect male reproductive health reveals alarming exceedances of acceptable

combined exposures. Due to the data gaps we had to cope with, this must be regarded as a minimum risk estimate. We predict substantial detrimental effects on semen quality due to current combined exposures to BPA, BPF, BPS, PCDD/F and paracetamol, with some contribution from phthalates. Although exposure reduction measures targeting BPA will lead to significant improvements of this situation, exposures to the remainder of the chemicals investigated here also present serious problems. Our analysis has the character of a prediction which could be verified in suitably designed epidemiological studies of semen quality. However, regulatory action such as a ban of BPA from food contact materials should not be delayed until such verifications are available which may take some time.

CRediT authorship contribution statement

Andreas Kortenkamp: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Visualization, Writing – original draft. **Martin Scholze:** Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing – review & editing. **Sibylle Ermler:** Formal analysis, Investigation, Writing – review & editing. **Lærke Priskorn:** Data curation, Methodology, Writing – review & editing. **Niels Jørgensen:** Data curation, Methodology, Writing – review & editing. **Anna-Maria Andersson:** Data curation, Methodology, Writing – review & editing. **Hanne Frederiksen:** Formal analysis, Data curation, Methodology, 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.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2022.107322.

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