"The difference between stupidity and genius is that genius has its limits."
Albert Einstein

Food for Thought ...

Thresholds of Toxicological Concern – Setting a Threshold for Testing Below Which There Is Little Concern

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Summary

Low dose, low risk; very low dose, no real risk. Setting a pragmatic threshold below which concerns become negligible is the purpose of thresholds of toxicological concern (TTC). The idea is that such threshold values do not need to be established for each and every chemical based on experimental data, but that by analyzing the distribution of lowest- or no-effect doses of many chemicals, a TTC can be defined – typically using the 5th percentile of this distribution and lowering it by an uncertainty factor of, e.g., 100. In doing so, TTC aims to compare exposure information (dose) with a threshold below which any hazard manifestation is very unlikely to occur.

The history and current developments of this concept are reviewed and the application of TTC for different regulated products and their hazards is discussed. TTC lends itself as a pragmatic filter to deprioritize testing needs whenever real-life exposures are much lower than levels where hazard manifestation would be expected, a situation that is called "negligible exposure" in the REACH legislation, though the TTC concept has not been fully incorporated in its implementation (yet). Other areas and regulations – especially in the food sector and for pharmaceutical impurities – are more proactive. Large, curated databases on toxic effects of chemicals provide us with the opportunity to set TTC for many hazards and substance classes and thus offer a precautionary second tier for risk assessments if hazard cannot be excluded. This allows focusing testing efforts better on relevant exposures to chemicals.

Keywords: toxicity limits, risk assessment, exposure, computational toxicology, alternative methods

1 Introduction

Except if you believe in homeopathy, there is a dose for any substance below which there is no expectation of biological activity and thus no concern of toxicity. Almost all toxicologists agree on this since Paracelsus wrote, "The dose makes the poison." The *de minimis* concept suggests a human exposure threshold for chemicals below which there is no significant risk to human health.

Many of our risk assessments are for this reason based on benchmark doses, no-(adverse)-effect-levels (NOEL, NOAEL), lowest-(adverse)-effect levels (LOEL, LOAEL), points of departure, derived no-effect-levels (DNEL), reference doses (RfD), etc. So, these effect thresholds – with and without incorporation of uncertainty factors – come in different flavors but, essentially, they do the same: They define a dose, below which nothing happens in test animals and, using safety and uncertainty factors, we can calculate doses that are extremely unlikely to have effects in humans.

The threshold of toxicological concern (TTC) concept aims to formalize this for direct comparison with exposure to a given

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substance. Hennes (2012) defined, "*The TTC is based on the concept that an exposure threshold value can be established below which a very low probability of an appreciable risk to human health (or the ecosystem) exists*". Our glossary (Ferrario et al., 2014) followed the very similar definition formulated by Duffus (2007), "*Threshold of toxicological concern (TTC): Human exposure threshold value for a group of chemicals below which there should be no appreciable risk to human health*". In a less than perfect world, this is an almost perfect way to pragmatically calculate limits of exposure for untested chemicals that promise to be safe.

Is there a threshold of toxic effects and do all toxic effects have one? A single molecule could, at least theoretically, create DNA damage, a mutation that is not necessarily repaired and can change the cell. However, there have been arguments over decades about whether mutagenicity and cancer do not follow this paradigm (Kirsch-Volders et al., 2000; Neumann, 2009) and similar arguments of no threshold have been made for teratogens (Gaylor et al., 1988) and even endocrine disruption (Kortenkamp et al., 2012), with the former largely defeated (Brent and Fawcett, 2007) while the latter is still a hot topic of discussion. They are seen by some as stochastic events that become less and less probable with declining dose, but the probability does not reach zero; in some cases, even effects found only at low doses that are not seen at higher ones are postulated, often called non-monotonous dose-response curves. However, outside academic discussion, in a real-world scenario, the probability of hazard at some point simply falls below the noise, i.e., the inevitable spontaneous development of such diseases.

For example, a recent, prominent study out of Hopkins suggests that two thirds of all cancers are due to chance (Tomasetti and Vogelstein, 2015; Tomasetti et al., 2017) – the authors concluded, across 32 cancer types, that 66% of cancer-promoting mutations arise randomly during cell division in various organs throughout life, 29% trace to environmental causes, and 5% are inherited. The environmental part, especially when you subtract smoking (Hartung, 2016), adds small risks to the random chance and is difficult to prove and thus to prevent for any given chemical, except in extreme exposure situations, for example at certain workplaces. So, without reentering the argument of threshold versus linear extrapolation, there is a practical threshold where the risk exerted is so small that there is no longer really concern. This is the basic idea of a threshold of toxicological concern (TTC).

Due to ever-improving analytical capabilities, very low levels of unexpected chemicals can now be detected in many products and in the environment. This improved analytical capability challenges industry and regulators to address with increasingly limited resources more and more issues associated with the detection of very low levels of chemicals in products. For example, there are about 10,000 food contact materials[1] that can possibly migrate into food. TTC help to set a limit determining whether these require further attention for testing and risk assessment: "*The threshold of toxicological concern (TTC) methodology provides a scientifically defensible, transparent approach for putting low-level exposures in the context of potential risk, as a tool to facilitate prioritization of responses, including potential mitigation*." (Felter et al., 2009).

The problem is exacerbated in case of chemicals for which little or no toxicological data is available (Koster et al., 2011). The TTC approach has been controversial, because it carries out risk characterization without the usual toxicity data; the validity

Tab. 1: Current regulatory use of TTC

Area	Authority	Reference
Food packaging migrants and flavoring agents	US FDA, JECFA, WHO	FDA, 1995, 2001; JECFA, 1998; WHO, 2000
Food flavorings and pesticide metabolites in groundwater; *Under discussion for:* food contact materials; impurities and breakdown/reaction products in food and feed additives; plant metabolites and degradants of pesticides; metabolites of feed additives; technological feed additives; flavoring substances in feed	EFSA	EFSA, 2012, 2016
Genotoxic impurities in (veterinary and human) pharmaceutical preparations and genotoxic constituents in herbal substances and preparations	EMEA, EMA	EMEA, 2004; FDA, 2008; EMA, 2006, 2013
Genotoxic and carcinogenic impurities in drugs	US FDA	McGovern and Jacobson-Kram, 2006; ICH guidance M5, 2015[a]
Within REACH registrations for industrial chemicals	ECHA	ECHA, 2016

[a] https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM347725.pdf

[1] https://www.kvcv.be/index.php/en/food-contact-materials

Tab. 2: Cramer classes
(Munro et al., 1999)

I.	Substances of simple chemical structure with known metabolic pathways and innocuous end-products, which would suggest a low order of oral toxicity.
II.	Substances less innocuous than substances in class I, but do not contain structural features suggestive of toxicity like those substances in class III. May contain reactive functional groups.
III.	Substances of a chemical structure that permit no strong initial presumption of safety, or may even suggest significant toxicity.

of the TTC approach is also critically dependent on the validity of the databases used, as we will discuss later.

Several reviews of the TTC concept (Kroes and Kozianowski, 2002; Kroes et al., 2005; Barlow, 2005; Munro et al., 2008; Hennes, 2012; Canady et al., 2013 and many more cited in the following) can give broader background than is the purpose of this article. Here, especially the challenges ahead for a broader use of TTC shall be addressed. TTC should be an integral part of the strategic development of safety sciences (Busquet and Hartung, 2017) as discussed in this series of articles (Hartung, 2017a) as part of the need to move away from animal experimentation (Hartung, 2017b). Regulatory use of TTC originated out of the US Food and Drug Administration (US FDA) already two decades ago, but regulatory approval is still rather limited (Tab. 1), with only two broadly accepted uses, i.e., low-level food constituents and drug impurities.

The TTC concept has found considerable interest in recent years, especially in Europe. A large ILSI-Europe workshop (Dewhurst and Renwick, 2013) and subsequent ILSI-Europe Threshold of Toxicological Concern Task Force[2], as well as several opinions by the European Agencies[3], and the scientific committees of Directorate General for Health and Food Safety (SCHER, SCCP, SCENIHR, 2008; EFSA, 2012) addressed the topic. In 2012, three independent non-food Scientific Committees of the European Commission were jointly tasked with evaluating potential applications of the TTC approach for human health risk assessment of chemical substances (EC, 2012, 2013). Their opinion focused on the potential applications of the TTC concept for cosmetics and other consumer products in relation to their mandates. They considered the TTC approach, in general, "*scientifically acceptable for human health risk assessment of systemic toxic effects caused by chemicals present at very low levels, as based on sound exposure information*". However, they emphasized the need for a high level of confidence in:

(1) the quality and completeness of the toxicity databases;
(2) the reliability of the exposure data for the intended use of the chemical; and
(3) the appropriateness of any extrapolations in order to apply the TTC approach in risk assessment.

Similarly, the European Food Safety Authority (EFSA) Scientific Committee concluded (EFSA, 2012) that the "*TTC approach can be recommended as a useful screening tool either for priority setting or for deciding whether exposure to a substance is so low that the probability of adverse health effects is low and that no further data are necessary*." TTC is also part of the ECHA guidance for testing[4] (ECHA, 2016, pp. 264-272), though in a more hesitant manner (see later). The emerging consensus might offer the opportunity for broader application of this very pragmatic approach to reduce testing and animal use.

2 The TTC concept and its emergence

Felter et al. (2009) credit Frawley (1967), who set out to "*determine a level of use of any food-packaging component which could be considered to be safe regardless of its degree of toxicity*" for the first TTC. The first TTC values were proposed for chemicals in food entering unintentionally from packaging or added in very low amounts, such as flavoring agents. The US FDA introduced a threshold of regulation (TOR) approach for indirect food additives (US FDA, 1995). The TOR represented a pragmatic way to address the safety of food packaging materials that had the potential to migrate into food at a level that was considered to be sufficiently low to be considered toxicologically insignificant, even in the absence of chemical-specific toxicity data. This was based on a statistical analysis of the Carcinogenic Potency Database (CPDB) of Gold et al. (1984, 1989) and the TOR of 0.5 ppb in the diet, corresponding to 1.5 µg/person/day (US FDA, 1995, 2001) or 0.025 µg/kg body weight/day, was set.

Two principal approaches have been used to develop (for food contact articles and flavoring substances): first, a general TTC, suggested to apply to all chemicals and all health effects, mainly based on carcinogenicity data, and second, a TTC based on structural information compared with toxicological data of chemicals ("the decision tree approach") for non-carcinogenic endpoints (Kroes et al., 2005; Munro et al., 2008), i.e., where TTC are deduced for categories of chemicals. Both approaches focused strongly on cancer studies, mutagenicity, and the underlying chemical reactivity. However, although derived from carcinogenicity data, the US FDA does not accept the use of this TOR for known carcinogens or for chemicals with structural alerts or other evidence of carcinogenicity. An ILSI workshop in

[2] http://ilsi.eu/wp-content/uploads/sites/3/2016/09/Threshold-of-Toxicological-Concern_TFonepager.pdf

[3] https://www.efsa.europa.eu/en/topics/topic/threshold-toxicological-concern

[4] https://echa.europa.eu/documents/10162/23047722/ir_csa_r7c_pbt_peg_en.pdf/3db0a474-02bb-4358-83fc-20e7ff81ef2c

2011 (Dewhurst and Renwick, 2013), however, considered the current TTC value to be adequate and fit for purpose for cancer because it is derived by linear extrapolation from the owest toxicological dose 50% (TD_{50}) for each compound in the largest available rodent carcinogenicity database.

Moving away from the cancer bioassays, analyzing a reference database of more than 600 substances tested in more than 2,900 sub-chronic and chronic toxicity studies, Munro et al. (1990, 1996, 1999) derived higher TTC values and linked them to structural chemical classes defined by Cramer et al. (1978) (Tab. 2). This classification is based on the single, potentially most toxic functional group present in the molecule. Most complex chemicals are therefore assigned to Class III (lowest TTC). More modern identifications of toxic chemophores and aggregate similarity measures have yet to be explored.

In Europe, the EU Scientific Committee on Food (SCF, 1996) first considered TTC use, raising questions on whether the initial TTC value of 1.5 µg/day, derived from the cancer database,

Tab. 3: Hazards for which TTC have been suggested

Hazard	**Value range (µg/kg bodyweight/day)** if not given otherwise; values given per person were divided by 60 kg	**References**
General toxicity (genotoxic substances)	0.0025 - 2 (depending on duration)	Rulis 1986, 1989; Kroes et al., 2005; Cheeseman et al., 1999; Felter et al., 2009; Müller et al., 2006
General toxicity (non-genotoxic substances)	0.025 - 1 (depending on Cramer classes)	Munro et al., 1996, 1999
General toxicity organophosphates including carbamates, organohalogens and remaining Cramer class III substances	0.30 - 4	Leeman et al., 2014
Repeat dose toxicity (oral)	0.63 - 60 (depending on Cramer classes and duration, OECD TG)	Munro et al., 1996, 1999; Bunke et al., 2006; Bitsch et al., 2006; Tluczkiewicz et al., 2011
Repeat dose toxicity (inhalation)	0.07 - 23 (depending on Cramer classes and duration, OECD TG)	Carthew et al., 2009; Tluczkiewicz et al., 2011; Bitsch et al., 2006; Munro et al., 1996, 1999; Escher et al., 2010; Bernauer et al., 2008
Genotoxicity	0.025 - 2	Rulis 1986, 1989; Kroes et al., 2005; Müller et al., 2006
Carcinogenicity (genotoxic)	0.0025	Kroes et al., 2005; Cheeseman et al., 1999
Carcinogenicity (non-genotoxic)	0.025 - 0.75 (depending on Ames test and acute toxicity)	Kroes et al., 2005; Cheeseman et al., 1999
Acute toxicity (inhalation)	4 - 1,000 µg/m^3	Grant et al., 2007; Escher et al., 2010
Neurotoxicity	0.3	Munro and Kroes, 1998; Kroes et al., 2000
Developmental toxicity	1[a] - 8 - 131 (depending on Cramer class) 0.5 - 1 µg/m^3 (inhalation)	Munro and Kroes, 1998; Bernauer et al., 2008; van Ravenzwaay et al., 2011; Laufersweiler et al. 2012
Reproductive toxicity	1 - 100	Bernauer et al., 2008; van Ravenzwaay et al., 2011, 2012, 2017
Estrogenic endocrine disruption	0.025	Kroes et al., 2000
Immunotoxicity	0.15 - 1,000	Kroes et al., 2000; Hartung and Corsini, 2013
Skin sensitization (dermal)	0.91 - 900 µg/cm^2	Safford, 2008; Safford et al., 2011; Keller et al., 2009

[a] derived differently, i.e., using the lowest NOAEL, not a 5th percentile and uncertainty factor 1,000, because of the small dataset.

would adequately cover neurotoxicity, developmental toxicity, endocrinological effects, and immunotoxicity. In response to the SCF (1996), Kroes et al. (2000) examined 81 chemicals from the Munro et al. (1996) database with data on developmental toxicity to determine if the distribution of NOELs for the developmental toxicity endpoint indicated more toxicity than the NOEL distribution from the chronic studies for Cramer Class III chemicals as a whole. They concluded that the distribution of NOELs from the developmental endpoint database was not significantly different from the one by Munro et al. (1996) for the Class III chemicals. The substances in the Munro reference database are of a wide variety of chemical structures although applicability of the values was specifically proposed for the safety evaluation of flavoring substances. To facilitate the application of the Cramer classification, the Joint Research Centre (JRC) of the European Commission later developed the software tool, Toxtree[5], which puts chemicals through the decision tree (see Patlewicz et al., 2008; Lapenna and Worth, 2011).

Munro et al. (1996) used the 5th percentiles of the lowest NOEL for each substance tested in chronic studies; NOELs of sub-chronic studies were divided by 3; and they then applied an additional assessment factor of 100. Bernauer et al., (2008), in contrast, used the lowest value and applied a safety factor of 100-1,000. The resulting TTC values are given in Table 3.

Munro et al. (1996) emphasized that substances should be chemically well-defined and without indication of possible genotoxic effects. Furthermore, they recommended that the TTC approach should in general not be used as an alternative to testing procedures required for regulatory approval.

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) built on the scheme and developed a decision tree for the evaluation of flavoring substances (JECFA, 1998; WHO, 2000). The TTC approach was reviewed at a 1999 workshop by ILSI Europe's TTC Task Force (Barlow et al., 2001), where some refinements were suggested.

A review by Kroes et al. (2005) resulted in the development of a TTC value for compounds with certain structural alerts for genotoxic carcinogenicity. Some chemical classes might need to be exempted, e.g., analyzing 31 organophosphorous insecticides in the Munro et al. (1996) database, Kroes et al. (2005) proposed a TTC value of 18 µg/person/day. Although this does not seem to have found general acceptance, the recent draft opinion of EFSA (2012) considers this value as sufficiently robust for the assessment of substances with anti-cholinesterase activity, such as organophosphates and carbamates.

A research project at the Fraunhofer Institute for Toxicology and Environmental Medicine looked at the RepDose (repeat-dose toxicity) database, which at the time contained over 500 chemicals tested in more than 1,400 repeat-dose, oral OECD guideline studies, and they pooled them with the chemicals of the Munro et al. (1996) database that were also tested according to the same guideline studies, i.e., more than 400 chemicals in 450 repeat-dose, oral studies (Bitsch et al., 2006; Tluczkiewicz et al., 2011). The derived TTC values are included in Table 3. The detailed evaluation of the underlying data showed that whilst there is a reasonable distinction between Cramer classes I and III, class II is not well defined. This could be addressed in the future to improve the validity of the TTC values that are derived from non-carcinogenic endpoints and are linked to the Cramer classes. A strategy to refine the current Cramer classification has been proposed (Tluczkiewicz et al., 2011).

Shortly later, Kalkhof et al. (2012) analyzed data from 813 industrial chemicals registered in the European List of Notified Chemical Substances, i.e., 756 chemicals tested according to OECD 407 (28-day repeat-dose) and 57 chemicals tested according to OECD 408 (90-day repeat-dose). The adjusted chronic NOAEL, i.e., derived by applying a factor of 6 for the 28-day and of 2 for the 90-day studies, were compared with Cramer class I and III values for the respective type of substances, thus confirming that the TTC values for these two Cramer classes are set conservatively.

In 2004, ECETOC proposed a targeted risk assessment approach for REACH, including a series of threshold values for a wide variety of organic and non-organic substances (both volatile and non-volatile), i.e., so-called generic exposure value (GEV) and generic lowest exposure value (GLEV) for acute and repeated dose toxicity (ECETOC, 2004) (category 1 and 1B carcinogens, mutagens and reprotoxicants were excluded). ECHA refers to this in their current guidance to industry (2016): "*The GEV is a generic threshold values [sic] for occupational exposure (and derived dermal values), derived from some most stringent Occupational Exposure Limits (OEL). The GLEV is based on classification criteria for repeated dose toxicity and extrapolation factors. It is noted that the derivation of GEV values was based upon an analysis of current published occupational exposure levels, and therefore also incorporated socio-economic and technical arguments in addition to the assessment factors applied to toxicological endpoints and other data on which the OELs were based*." As ECHA (2016) notes "*This approach has not been peer reviewed nor accepted by regulatory bodies*."

3 Threshold setting in toxicology

Following Paracelsus, considered the founding father of toxicology, "*All things are poison and nothing is without poison; only the dose makes a thing not a poison*". If so, all toxicology is about defining the limits of safe use of substances. The author has argued elsewhere that the statement that everything is poisonous is quite misleading as not all substances can produce toxic effects in animals in doses that can be practically applied (Luechtefeld et al., 2016a). For example, only about 20% of substances are acutely toxic up to the common limit of 2 g/kg bodyweight (Luechtefeld et al., 2016b). Imagine a human swallowing 100 to 200 g of pure chemical… But Paracelsus is right about the fact that for those chemicals that are poisonous, it is

[5] http://ihcp.jrc.ec.europa.eu/our_labs/computational_toxicology/qsar_tools/toxtree

the dose that makes them so. Thus, toxicology is about identifying substances with a hazard potential and defining thresholds for their safe use.

However, these thresholds are determined in experimental (animal) models, which necessarily differ from human real-life exposures. Uncertainty or assessment factors were therefore introduced to err on the side of safety. Some of the discussion around such factors is reflected below, but their adequacy is not really the point of this article as they are broadly accepted and used in the risk assessment community.

Another interesting threshold used in toxicology is the percentage of substance in a product or as a contaminant of a chemical that warrants an assessment: typically, 1-10% depending on the regulation. These limits are quite arbitrary, pragmatically limiting testing demands rather than being science-based. Most evidently for allergens, but also for highly toxic or carcinogenic substances, these limits are difficult to justify.

3.1 Cancer TTC

As mentioned above, TTC was developed originally using a cancer database but is controversially discussed for exactly these threshold mechanisms and not broadly accepted, at least not for genotoxic carcinogens. Dewhurst and Renwick (2013) summarize that it was considered adequate to move from 0.15 to 1.5 µg/person/day based on an absence of alerts for genotoxic carcinogenicity, but greater evidence of the absence of DNA reactivity, i.e., a "non-threshold" mode of action, was necessary before moving to the Cramer class tiers. To this end, a transparent, consistent and reliable means to identify structural alerts needs to be produced as current tools such as DEREK and TopTree can give disparate results. To move from 1.5 to 90 µg/person/day should require a weight of evidence that the compound is not a suspect DNA reactive carcinogen, rather than just the absence of data.

3.2 Non-cancer TTC

Since the 1.5 µg/person/day value derived by Munroe et al. (1996) is very conservative, it is often considered to be a general threshold of no concern, implying that it would be applicable to any chemical of unknown toxicity. Barlow et al. (2001) reported on an ILSI Europe workshop that had looked at some potentially sensitive non-carcinogenicity endpoints such as immunotoxicity, developmental toxicity, neurotoxicity and developmental neurotoxicity, endocrine active compounds, and allergenicity. They concluded that the large margins of safety built into this TTC would probably also cover these endpoints, except for allergenicity. In the meantime, TTC for skin sensitization are, however, quite well established too (Basketter et al., 2002; Safford, 2008; Keller et al., 2009; Safford et al., 2011, 2015).

A number of studies expanded the concept also to inhalation toxicology as an alternative route of administration (Munro et al., 1996, 1999; Bitsch et al., 2006; Grant et al., 2007; Bernauer et al., 2008; Carthew et al., 2009; Escher et al., 2010; Tluczkiewicz et al., 2011). Recently, Schüürmann et al. (2016) derived structural alerts that discriminate between high- and low-toxic compounds for inhalation repeated-dose TTC. Furthermore, they identified physicochemical parameters related to the inhalation-specific bioavailability and explored their use as predictors of high vs. low toxicity.

Kroes et al. (2005) developed a separate threshold for organophosphates and EFSA (2012) suggested that carbamates with anti-choline esterase activity can be included in this TTC. Leeman et al. (2014) focused on these thresholds and developed TTC for lifetime exposure for organophosphates including carbamates, the group of organohalogens and the remaining Cramer class III substances, being 0.30, 1.5 and 4.0 µg/kg bodyweight/day, respectively.

Cheeseman et al. (1999) extended the TTC concept by proposing a tiered approach based on structure-activity relationships, genotoxicity and short-term toxicity data. They further analyzed the databases to define subsets of chemical substances based on the results of the Ames assay, structural alert classes and lethal dose 50% (LD_{50}) values, and thus derived higher threshold levels for less potent substances. Assuming linearity in the dose-response relationship when extrapolating to low doses, even for non-genotoxic carcinogens, still represents a highly conservative approach.

Analyzing data on 91 chemical substances from the EU existing chemicals program on fertility or developmental toxicity, Bernauer et al. (2008) derived TTC values for reproductive toxicity. For oral exposure, 58 NOAEL for fertility and 62 NOAEL for developmental toxicity were found. Because of the limited number of data points, the lowest value in the distribution was used to derive the thresholds as opposed to identifying a percentile-based value. Applying an overall assessment factor of 1,000 (10 for interspecies differences, 10 for human variability, and 10 for uncertainty from a small dataset and severity of the health effects), the TTC values included in Table 3 were obtained. A company-internal database from BASF served to derive further TTC values for reproductive toxicity endpoints for oral exposure in rats (van Ravenzwaay et al., 2011). They analyzed 93 prenatal developmental toxicity studies according to OECD 414 using the 5th percentile to derive TTC for developmental and maternal toxicity (Tab. 3) resulting in a TTC of 10 µg/kg bw/day. Furthermore, using either maternal toxicity data of the same substances or expanding to include the Kroes et al. (2005) data, a TTC of 8 µg/kg/day was obtained. The same group (van Ravenzwaay et al., 2012) identified 104 rabbit studies with values for maternal and developmental toxicity (48 from BASF, 56 from literature) using the 5th percentile for developmental toxicity of these mostly active ingredients, a TTC value of 4 µg/kg bw/d was calculated using a safety factor of 500 to account for the relatively small database. Laufersweiler et al. (2012) expanded this approach to 300 chemicals with reproductive and developmental data, deducing a TTC of 6 µg/kg bw/day. These reports already showed very consistent TTC for reproductive and developmental toxicity. The fact that this series of independent assessments resulted in very similar NOAEL thresholds is very reassuring.

Our own recent work making the REACH registration database machine-readable (Luechtefeld et al., 2016a) allowed a further expansion by teaming up with BASF (van Ravenzwaay et al., 2017): A total of 480 chemicals tested in rats and 110 in rabbits were obtained and used for evaluation. The

5th percentile of the evaluated studies in rats of the relevant NOAEL for maternal toxicity is 10 mg/kg bw/d (based on 434 values) and for developmental toxicity is 11.5 mg/kg bw/d (based on 469 values). For the 110 rabbit studies, the 5th percentile for maternal toxicity NOAEL is 5.2 mg/kg bw/d and for developmental toxicity 10 mg/kg bw/d. With the now greatly enlarged database, an uncertainty factor of 100 appears to be justified. This contributes to a remarkably higher TTC of 100 µg/kg bw/day for rats and 95 µg/kg bw/day for rabbits for reproductive toxicity compared to other endpoints. The fact that developmental and maternal toxicity hardly differ is also quite remarkable.

3.3 TTC for environmental endpoints

The application of TTC for environmental assessments is so far rather limited. De Wolf et al. (2005) analyzed environmental toxicological databases for acute and chronic endpoints. 5th percentile values were derived by stratifying based on mode of action (MOA; 1 = inert chemicals; 2 = less inert chemicals; 3 = reactive chemicals; 4 = specifically acting chemicals). A preliminary analysis showed in case of MOA1 or MOA2, a TTC even higher than 0.1 µg/l. A significantly lower TTC resulted for MOA4. Gross et al. (2010) discussed this in a workshop for one of the more controversial applications in human health, i.e., endocrine disruption. Furthermore, Sorell (2016) recently explored the TTC concept for three drugs found frequently as contaminants in the environment, but could only recommend this compared to other approaches on a case-by-case basis.

3.4 TTC for food additives and contaminants

Chemicals get into food either as contaminants from the environment (air, soil and water), as unexpected formation of chemicals during processing and preparing foods, as naturally present chemicals in the foods, as accidental release of chemicals used in food production, or as unrecognized failure of food quality control, e.g., intentional adulteration of foods or ingredients (Felter et al., 2009). Since the origin or source of a chemical (e.g., whether it is intentionally used as a food packaging material or is added as a flavor ingredient versus whether it is present as a contaminant) has no bearing on its inherent toxicity, a generally applicable threshold approach based on the potential exposure is considered to be appropriate for any low-level exposure.

In 1958, the Food Additives Amendment to the Federal Food, Drug and Cosmetic Act (US FDA, 1958) defined that contact materials and their components that might migrate unintentionally into food were included in the definition of a food additive. This, in combination with the emerging more sensitive chemical analytical methods, prompted a need for an FDA policy to handle low dose exposure. While still protecting public health if the substance turns out to be a carcinogen, the US FDA wanted to be able to waive requested tests in certain cases, and to be consistent in this waiving procedure (TemaNord, 2005). During several years discussions went on concerning how to establish a safe level, which, as described in the introduction, was introduced in the 1980's as a "threshold of regulation" for food contact materials at a level of 0.5 ppb in the diet (Rulis, 1986, 1989). The first to use the TTC for flavoring agents was the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 1993, 1995, 1999). The TOR approach was later expanded into a tiered TTC decision tree (Kroes et al., 2005; Munro et al., 2008). Both JECFA and EFSA have been using TTC for evaluating risk assessment of flavoring agents with very low levels of exposure through the diet (JECFA, 1997; EFSA, 2012; Renwick, 2004).

Most food products are very complex, including many natural materials of complex and variable composition. It is unrealistic to identify and quantify the complete mixture of substances for risk assessment purposes. Since many of these substances, often only identified as a peak in a mass-spectrometer, are found in low abundance, a TTC concept for handling their risk assessment has been suggested (Rennen et al., 2011).

Most critically, Grob (2002) demonstrated the problem of analysis of migrates from food-packing materials, listing analytical requirements and problems to be dealt with: According to this article, consumers often ingest more than 100 µg of an unidentified migrant from a single packed food. It is concluded that many food-packing materials may not correspond to the safety called for by law, and that analysis down to the TTC (1.5 µg/person/day) seems difficult or impossible.

3.5 TTC for pharmaceuticals

The synthesis of medicines often requires reactive reagents and results in the formation of intermediates and by-products. Low levels of these are often present in the final product as impurities with possibly unwanted toxicities including genotoxicity and carcinogenicity. Dolan et al., (2005) suggested acceptable daily intake values (ADIs) for compounds with limited or no toxicity information to support pharmaceutical manufacturing operations by TTC: 1 µg/day for compounds that are likely to be carcinogenic, 10 µg/day for compounds that are likely to be potent or highly toxic, and 100 µg/day for compounds that are not likely to be potent, highly toxic or carcinogenic. Most recently, TTC were adapted also for impurities, residual materials, and contaminants in vaccines (White et al., 2016).

For the purpose of assessing (genotoxic) impurities for both human and veterinary medicines, the TTC concept is well established. Kirkland and Snodin (2004) report early regulatory developments for genotoxic impurities, discussing the content of the *Position Paper on the Limits of Genotoxic Impurities* that was published in 2002 by the Safety Working Party (SWP) of the European Committee for Proprietary Medicinal Products (CPMP). An important paper dealing with genotoxic impurities was published by Müller et al. (2006), which was developed by an expert group of the Pharmaceutical Research and Manufacturing Association (PhRMA); it summarizes several innovative approaches for determining, testing, and controlling potential genotoxic impurities. In consequence, the TTC decision tree has also been recommended as a tool to evaluate low-level exposures associated with contaminants in pharmaceuticals by the respective European agency EMEA, now EMA, for impurities in pharmaceutical products (EMEA, 2004; EMA 2006, 2007, 2008) and US FDA (2008). Delaney (2007) has argued that higher TTC would be defendable as the values were taken over from the food sector where they were based on databases with potent carcinogens, which can be excluded to be present in medicinal products.

3.6 TTC for cosmetics and other consumer products

The European cosmetics regulation has banned animal testing for this industry, starting with the 7th amendment of the legislation in 2002 (Hartung, 2008). This was about the time when TTC discussions became more prominent in the food sector. Thus, and given the fact that a lot of food materials are used in cosmetics, it was not surprising that this sector explored the suitability of TTC for their products' safety assessments (Kroes et al., 2007). However, the relevance of the chemical domain that supports the use of TTC has been challenged, especially when considering classes of chemicals with specific uses, e.g., personal and household care products and cosmetics (SCCP, 2008). A workgroup of the European trade organization Colipa, now Cosmetics Europe, showed good coverage of the product ingredient structures and confirmed that the NOAELs for the ingredient chemicals are similar in range to the original dataset, supporting the use of the TTC for ingredients in consumer products (Blackburn et al., 2005).

The COSMOS consortium within SEURAT[6], a 3.4 million € cluster of research jointly funded by the European Commission and Cosmetics Europe, has prepared a new Cosmetics Inventory combining the EU COSING database[7] and the list from the US Personal Care Products Council (PCPC) (PCPC, 2011). At time of download, the COSING inventory consisted of 9,286 unique CAS Registry Number (CAS RN) – a unique numerical identifier assigned by Chemical Abstracts Service (CAS) to every chemical substance described in the open scientific literature – and 19,390 unique International Nomenclature of Cosmetic Ingredients (INCI) names. The PCPC inventory lists 3,716 unique CAS RNs and 3,657 unique INCI names. The consortium also developed a new toxicity database enriched with oral repeated dose studies for cosmetics-related chemicals (including US FDA, US EPA, EU SCCS, EU ECHA, US National Toxicology Program, and literature publications). A new non-cancer TTC database for cosmetics-related chemicals has been compiled by augmenting the COSMOS database with substances from the Munro dataset found in the Cosmetics Inventory. The resulting TTC database contains over 580 chemical structures with NOAEL (Yang et al., 2013). The consortium stated in several presentations that the chemical space of the new TTC database has been compared with existing TTC databases to demonstrate that the coverage is suitable for the assessment of cosmetics products. The TTC database is available as a download from the website and shall serve as a resource for alternative methods, but the key results as to TTC have only been submitted (personal communication Mark Cronin, Liverpool) except for a preliminary report (Worth et al., 2012) and the SEURAT-1 annual report books[8]. Williams et al. (2016), as part of COSMOS, combined the TTC with an algorithm to predict skin penetration, which further refines the TTC concept for cosmetics.

3.7 TTC for industrial chemicals

Over the last decade, safety testing of industrial chemicals already on the market for decades has received a lot of attention, especially with the pioneering REACH legislation (Hartung, 2010). The enormous task and the fact that REACH testing needs are very much exposure-driven, should have opened industrial chemicals up for TTC use. Combes et al. (2003) already suggested integrating a TTC concept into the REACH procedure in order to minimize testing needs, however, without developing a clear strategy. Concern of member states dampened the application of TTC. The Nordic Council of Ministers in 2005 came to the conclusion that application of the TTC principle at the higher tonnage levels within REACH would be premature and raised some concerns, for example that the TTC concept has not been evaluated for the diverse group of industrial chemicals and for different routes of exposure (Bernauer et al., 2008).

Still, the REACH principle that negligible exposure allows waiving of testing represents, at least theoretically, a premier entry port for TTC. According to the regulation, aside from waiving criteria such as technical feasibility, such exemption from conducting individual toxicity tests ("waiving") is possible in cases where exposure is negligible (REACH Annexes VIII-XI). However, it is difficult to define what constitutes "no exposure" and the REACH Annexes VIII-XI use different terms: "no relevant exposure", "limited exposure", "no exposure", "no significant exposure" and "unlikely exposure", but refrain from defining the level of exposure at which exposure is thought to be so small that no risk can result irrespective of the inherent toxicity of the chemical.

Bernauer et al. (2008) from the German Federal Institute for Risk Assessment suggest establishing cut-off criteria for "relevant" (detrimental) exposure based on the TTC principle. They propose to employ an endpoint-specific TTC, starting from a comparison of the tentative external exposure to the specific TTC. This promising strategy enables the assessment of what "no relevant exposure" is and safeguards an appropriate level of protection of the general population demonstrated for reproductive toxicity endpoints. Similar arguments in favor of TTC in REACH were made by Rowbotham and Gibson (2011).

The author had the privilege to coordinate on behalf of the European Commission the development of REACH test guidance for industry. Appendix R.7-1 to Chapter 7C provides a brief summary of the approaches for deriving a TTC, their limitations and the chemicals that should be excluded. A schematic diagram illustrates how the concept of TTC may be used in REACH (Figure R.7.13-1 in Technical Guidance Chapter R.7C) (ECHA, 2016). Box 1 reproduces the current ECHA TTC guidance as to REACH.

6 http://www.cosmostox.eu/about/seurat/

7 http://ec.europa.eu/consumers/cosmetics/cosing/

8 http://www.seurat-1.eu/media/download_gallery/SEURAT-1_Annual-Report_Vol6_LR.pdf

Box 1:
Potential TTC use within REACH
(Excerpt from Chapter R.7c: Endpoint specific guidance 268 Draft Version 3.0 (ECHA, 2016))

It is feasible that within REACH the TTC concept may be of use for the chemical safety assessment at tonnage levels triggering limited information on repeated dose toxicity and/or reproduction: REACH clearly indicates the need for non-testing methods and provides the opportunity of waiving testing based on exposure considerations. When clearly documented and justified the following options could apply.

REACH Annex VII
The testing requirements specified in Annex VII would normally not trigger toxicity testing involving repeated exposures and the information at this tonnage level do provide insufficient information to determine a dose descriptor or any other starting point for the derivation of a DNEL for use in an assessment of the human health risks associated with repeated exposures. Although non-testing or *in vitro* methodologies may give insight in the toxicological properties of a substance, generally such methods are insufficiently specific to provide quantitative information on the potency and/or threshold of an adverse effect. In such a case the threshold derived from the TTC methodology might provide a reference value to assess the significance of the human exposure.

REACH Annex VIII-X
At these tonnage levels there may be circumstances triggering an adaptation of the REACH requirements that may lead to waiving of the repeated dose toxicity study and, consequently, the generation of a substance-specific dose descriptor or another starting point for the derivation of a DNEL:

- in Annex VIII, repeated dose toxicity (28 d test, 8.6) and reproductive toxicity testing (8.7) may be waived 'if relevant human exposure can be excluded in accordance with Annex XI section 3.
- in Annex IX and X testing could be waived in case there is no significant exposure, and there is low toxicity, and no systemic exposure.

In a case-by-case consideration, the appropriate threshold derived from the TTC methodologies agreed upon by the relevant regulatory body might be considered as a starting point to assess the significance of the human exposure. The level chosen will be critical to ensure a level of sufficient protection.

Final remark
Independent of the approach used in risk assessment of industrial chemicals it is important to maintain a sufficient level of protection. In the striving for alternatives to animal testing one suggested approach is the use of generic threshold values. However, application of TTC would imply that limited data may be generated and thus, that the level of protection might be influenced. From information on flavouring substances in the diet the TTC concept seems to be reasonable well based with respect to general toxicity and the particular endpoints examined. However, the possible application of TTC on industrial chemicals needs to be carefully considered. There may be some important differences between industrial chemicals and substances used for food contact articles or flavourings, such as differences in use pattern and composition (for a further discussion see Tema Nord, 2005; COC, 2004).

However, it appears that the TTC concept is not as widely applied in REACH registrations as it could be. The subtitle "*Potential Use…*" of the guidance already indicates some hesitance and, overall, the recommendation is "*the possible application of TTC on industrial chemicals needs to be carefully considered*". The guidance itself stresses that additional scientific and regulatory discussions on TTC values and their derivation are needed before integration into the guidance can take place. Unfortunately, this has not really taken place and with the last deadline in 2018 approaching, this has to be considered a missed opportunity.

We will have to see to what extent TTC will find their way into the implementation of the US Toxic Substance Control Act reauthorization of 2016 – the respective ruling is ongoing – and other industrial chemical legislations mushrooming worldwide.

3.8 TTC for pesticides

The very extensive requirements for safety data for pesticides (plant protection products) and other biocides so far leaves little room for TTC use. Terry et al. (2015) used relative potency factors and TTC to assess hazard and human risk assessment profiles of environmental metabolites of a pesticide, reducing the need for *in vivo* studies. Melching-Kollmuss et al. (2010) use the TTC reasoning to argue that suggested EU limits for tolerable concentrations of ground water metabolites ("non-relevant metabolites" without targeted toxicities and specific classification and labeling) derived from active ingredients of plant protection products (PPPs) are too low: Risk assessments were at the time discussed for "non-relevant metabolites" above 0.75 µg/l. They show that a TTC approach leads to a value of 4.5 µg/l. The argument was later taken up by a broadened industry group (Laabs et al., 2015). This shows how a broadly accepted TTC concept could bring consistency into regulations and streamline threshold setting.

4 The relation of TTC to other computational tools

The different computational toxicology tools (Hartung and Hoffmann, 2009) are benefitting from the increasingly available large-scale toxicological databases. However, they make use of these in very different ways. TTC is an approach based on exist-

ing data, notably NOAEL of some kind. While computers are typically used to mine the databases, TTC are not computational tools predicting properties of individual (untested) substances. This makes them very different from (Q)SAR and read-across. As these techniques are typically restrained to identify hazard, they cannot provide the NOAEL needed to derive TTC. Their use scenarios are therefore very different: For example, the European REACH legislation (Hartung, 2010) is very open to (Q)SAR but does not foresee TTC to the same extent, although REACH strongly improves the exposure assessment aspect; on the contrary, EFSA uses TTC to some extent, but much less (Q)SAR. Lo Pilparo et al. (2011) on behalf of EFSA surveyed the use of computational tools for food safety with a strong focus on (Q)SAR: "*…the majority of key players in the food safety field either do not use (Q)SAR methodology at all or use it in a very limited way mainly because of a lack of expertise. … to support priority setting exercises or to fill information gaps on possible health concerns during the management of a food crisis in food industry (e.g. if a contaminant is found in food). At present, (Q)SAR is not used routinely to fill data gaps in the pre-marketing assessment of food additives, food contact substances, or pesticide and pesticide metabolite residues. However QSARs are currently being explored, developed and utilised by regulatory authorities for risk assessment purposes such as EFSA. Some organisations, however, are very experienced in the use of QSAR, notably government authorities such as the FDA Center for Food Safety and Applied Nutrition (CFSAN), FDA Center for Drug Evaluation and Research (CDER) and the EPA (OCSPP), as well as some companies (e.g. Nestlé). … Despite this variable situation, (Q)SAR analysis is widely perceived as a potential useful tool to support regulatory assessments in the field of food safety, and this justifies further exploration and development*."

Similarly, the use of (Q)SAR in REACH has not met expectations (Hartung, 2016) as the regular reports on the use of alternatives under REACH show[9]. This does not say that these methods could not be used more extensively, but obviously the process is not encouraging this enough at the current state of science. The generation of TTC values based on (Q)SAR is at this stage, where NOAEL prediction is hardly possible, not foreseeable.

Read-across is traditionally not a computational approach, but a rather manual data-gap filling based on circumstantially available data on similar compounds (Patlewicz et al., 2014). Its broad use under REACH and possibly new regulations elsewhere prompted the development of Good Read-Across Practice only most recently (Ball et al., 2016; Zhu et al., 2016). The TTC concept could, however, be seen as an extension of approaches such as read-across and chemical categories as it uses other chemicals' data to intrapolate for an untested chemical.

With the availability of toxicological "big data", a fusion of read-across and (Q)SAR as more automated read-across by machine-learning is now possible (Hartung, 2016). The first commercial solution was recently released by Underwriters Laboratories (UL)[10]. Similar to (Q)SAR, read-across can synergize with TTC by establishing a probability of hazard. Notably, the automated approaches also can assign a measure of confidence to this hazard assessment. As automated read-across has not yet been shown to estimate potency or NOAEL, it is not directly suited to generate TTC estimates though.

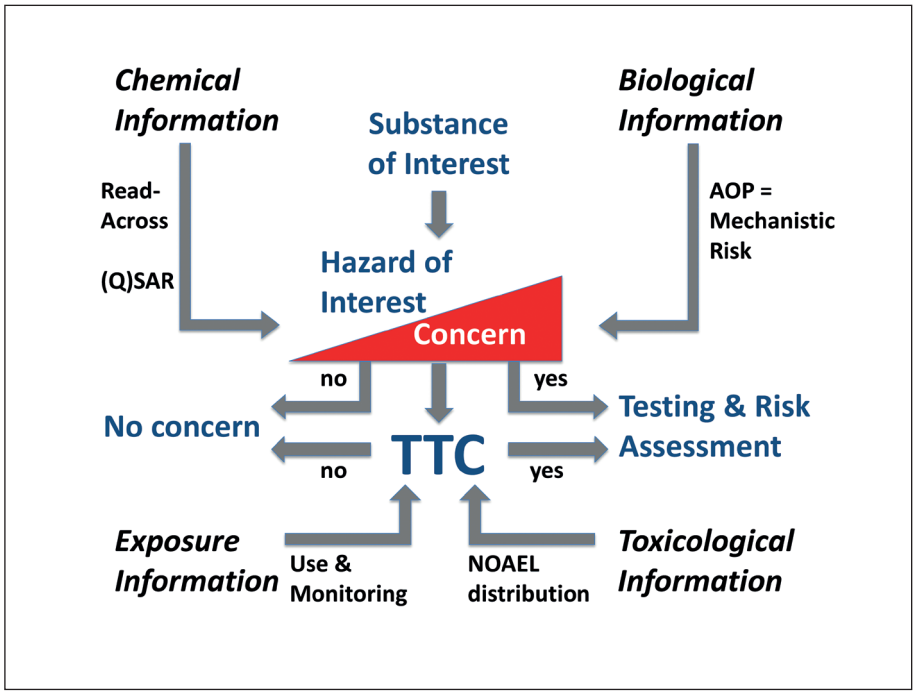

Fig. 1: The synergy of information sources to reduce safety testing requirements
The figure visualizes the synergy of deriving on the one hand (upper part) hazard prediction from chemical (structural and physicochemical) information as well as biological (AOP = Adverse Outcome Pathways and other mechanistic) information and, on the other hand, TTC (lower part), which are deduced from toxicological legacy information, and compared with the exposure information. TTC lend themselves as complementary to hazard identification, especially when hazard cannot be excluded, but concern also does not inevitably lead to testing and risk assessment. The TTC will then help to drive the decision to either side, i.e., no concern or need for testing and risk assessment.

[9] https://echa.europa.eu/documents/10162/13639/alternatives_test_animals_2017_summary_en.pdf/487e2516-0ad0-90a2-a923-96417ffd6b6b

[10] https://www.ulreachacross.com

As discussed further below, however, there is discussion that TTC should only be applied for endpoints where there is a sufficient probability of hazard. Here, both (Q)SAR and read-across could be very valuable (Fig. 1). However, we can also reason that the two approaches of showing that hazard is unlikely and that exposure would not be relevant anyway, strongly synergize to reduce concerns.

5 Challenges for TTC application

Table 4 lists a number of challenges, which will be detailed in the following. The necessary discussion could lead to a Good TTC Practice as a consensus of the practitioners from the regulating and regulated side on when and how to carry out TTC and how to report it.

Tab. 4: Eleven challenges to the broad application of TTC

Challenges to the TTC approach	Comment
(1) Can a TTC with sufficient margin of safety but still waiving a substantial number of tests in general be established?	There seems to be increasing consensus that this is possible. However, the obvious trade-off is to set the TTC without compromising safety but allowing enough substances to be deprioritized. Combination with probability of hazard, which is effectively the purpose of using the Cramer classes, might accommodate this.
(2) When to use TTC?	TTC should be used where it satisfies an information need, i.e., its sound basis to do so has been shown. The applicability domain has to be defined, especially for which substances it does not work. As this is mainly due to the lack of these substance classes in the TTC database, this requires continuous update.
(3) Access to data to derive TTC and data quality	TTC are as good as the data they are deduced from. Big (high-quality) data makes the difference – this requires a central, curated, maintained searchable database. Data aggregation over many substances helps to some extent with the reproducibility problem of animal test data. It is critical that enough similar substances are part of the database (applicability domain).
(4) Consensus on setting the TTC-NOAEL – is the 5th percentile good enough?	The question is, what happens below the 5th percentile? Are these errors or unusually toxic substances with understandable mechanisms? This requires database curation and substance-by-substance review.
(5) Consensus on uncertainty factors for TTC – is the broadly used 100 sufficient?	This is probably a borderline choice: Factor 100 is already the minimum when starting with a measured NOAEL. Here we start with a value that is too high in 5% of the cases.
(6) Dependence on exposure does not allow closing the chapter of risk assessment	Exposure is not the strong part of chemical risk assessment and also highly variable between individuals and over time. Regular reviews of changes in exposure patterns will require revisiting a TTC-based decision regularly. Not very attractive…
(7) How endpoint-specific are TTC?	One appeal of TTC is its use for endpoints other than the database they were derived from. This astonishingly seems to work out quite well (probably because the approach is so conservative), though some endpoints have been excluded in the past. Making TTC endpoint-specific is a chance to achieve higher thresholds for certain endpoints.
(8) Can additional biological or computational information for a given substance improve TTC?	This has been done so far for genotoxicity information (especially the Ames tests), but other information on the likelihood of any hazard (biological, (Q)SAR, read-across) or metabolism could improve predictions.
(9) Should we develop an internal TTC, i.e., one based on blood concentrations of the substances associated with no adverse effects?	This would obviously take kinetics out of the picture, which is little reproducible between species anyway. It allows comparing to biomonitoring studies and *in vitro* effects of substances more easily. However, when to measure? And is it peak concentrations or area under the curve that is relevant for a given substance class?
(10) Adaptation to less-than-lifetime exposure	Due to the fact that TTC were first derived from cancer bioassays, they represent lifetime exposures. Adaptation for other exposure durations is controversial, but makes a lot of sense, e.g., for drug contaminants.
(11) Can TTC be applied to mixtures?	They actually are already applied mostly to mixtures (additives and contaminants in food and drugs), but the question how cocktail effects impact on TTC is well warranted.

Challenge 1: Can a TTC with a sufficient margin of safety – but still waiving a substantial number of tests – in general be established?

This question needs to handle the distinction between a threshold of toxicity and a threshold of concern: It is on the one hand asking which toxic hazards show a threshold, but even in the absence of this, on the other hand, there might be simply no concern below some point because the probability of hazard is so low. For either, the question is whether such a threshold is sufficiently high that a large number of chemicals, due to their use and exposure scenarios, fall below this threshold.

The National Research Council report on Science and Decisions (NRC, 2009) proposes harmonizing dose-response approaches for cancer and non-cancer endpoints, and for non-cancer quantitative risk assessment this would usually take the form of a low-dose linear no-threshold dose-response curve. The soundness of this recommendation has been questioned (Rhomberg et al., 2011; Bogen, 2016). "*If most endpoints for most agents are assumed to have non-zero low-dose risks, then the critical-effect concept, choosing the one endpoint on which to calculate acceptable doses, loses its basis. All regulatory decisions, since they entail substituting some exposures (and their attendant risks) for others, become risk-risk trade-off decisions, and equity questions are raised since risk transfer is inevitably involved. A valid basis for estimating low-dose linear components is not evident, and upper-bound approaches fail to be reliably public health-protective owing to the risk trade-off decisions that need to be faced*" (Rhomberg et al., 2011). What Lorenz Rhomberg and colleagues are voicing here is the consequence of giving up the safe dose concept, which would annihilate a lot of the risk assessment currently done and also severely impair the TTC concept.

Even more important, however, is how we arrive at sufficiently high TTC that they are of practical use. For cancer, for example, a threshold of concern has been suggested, where exposure leads to one additional death in a year in one million exposed persons. This approach can be judged very differently at an individual vs. a population level. It might be fine to take a risk of one in a million to contract cancer from a product I am using, but imagine a product that the 300 million Americans or 500 million Europeans are using – 300 to 500 people killed per year means quite a scandal. Similarly, we might ask, is it acceptable to use the 5th percentile? Doesn't this mean that we are underestimating toxicity in one of 20 cases?

Yes, but such calculation still makes sense, first of all because we can practically carry it out: If asked for the 1st percentile or even the 0.1st one, we would need an enormous number of substances to robustly calculate this, much more than we currently have available. The same holds true for a population risk with lower probability. The higher safety for consumers and patients comes from other additional elements:

- Our methods are precautionary and rendered very sensitive, e.g., by dosing schemes, exposition routes, choice of species.
- We include uncertainty factors on top of our estimates.
- Most NOAEL probably represent false-positive findings anyway because of the multiple-testing fallacy.

The last point needs explanation: In a 28-day repeat dose study we measure about 40 endpoints, in a cancer bioassay 60 and in a two-generation reproductive toxicity study 80. This is called "multiple testing" by statisticians. If we apply for each endpoint the normal 5% significance level, there will always be some false positives (theoretically one in twenty endpoints), even for the most innocent (inert) chemical. We can correct for this mathematically, but then we need many more animals per group, which we cannot easily do for cost and animal welfare reasons. This is part of the reason for the lousy reproducibility of these tests – false-positives cannot be repeated. We have discussed this previously in several publications, most extensively for the cancer bioassay with its mere 57% reproducibility (for $1 million per study!) in Basketter et al. (2012). For the TTC discussion, repeat-dose studies are certainly most relevant – the cancer database was probably used first of all because it was available and the approach followed the line "if you can do it here, you can do it anywhere".

A very interesting study by Wang and Gray (2015) sheds light on the reproducibility of repeat-dose studies: They analyzed 37 chemicals studied in cancer bioassays in mice and rats in the US National Toxicology Program; they, however, analyzed the non-cancer endpoints as these animals also undergo extensive further evaluations. They asked, how reproducible are the findings between animals, genders, mice and rats as well as with earlier reported long-term repeat-dose studies of the same substances? The answer: not at all! In their words: "*Overall, there is considerable uncertainty in predicting the site of toxic lesions in different species exposed to the same chemical and from short-term to long-term tests of the same chemical*." This confirms that our databases are full of false-positives (Hoffmann and Hartung, 2005). In many cases, this is even quite convenient – it gives a point of departure for a risk assessment and, whether true or not, it is at least precautionary. And as most exposures used in the animal tests are far higher than exposures in humans, we "live happily ever after" with the fairy tale of a toxic effect. The consequences for chemophobia and discomfort of placing a product with a putative looming toxicity just at higher doses (or possibly longer duration of exposure) on the market left aside, this actually means a large of safety margin for the TTC.

Challenge 2: When to use TTC?

The extensive EFSA (2012) Scientific Committee analysis and opinion states "*In principle, the science supports the application of the TTC approach in any area of chemical risk assessment for which human exposures are low, whether exposure is from deliberate addition or due to contamination. However, for substances for which EU legislation requires the submission of toxicity data, the TTC approach would not be used*." So, after recommending TTC for any chemical risk assessment, they pour a lot of water into the wine by advising against it when data submission is required. Either the approach works or not, and if it works, it should help to reduce data generation also, and especially, for regulatory purposes.

There are substance-classes that are currently considered as excluded from applying the TTC concept. For some of them,

e.g., heavy metals, the reason for their exclusion is that they are not covered in the databases underlying TTC values. It appears obvious that a TTC cannot be deduced for substance classes that were not part of the training set, at least not without explicit discussion. It is necessary to define the chemical domains covered (applicability domain) or, more importantly, those that are not covered. The EFSA (2012) opinion on the TTC approach produced the most comprehensive list of substances for which TTC currently should not be used:

1. High potency carcinogens (i.e., aflatoxin-like, azoxy- or N-nitroso-compounds, benzidines, hydrazines).
2. Inorganic substances
3. Metals and organometallics
4. Proteins
5. Steroids
6. Substances known or predicted to bioaccumulate
7. Nanomaterials
8. Radioactive substances
9. Mixtures of substances containing unknown chemical structures

Leeman et al. (2016), however, evaluated the exclusion of bioaccumulating substances and found no such need. The reasons for most other exclusions are that these substances were not represented in the TTC database. This means that future expansions of the database may allow extending the approach but also that we need a centralized institution to track such progress, likely to be combined with TTC validation efforts.

Challenge 3: Access to data to derive TTC and data quality

The TTC databases used so far are based mainly on repeat-dose studies or cancer bioassays. This bias could mean that other toxicological endpoints are not represented adequately, e.g., reproductive/developmental toxicity. Dewhurst and Renwick (2013), however, report that this has not been the experience, but further analysis might be warranted. For example, earlier analyses (Barlow et al., 2001; Kroes et al., 2005) concluded that it was premature to consider TTC for endocrine disrupting chemicals. Overall, an EFSA project (Bassan et al., 2011) concluded that the Munro et al. (1996) database is broadly representative of the "world of chemicals".

It is highly desirable to have a centralized TTC database that would be continually maintained to allow inclusion of new data as they become available. Standard and quality checks would need to be performed. For example, the carcinogenicity database (CPDB) of Gold et al. (1984, 1989), extended by Cheeseman et al. (1999), contains many substances later not confirmed as carcinogenic. This overall TTC database should be in a searchable format that enables independent investigation. An agreement on the method of dose-response analysis and the appropriate dosemetric should be harmonized.

There are some shortcomings in the TTC databases, e.g., as outlined earlier, the classification class II according to Cramer et al. (1978) and used by Munro et al. (1999) to define related TTC values has been found to be not well defined; there are considerable overlaps with classes I and III. A re-evaluation of the underlying repeat-dose toxicity data of the Cramer classification, thereby also including other data compilations like RepDose, has recently been undertaken and an improvement proposed (Tluczkiewicz et al., 2011). Munro et al. (1996) and RepDose (Escher et al., 2010; Batke et al., 2011) databases contain few chemicals in Cramer Class II, suggesting that this class may be of limited practical utility.

A question already raised in the ILSI workshop (Dewhurst and Renwick, 2013) is whether the current delineation between the cancer and non-cancer tiers in the Kroes et al. (2005) scheme, which is based on the presence of structural alerts for genotoxicity, is adequate. The draft opinion of SCHER, SCCP, SCENIHR (2008) proposed establishing on the contrary a new, separate database for substances classified as human carcinogens, or probable or likely human carcinogens. It is not clear whether such separation is actually desirable, but coverage of these classes and clear distinction from the non-carcinogens is needed. The TTC cancer database does not include considerations of mode of action or the human relevance of the mode of action or tumor site. The current TTC scheme might not always cover the production of reactive metabolites. Another aspect not covered is effect strength, which not only manifests as minimal active concentration but also as extent of damage.

But, all together, the enormously increasing availability of toxicological data (Hartung, 2016; Zhu et al., 2016) fuels the opportunities for TTC. There is still a lot to be done as to general data-sharing, data curation, machine-readability, harmonized ontologies (Hardy et al., 2012a,b), etc. The increasing discussion of reproducibility and relevance of animal test data also needs to be addressed (Hartung, 2013).

One aspect as to the harmonization of the database raised in the workshop reported by Dewhurst and Renwick (2013) is whether TTC should be expressed on a molar basis, which was supported in scientific terms because (Q)SAR usually apply this. The workshop recognized that such a change would result in a "*need to convert all the NOAELs in the databases to a molar basis and derive the TTC values on the distribution of molar-based NOAELs. Although this was considered not to be a difficult task, application of molar TTC values would require that analytical and exposure data were also expressed on a molar basis. Adoption of a molar basis would result in the TTC approach being different to existing regulatory approaches used in setting reference doses for chemicals with extensive databases*."

Another aspect raised by EFSA (2012) is that for application of the TTC approach to the whole population including infants and children, all TTC values should be converted to corresponding values that take body weight into account (as done in Tab. 3).

Challenge 4: Consensus on setting the TTC-NOAEL

Why the 5th percentile? If we had 12 fingers, it would probably be the 6th... Why not use the lowest value? Simply because the extreme outliers otherwise have too much impact. These are the "Botulinum toxins among chemicals", but they require optimization of biological activity either by evolution or drug development processes; their occurrence by chance is rather low.

However, simple mistakes in dosing or reporting of doses are not very rare. Besides avoiding the impact of outliers, the 5th percentile can be calculated quite robustly. We have to keep in mind that there is a minimum number of NOAEL (n) to calculate percentiles of the distribution – for a 5th percentile at least 20, so that the 5th percentile is between number 19 and number 20. To get a robust 5th percentile, we thus need considerably more than 20 NOAEL. Schoonjans et al. (2011) show how small sample sizes can lead to errors in calculating percentiles. The large standard deviations of the observed differences in the 5th percentile illustrate the large statistical uncertainty associated with the estimated percentiles in small sample sizes. They also compare different methods to calculate percentiles. Therefore, they recommend log-transforming the data and stress the importance of reporting percentiles with their 95% confidence interval. It might be necessary to add an uncertainty factor if 5th percentiles are derived from smaller numbers of NOAEL.

Challenge 5: Consensus on uncertainty factors for TTC

The derivation of a TTC depends critically on the use of uncertainty factors, not very different from traditional risk assessment (Dourson et al., 1996; Dorne and Renwick, 2005; Dankovic et al., 2015). Their use in establishing exposure limits goes back at to the 1950's, when Lehman and Fitzhugh (1954) proposed a factor of 100 (referred to as a "margin of safety") for extrapolating from animal data to safe levels for humans for food additives and pesticide residues. Six key uncertainties to be considered include:

1. Inter-species variability when extrapolating from animal studies to humans
2. Response variability in humans (e.g., susceptible subpopulations)
3. Uncertainty in estimating a no-effect level from a dose where effects were observed
4. Extrapolation from shorter duration studies to a full lifetime exposure
5. Insufficiencies in the health effects database, i.e., the most sensitive adverse effect may not have been evaluated
6. A modifying factor is used by some organizations to account for other remaining uncertainties (typically related to exposure scenarios or accounting for the interplay among the five areas noted above).

Typically, for TTC only the first two uncertainties are taken into account, thus the traditional factor 100, i.e., 10 each for inter-species and inter-individual variability. Uncertainties (3-5) are mitigated by using NOAEL, assuming human lifetime exposure without correction for shorter exposures and basing the TTC deduction on many substances and endpoints. Uncertainties from exposure scenarios (6) might, however, need further consideration (see below), a discussion toxicologists too often neglect.

Factor (2) includes susceptible populations; EFSA (2012) with its Scientific Committee considered for example whether the TTC approach could be applied to young infants under the age of 6 months, in whom not all metabolic and elimination processes are yet mature. As the toxicokinetic differences between young infants and children or adults are transient and generally not more than 2- to 5-fold, the committee concluded that TTC can be applied, but where the estimated exposure is in the range of the TTC value, additional consideration needs to be given.

Is the current uncertainty factor good enough? Probably. As discussed already under challenge 1, the assumption that our chemical of interest is among the 5% worst guys is already quite precautionary, but several additional indirect safety factors are included in the approach.

Challenge 6: Dependence on exposure does not allow closing the chapter on risk assessment

Adoption of the TTC approach, while reducing animal use for testing purposes and reducing costs to industry, would, however, place much more reliance on the development of reliable exposure assessment. As TTC cannot exclude the hazard, the risk assessment is vulnerable to exposure changes. But who tracks these and comes back to the risk assessment if the combined exposure to all sources exceeds the TTC? This would in doubt need to be a manufacturer liability, but what to do if there are several manufacturers?

Manufacturing is also not exposure, so who controls the downstream use? REACH established the substance information exchange forum (SIEF) of all co-registrants of a given substance (one substance, one registration principle). Does use of TTC imply that these consortia have to maintain their information exchange to monitor whether TTC are exceeded? SIEF forever! This is burdensome and through the costs, which will at some point compare to costs of testing, will become less and less attractive.

The situation is not very different in other areas such as food additives.

Challenge 7: How endpoint-specific are TTC?

By using an endpoint considered very sensitive (cancer), the first TTC were produced very conservatively. Very sensitive mechanisms (e.g., choline esterase inhibition) and endpoints (e.g., allergenicity) where furthermore excluded. Hennes (2012) remarked "*The reference to toxicological endpoints from which TTC values were derived is however not of particular significance in the application of the TTC approach, unless it could be identified whether a compound was likely to produce the effect of concern*." However, this is not playing to the full potential of TTC, which could nowadays with larger databases be derived endpoint-specifically and allow for many endpoints to set higher threshold doses, i.e., broader applicability to more chemicals.

Pragmatically, a very low general TTC might be applied first and then a lower endpoint-specific one if exposure exceeds the former.

Challenge 8: Can additional biological or computational information for a given substance improve TTC?

Historically, TTC for genotoxic carcinogens have been treated separately. For example, the inclusion of Ames data on chemicals with structural alerts for genotoxicity provided such dis-

tinction. Felter et al. (2009) remarked: "*Because Ames data are often the only data available in the publicly available literature or easily generated for newly identified chemical contaminants, it is important that the tiered approach offers a way to integrate these data into the appropriate TTC-based exposure tier*."

Another type of biological information of high relevance is metabolism. The computational tools are still rather limited, but a genotoxic metabolite of a non-genotoxic mother compound, for example, would strongly affect TTC values in the current use. A number of reviews on metabolic prediction tools are available (Boobis et al, 2002; Kulkarni et al, 2005; Norinder and Bergström, 2006; Mostrag-Szlichtyng and Worth, 2010; Tsaioun et al., 2016). The EFSA (2012) Scientific Committee judged: "*it is not straightforward to apply such tools in a regulatory context, and further work in this area is needed for practical application to the TTC approach. In particular, there is a need to develop tools capable of quantitatively predicting metabolite and degradate formation*."

An especially interesting combination is the one of TTC with read-across or (Q)SAR, especially as both are fast and not costly. Establishing a probability of hazard using these *in silico* tools as discussed above can synergize with TTC (Fig. 1): If hazard cannot be excluded with high confidence, TTC should allow to demonstrate whether exposure is relevant for such hazard to manifest.

Challenge 9: Should we develop an internal TTC, i.e., one based on blood concentrations of the substances associated with no adverse effects?

Partosch et al. (2015) addressed the risk assessment of substances with a low absorption (by the oral route, or through skin). Here, the internal exposure, i.e., the bioavailable fraction of the dose, is more relevant than the external exposure. The European REACH legislation allows that tests might not be necessary for substances with negligible absorption, i.e., with low internal exposure. In order to derive internal TTC values, the external NOAEL was multiplied by the bioavailability of the individual chemical predicted using an *in silico* prediction tool (ACD Percepta). This intriguing concept might allow narrowing down interspecies differences (thus uncertainty), which are very pronounced for bioavailability (Grass and Sinko, 2002).

Challenge 10: Adaptation to less-than-lifetime exposure

Whereas the existing TTC exposure limits assume a lifetime of exposure, human exposure to unintended chemicals in food is often only for a limited time. Recommendations are made to refine the approach for less-than-lifetime exposures (Felter et al., 2009). A staged TTC (Müller et al., 2006) has been proposed for drug impurities, adapting acceptable daily intake values of 1.5 µg/day for lifetime intake to 120 µg/day for 1 month as virtually safe doses. This means that the TTC is adjusted by taking into consideration both the dose and the duration of the clinical trials, resulting in a lower TTC for higher doses and a higher TTC for shorter duration. The rationale behind this is that clinical studies are conducted with limited duration of dosing and the total exposure is relatively low.

The conservative risk estimate for the original TTC means that this approach results in large safety margins for all except the most potent carcinogens. The staged TTC values should apply to all stages of development and to each individual compound in cases where several genotoxic impurities are present with the exception of highly potent carcinogens. Both concepts were introduced with the intention of providing the industry with some flexibility and a risk mitigation strategy during the development phase of a new drug. The staged TTC approach is also an essential element of the EMA and US FDA guidelines on genotoxic impurities (EMA, 2006; US FDA, 2008).

These intake levels are estimated to give an excess cancer risk of 1-10 in a million people over a lifetime, and are considered extremely conservative ("as low as reasonably practicable" (ALARP) principle) given the current lifetime cancer risk in the population of over 25%[11]. In its review of TTC, the EFSA Scientific Committee was not confident about the general applicability of adjusting the TTC value for substances with a structural alert for genotoxicity or for non-cancer endpoints for shorter than chronic durations of exposure; it therefore recommended that the issue be addressed case-by-case (EFSA, 2012).

Challenge 11: Can TTC be applied to mixtures?

Recently, a discussion has started on whether TTC values can also be useful for the assessment of combined exposures to chemicals, i.e., a first estimate of a combined exposure could be compared to the TTC (SCHER, SCCP and SCENIHR, 2008). This was based on analysis of the applicability of the TTC approach for a screening-level assessment of chemical mixtures by Boobis et al. (2011) and Price et al. (2009). As this is still a relatively new concept, according to SCHER further evaluation, possibly including example cases, would be of value. This judgement is quite surprising as the established uses for TTC are for mixtures, i.e., additives and contaminants in food and drugs. Also, the use for pesticide metabolites falls into this category. However, certainly with increasing understanding of cocktail effects of chemicals, we will have to adapt our TTC assessments to these possible confounding factors.

6 Conclusions

First of all, it should be clearly stated that the first priority is always to avoid the occurrence of unintended chemicals in products and the environment and, if detected, to take steps to remove such chemicals as appropriate. TTC must not be an excuse to allow pollution.

Second, TTC are meant to be a "screening" tool for risk managers to make rapid, scientifically defensible, consistent, and

[11] http://seer.cancer.gov/statfacts/html/all.html

transparent decisions as to the urgency of responses needed. It is a probability-based screening tool and thus it does not offer complete certainty. The goal is to establish approaches that are protective of public health without unnecessarily alarming consumers or disrupting trade, and that reflect a prudent and responsible use of limited resources (Felter et al., 2009). The TTC limit must therefore be conservative, which also means that exposures exceeding this level are not necessarily a health concern but indicate the need for further evaluation.

The discussion of TTC has now been going on for more than two decades and regulatory implementation is still rather slow despite the fact that no wrong decision based on TTC has been reported, to the best of the author's knowledge. The remaining challenges and hurdles should be tackled, but it is irresponsible to carry out testing on a large scale if such rather simple limits could help prioritize resources for other cases.

An obvious area of application is endocrine disruption, where the central obstacle to broad application of TTC is the discussion around non-monotonous dose-response curves (see summary by Swedish Chemicals Agency KEMI[12]; Vandenberg et al., 2012; Rhomberg and Goodman, 2012; Lagarde et al., 2015), i.e., the idea that lower concentrations of a substance can have effects not seen at higher concentrations. This is highly controversial, but at least for estrogenic and androgenic effects seems rather unlikely, as we have in comparison enormous endogenous hormone activities, making it unlikely that such low-dose-effects could manifest, i.e., adding a drop of water to the bucket.

Upon request of our board, the Center for Alternatives to Animal Testing (CAAT) is currently establishing the TTC Collaboration, but we have also happily joined current new initiatives by EFSA. A major step forward would be an explicit formulation of a Good TTC Practice, which states what should be done, how it should be done, and how it should be documented and reported. Such a consensus document would further push the limits of TTC use. In this sense, I would like to close with a quote by the American political scientist and economist Herbert Simon "*One finds limits by pushing them*". Let's push!

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Conflict of interest

The author consults Underwriters Laboratories (UL) on computational toxicology, especially read-across, and has a share of their respective sales.

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