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Soil Dioxin Relative Bioavailability Assay Evaluation Framework

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1. Introduction

The Risk Assessment Guidance for Superfund (RAGS) Part A (USEPA, 1989) discusses making adjustments to Superfund site-specific risk assessments when the medium of exposure in an exposure assessment differs from the medium of exposure assumed by the toxicity value (cancer slope factor, reference dose value, etc.) based upon site-specific bioavailability data. An important consideration in assessing risks from exposures to dioxin in soil is whether an adjustment is needed in the application of the oral cancer slope factor (CSF) and/or oral chronic reference dose (RfD) for 2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDD). This adjustment would account for differences in the bioavailability of TCDD (and toxicologically related polychlorinated dibenzo-p-dioxins [PCDD] and polychlorinated dibenzofuran congeners [PCDF]) in soil and in the test medium used in the critical study(s) on which the CSF and/or RfD were based (e.g., dietary exposure vs. exposure to soil). An adjustment would be considered appropriate if evidence were sufficient to indicate that the relative bioavailability (RBA) of the PCDD/F mixture in soil was less than 100%.

EPA recently compiled and summarized studies conducted to estimate relative bioavailability (RBA) of TCDD and PCDD/F in soils (USEPA, 2010). Nine studies were identified that collected data on soil RBA based on bioassays conducted in guinea pigs (McConnell et al., 1984; Umbreit et al., 1986; Wendling et al., 1989), rabbits (Bonaccorsi et al., 1984); rats (Budinsky et al., 2008; Finley et al., 2009; Lucier et al., 1986; Shu et al., 1988) or swine (Budinsky et al., 2008; Wittsiepe et al., 2007). These studies used various experimental designs for dosing animals, metrics for estimating bioavailability, and data reduction methods for calculating soil absolute bioavailability (ABA) or RBA (Table 1). The extent to which variations in experimental design affects RBA estimates has not been rigorously evaluated. Only one study has compared RBA estimates for the same test materials in more than one assay; the outcome was dissimilar estimates of RBA for 2 soils based on a single dose rat bioassay and a multiple dose swine assay (Budinsky et al., 2008).

The current status of methods for estimating RBA of PCDD/F in soil can be considered as being in the early development phase. Although various methods have been explored, no single methodology has been determined to be optimal; furthermore, advancements and refinements of methodologies is expected to continue to progress towards the establishment of standard procedures. The evolution of varying methodologies into generally accepted and validated methods for use in risk assessment occurred in the history of the development of the juvenile swine assay for soil lead RBA (USEPA, 2007a,b).

Until standard procedures for estimating RBA of PCDD/F in soil are established, there is a need for a consistent approach to evaluate the strengths and weaknesses of assays designs that are proposed or implemented to support in risk assessments. This report offers a framework for making such evaluations. Specific design parameters that should be subject to evaluation are identified and relevant scientific literature is cited where more in depth discussion can be found. Whenever possible, minimal requirements for study designs are proposed. This report also identifies issues that have yet to be resolved regarding how RBA assays should be designed and which could be objectives of further research to develop RBA assays for soil PCDD/F and applications to risk assessment.

2. RBA Assay Requirements

This report is organized into subsections that discuss important experimental design features that should be considered in evaluating the potential utility of a given RBA assay design to support risk assessment. Minimal requirements are identified at the start of each subsection and are followed with discussions of the rationale for the requirements.

2.1. Application of RBA to Risk Assessment

Requirement 1: PCDD/F risk assessment requires estimates of the RBA for soil TEQ (RBA$_{TEQ}$)

Exposures from soil are almost always to mixtures of PCDD/F congeners that have varying toxic potency and, very likely, different RBA (USEPA, 2010). Variations in toxic potency of the congeners are accounted for in risk assessment by assigning Toxicity Equivalence Factors (TEF) to concentrations of PCDD/F in soil, with TEF reflecting the relative toxic potency of each congener, relative to 2,3,7,8-TCDD (TCDD, Equation 1).

$$C_{TEQ} = \sum C_i \cdot TEF_i \qquad \text{Eq. (1)}$$

where C_{TEQ} is the concentration of 2,3,7,8-TCDD Toxic Equivalents, C_i is the concentration of congener i, and TEF_i is the TEF of congener i. The C_{TEQ} value is used in the appropriate equation for average daily intake (ADI_{TEQ}), which is then used in the appropriate risk equation (e.g., Equations 2 - 4):

$$ADI_{TEQ} = C_{TEQ} \cdot IR_S \qquad \text{Eq. (2)}$$

$$HQ = \frac{ADI_{TEQ}}{RfD_{TCDD}} \qquad\qquad \text{Eq. (3)}$$

$$CR = CSF_{TCDD} \cdot ADI_{TEQ} \qquad\qquad \text{Eq. (4)}$$

where IR_S is the soil ingestion rate, HQ is the hazard quotient, RfD is the reference dose, CR is the cancer risk, and CSF is the cancer slope factor. The corresponding adjustments for RBA would be (Equations 5 and 6):

$$HQ = \frac{ADI_{TEQ} \cdot RBA_{TEQ}}{RfD_{TCDD}} \qquad\qquad \text{Eq. (5)}$$

$$CR = CSF_{TCDD} \cdot ADI_{TEQ} \cdot RBA_{TEQ} \qquad\qquad \text{Eq. (6)}$$

where RBA_{TEQ} is the RBA for total TEQ in the soil.

2.2. Calculating RBA_{TEQ} of PCDD/F in Soil

Requirement 2: Calculation of RBA$_{TEQ}$ requires quantification of the total TEQ external dose and total TEQ internal dose, as well as the excretion fraction for TEQ (or experimental designs that ensure that the excretion fractions for TEQ are the same when administered in the soil or reference material).

The general form of the calculations used to estimate RBA for PCDD/F is given in Equations 7 and 8:

$$RBA = \frac{ABA_{TM}}{ABA_{RM}} \qquad\qquad \text{Eq. (7)}$$

$$ABA = AF = \frac{ID}{ED} \cdot \frac{1}{(1-EF)} \qquad\qquad \text{Eq. (8)}$$

where ABA_{TM} and ABA_{RM} are *absolute bioavailability* for PCDD/F in the *test material* (e.g., soil) and *reference material* (e.g., PCDD/F in a suitable vehicle), respectively; AF is the absorbed

fraction of the dose; *ID* and *ED* are the *internal dose* (e.g., body burden) and *external dose*, respectively, of the test or reference material; and *EF* is the fraction of the absorbed dose eliminated by metabolism and excretion. Although the elimination fraction (*EF*) appears in the expression for absolute bioavailability (*ABA* in Equation 8), it does not need to be considered in the calculation of RBA (Equation 7), as long as the elimination fractions are similar for the PCDD/F absorbed from the test material and reference materials (i.e., $EF_{TM} = EF_{RM}$). However, if EF_{RM} were to exceed EF_{TM}, Equation 7 will overestimate RBA. If EF_{RM} were less than EF_{TM}, Equation 7 will underestimate RBA. The validity of the assumption of equal elimination fraction of the test and reference materials is an important issue in the estimation of RBA for PCDD/F congeners, because the metabolic elimination of PCDD/Fs is dose-dependent. Dose-dependency derives from the induction of cytochrome P450 (CYP450), which is the primary mechanism for metabolic elimination of PCDD/F. This issue is addressed further in the data analysis sections of this report.

The units of *ID* and *ED* in Equation 8 can be either congener mass (i.e., g or moles congener) or TEQ (i.e., g or moles TCDD equivalents). When expressed in units of TEQ, the RBA outcome is RBA_{TEQ}, which is the parameter needed to estimate RBA-adjusted risk in Equations 5 and 6. Equation 8, expressed in units of TEQ, is applicable to a single congener (e.g., TCDD) in soil or to a mixture of congeners. However, when applied to the mixture of congeners, the parameters *ID* and *ED* become sums of the TEQs for individual congeners that make up the ID or ED (Equation 9):

$$ABA = AF = \frac{\sum ID_{TEQ,i}}{\sum ED_{TEQ,i}} \cdot \frac{1}{(1 - EF_{TEQ})} \qquad \text{Eq. (9)}$$

The data requirements for Equation 9 are quantification of the total TEQ external dose and total TEQ internal dose. The elimination fraction for TEQ does not have to be quantified if the experimental design ensures that $EF_{TM} = EF_{RM}$.

2.3. RBA$_{TEQ}$ in Soil for Noncancer Risk Assessment

Requirement 3: For noncancer risk assessment two RBA estimates are needed: (1) RBA for TEQ in corn oil relative to TCDD in corn oil (ABA$_{TEQ,corn\ oil}$/ABA$_{TCDD,corn\ oil}$); and (2) RBA for TEQ in soil relative to TEQ in corn oil (ABA$_{TEQ,soil}$ /ABA$_{TEQ,corn\ oil}$).

The current chronic oral RfD, 0.7 pg TCDD/kg/day is based on epidemiology of cohorts from Seveso, Italy cohort (U.S, EPA, 2014). These cohorts experienced relatively high acute multi-pathway exposures (inhalation, dermal, soil ingestion, ingestion of contaminated produce) shortly after an industrial accident (explosion) dispersed TCDD into the Seveso community. The dose metric in the dose-response modeling that supports the RfD is blood TCDD. The Point of

Departure (POD) was translated into an average daily intake by use of a PBPK model which was calibrated to achieve an oral bioavailability of 87% (based on an ingestion balance study conducted in a single individual who ingested a single dose [^{3}H]TCDD dissolved in corn oil; Poiger and Schlatter, 1986).

Based on the above considerations, the proposed RfD assumes 87% absolute bioavailability of TCDD from corn oil (ABA$_{corn\ oil}$=87%). Therefore, the appropriate RBA for TCDD in soil would be (Equation 10):

$$RBA_{TCDD,soil} = \frac{ABA_{TCDD,soil}}{ABA_{TCDD,corn\ oil}}$$
Eq. (10)

and the appropriate application of the RBA to the TCDD Hazard Quotient (HQ) would be (Equation 11):

$$HQ_{TCDD} = \frac{ADI_{TCDD,soil}}{RfD_{TCDD}/RBA_{TCDD,soil}} = \frac{ADI_{TCDD,soil} \cdot RBA_{TCDD,soil}}{RfD_{TCDD}}$$
Eq. (11)

However, EPA assesses risks for total TCDD TEQ in soil, not just for TCDD alone. Therefore, the *RBA$_{soil}$* in Equation 11 must represent the *RBA$_{soil}$* for TEQ (*RBA$_{TEQ}$*) and not just the RBA for TCDD.

This raises several problems. The 87% ABA assumption used in the basis for the RfD represents the bioavailability of TCDD and would not necessarily apply to the bioavailability of TEQ for a mixture of dioxin congeners because bioavailability appears to be dependent on chlorination (USEPA, 2010). Therefore, the appropriate RBA adjustment for TEQ in soil would be (Equation 12):

$$RBA_{TEQ,soil} = \frac{ABA_{TEQ,soil}}{ABA_{TEQ,corn\ oil}} \cdot \frac{ABA_{TEQ,corn\ oil}}{ABA_{TCDD,corn\ oil}}$$
Eq. (12)

and the appropriate application of the RBA to the TEQ Hazard Quotient (HQ) would be (Equation 13):

$$HQ_{TEQ} = \frac{ADI_{TEQ,soil}}{RfD_{TCDD}/RBA_{TEQ,soil}} = \frac{ADI_{TEQ} \cdot RBA_{TEQ,soil}}{RfD_{TCDD}}$$
Eq. (13)

Operationally, this translates into two requirements for a soil RBA bioassay for TEQ to be used in noncancer risk assessment: (1) estimate of RBA for TEQ in corn oil relative to TCDD in corn oil (ABA$_{TEQ,corn\ oil}$/ABA$_{TCDD,corn\ oil}$); and (2) estimate of the RBA for TEQ in soil relative to TEQ in corn oil (ABA$_{TEQ,soil}$ /ABA$_{TEQ,corn\ oil}$).

2.4. RBA$_{TEQ}$ in Soil for Cancer Risk Assessment

Requirement 4: For cancer risk assessment in which the oral slope factor (OSF) is based on exposures to TCDD in food, two RBA estimates are needed: (1) estimate of RBA for TEQ in food relative to TCDD in food (ABA$_{TEQ,food}$/ ABA$_{TCDD,food}$); and (2) estimate of the RBA for TEQ in soil relative to TEQ in food (ABA$_{TEQ,soil}$ /ABA$_{TEQ,food}$).

Based on logic similar to that described above for the noncancer risk assessment, if the OSF is based on a bioassay or a human epidemiology study in which exposure was to TCDD in food, the appropriate RBA adjustment for soil TEQ for use in cancer risk assessment would be (Equations 14 and 15):

$$RBA_{TEQ,soil} = \frac{ABA_{TEQ,soil}}{ABA_{TEQ,food}} \cdot \frac{ABA_{TEQ,food}}{ABA_{TCDD,food}} \qquad \text{Eq. (14)}$$

$$CR_{TEQ} = \frac{ADI_{TEQ} \cdot OSF_{TCDD}}{RBA_{TEQ,soil}} \qquad \text{Eq. (15)}$$

Operationally, this translates into 2 requirements for a soil RBA bioassay for TEQ for use in cancer risk assessment: (1) estimate of RBA for TEQ in food relative to TCDD in food (ABA$_{TEQ,food}$/ ABA$_{TCDD,food}$); and (2) estimate of the RBA for TEQ in soil relative to TEQ in food (ABA$_{TEQ,soil}$ /ABA$_{TEQ,food}$).

2.5. Selection of Animal Model for Predicting RBA in Humans

Requirement 5: There is no general consensus on the preferred animal model for estimating RBA for PCDD/F. RBA assays for congener mixtures in soil have been conducted in rats and swine, and these two assay yield different estimates of RBA$_{TEQ}$.

Differences are evident between RBA estimates for test soils assayed in swine and rats (USEPA, 2010). This included large differences in the average RBA values for the same test material assayed in swine and rats (Budinsky et al., 2008), as well as regression coefficients for the effect of congener chlorine content on RBA that are in opposite directions. RBA varies with congener chlorination. The direction of the relationship (i.e., positive or negative slope) is not the same

when estimated based on data from swine or rat assays. Data from swine assays indicates an increase in RBA with increasing chlorine content (Budinsky et al., 2008; Wittsiepe et al., 2007), whereas, data from rat assays indicates a decrease in RBA with increasing chlorination (Budinsky et al., 2008; Finley et al., 2009). These differences suggest substantially different RBA estimates may be obtained depending on the animal model used. The dependence of RBA on congener chlorination suggests that soil RBA will depend on the congener composition of the soil (as well as the bioassay used to estimate RBA).

2.6. Dosing Regimen

Requirement 6a: External doses of TEQ should not exert overt systemic toxicity that alters PCDD/F distribution or impairs elimination (metabolism or excretion). External dose should be well below the LD_{50} and preferably, well below to LD_{01}.

Requirement 6b: Multiple dose levels of TEQ should be administered to allow and evaluation of the dependence of RBA on dose.

Requirement 6c: External doses of TEQ delivered in the test (e.g., soil) and reference material (e.g., corn oil) must result is similar (or overlapping ranges) of internal doses of TEQ. This is needed to prevent different levels of induction of CYP450 and different elimination fractions of TEQ for the test and reference material.

Requirement 6d: There is no general consensus as to whether single doses or repeated doses should be administered. Regardless of the dosing schedule, a sufficient cumulative (and non-toxic) external dose must be delivered to allow quantification of the internal dose of administered congeners that comprise ≥95% of the administered TEQ.

Requirement 6e: For assay of RBA of PCDD/F in soils, the administered soil should be the <250 μm fraction

As noted previously in reference to Equations 7 and 8, measurement of the elimination fraction (*EF*) is not needed in the calculation of RBA as long as the elimination fraction is not different following administration of the PCDD/F dose in test or reference materials. However, because the internal TEQ dose (e.g., liver dose) can induce CYP450 (which increases elimination rate), the elimination fraction may vary with internal TEQ dose. Therefore, dosing regimens for the test and reference materials should be matched to achieve similar internal TEQ doses (Finley et al., 2009; USEPA, 2010). Establishing internal dose equivalents for TEQ requires forehand knowledge of TEQ RBA for the test material of interest, which, of course, will not be known (if it were, there would be no need to assay the test material). Therefore, administering multiple dose levels of TEQ is recommended to achieve overlap of the corresponding internal TEQ doses.

Use of multiple dose levels will allow evaluation of the external dose-internal dose relationship and detection of any nonlinearities that might suggest dose-dependence of elimination kinetics. The RBA can be calculated from the regression relationships for the reference and test materials (USEPA, 2007b).

Calculation of RBA for total TEQ in the test material requires that the internal doses of TEQ contributed from each administered congener be quantified and summed (Equation 9). To achieve this, the administered dose of each congener must be sufficient to achieve a corresponding internal dose that is above the detection limit. Those congeners that are below the detection limit must be assigned values that will introduce uncertainty into the RBA estimate (e.g., one half detection limit). There is no general consensus as to whether single doses or repeated doses should be administered. Given the relatively slow elimination kinetics, it is unlikely that steady state conditions are feasible. However, repeated dosing will allow the accumulation of the more rapidly eliminated congeners and congeners having low RBA, and may improve detection and quantification of these congeners in the internal dose. Whether or not single or repeated dosing is feasible will depend, in part, on the animal model selected. Detection and quantification of all congeners in the internal dose may not always be possible for congeners have very low RBA. Minimum objectives for quantification of the internal dose should be established in the study design and results evaluated against these objectives. As a general default, the administered doses should ensure detection of $\geq 95\%$ of the administered TEQ.

In risk assessment applications, the grain size fraction that is most likely to adhere to human skin is typically of primary importance. It is generally accepted that for moisture contents found in typical surface soils, this is the <250 μm fraction (Berstrom et al., 2011; Kissel et al., 1996; Siciliano et al., 2009; Yamamoto et al., 2006). Therefore, unless a strong argument can be made for an alternative, the assay should estimate the RBA for the <250 μm fraction.

2.7. Measurement of Internal TEQ Dose

Requirement 7: Tissues selected for assay of PCDD/F congeners should provide reliable predictions of the TEQ body burden. There is no general consensus regarding which tissue would satisfy this requirement, and it is likely to vary across animal species. Ideally, if whole body (gastrointestinal tract excluded) is not analyzed for TEQ, selected tissues should include those that collectively contribute $\geq 50\%$ of total body burden. At a minimum, this should include liver and adipose.

Calculation of RBA for TEQ requires quantification of the relationship between the administered (external) dose and internal dose (Equations 7 and 8). Absorbed PCDD/F is widely distributed and partitions into tissue lipid. Therefore, the internal dose is the total TEQ body burden

(excluding unabsorbed TEQ in the gastrointestinal tract). Ideally this could be achieved by quantifying the entire body burden of administered PCDD/F congeners, however, this may not be feasible for most animal models. In most mammalian species in which the whole body distribution of PCDD/F (e.g., TCDD) has been studied, that largest fractions of the body burden ≥50% reside in liver and adipose (USEPA, 2003). RBA assays of congener mixtures have measured internal dose as PCDD/F concentrations or burdens of liver (Finley et al., 2009), liver plus adipose combined (Budinsky et al., 2008) or combined adipose, blood, brain, liver, and muscle (Wittsiepe et al., 2007). Given the relatively large contribution of adipose and liver to body burden, at a minimum, these two tissues should be assayed. Estimation of the PCDD/F burdens in tissue requires measurement of the PCDD/F concentrations and the total mass of each tissue. This is easily accomplished for the liver but is more difficult for adipose. The experimental design should address how the adipose mass (or volume) is to be estimated and, if not actually measured, what assumptions are to be made about it mass or volume.

2.8. Confidence in RBA Estimates

Requirement 8: The study design must provide: (1) statistical confidence limits on the estimate (e.g., 95% confidence limits) of the RBA and; (2) an evaluation of reproducibility of RBA estimates when the same test materials are assayed.

The RBA calculation shown in Equation 7 is typically a ratio of mean ABA values obtained from a sample of measurements of ABA from a group or groups of animals that received doses of the test or reference material. The resulting RBA from Equation 7 represents an estimate of the mean RBA. Estimating confidence limits on the mean RBA requires estimating the confidence limit on a ratio of mean values for ABA, where each mean has an associated uncertainty that must be estimated from the sample distributions. Several different computational strategies for calculating confidence limits on the RBA from single or multiple dose level assays of PCDD/F have been described (USEPA, 2007a; 2010). These include application of Fieller's theorem and bootstrap methods. The statistical design for estimating confidence on the RBA should be articulated in the study design.

In addition to confidence limits on each RBA estimate, reproducibility of RBA estimates should be evaluated. The only way to accomplish this is to assay the same test material several times and compare outcomes. Where this is not feasible (e.g., budget limitations) the study design must address how uncertainty in the reproducibility of the assay would be addressed in any application of the RBA estimate to risk assessment.

2.9. Soil Characterization

Requirement 9: Study designs intended to estimate RBA of PCDD/F in soils should include a characterization of the soil, including a complete analysis of PCDD/F congeners, as well as soil characteristics. Minimum soil characteristics should include total solids, pH, total organic carbon, and grain size distribution.

The expectation is that adherence of PCDD/F to constituents of soil (e.g., organic carbon) is an important determinant of RBA. The soil characteristics that most greatly influence PCDD/F RBA have not been identified. However, an important objective will be to utilize data obtained from soil RBA studies, data on soil characteristics, and *in vitro* extraction methods to establish methods to predict RBA that circumvent the need for expensive animal bioassays. Therefore, collection of data on the characteristics of soils (composition, mineralogy) that are assayed is highly desirable. At a minimum, soil should be evaluated for PCDD/F congener composition, total solids, pH, total organic carbon, and grain size distribution.

3. Summary and Conclusions

This report provides the basis for minimum requirements of assays intended to estimate RBA of PCDD/F in soils for applications to risk assessment. Given that the methodology for assaying PCDD/F RBA in soils is evolving, greater experience with various experimental designs is likely to prompt modifications to the requirements identified in this report. The minimal requirements identified in this report are summarized in Table 2.

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Table 1. Reported Variations in Experimental Designs for TCDD or PCDD/F RBA Assays

Experimental Design Parameter	Implemented Design
Animal models	• Guinea pig • Rabbit • Rat • Swine
Soil test materials	• In situ contaminated soil • Laboratory spiked soil
Dosages	• Subtoxic • Systemically toxic • Similar tissue levels of PCDD/F achieved in animals that received soil and reference • Similar tissue levels of PCDD/F achieved in animals that received soil and reference
Dosing regimens	• Single dose • Repeated dose • Single dose level • Multiple dose levels
Dose vehicles for soil	• Aqueous suspension • Food mix (e.g., dough ball)
Dose vehicles for reference	• Acetone/corn oil • Acetone/hexane • Corn oil • Gum acacia
Measured bioavailability metrics	• Liver TCDD • Liver PCDD/F • Adipose, liver PCDD/F • Adipose, blood, brain, liver, muscle PCDD/F
Interval between dosing and tissue collection	• 1 day • 6 days • 7 days • 30 days • 60 days
Data reduction methods	• Soil:reference tissue concentration ratio • Soil:reference slope ratio for dose-tissue PCDD/F • Absolute bioavailability based on intravenous reference dosing

Based on Bonaccorsi et al., 1984; Budinsky et al., 2008; Finley et al., 2009; Lucier et al., 1986; McConnell et al., 1984; Shu et al., 1988; Umbreit et al., 1986; Wendling et al., 1989;Wittsiepe et al., 2007)

Table 2. Minimum Experimental Design Requirements for PCDD/F RBA Assays

Design Parameter	#	Requirement
RBA for TEQ	1	PCDD/F risk assessment requires estimates of the RBA for soil TEQ (RBA_{TEQ})
Calculating RBA_{TEQ} in soil	2	Calculation of RBA_{TEQ} requires quantification of the total TEQ external dose and total TEQ internal dose, as well as the excretion fraction for TEQ (or experimental designs that ensure that the excretion fractions for TEQ are the same when administered in the soil or reference material).
RBA_{TEQ} in soil for noncancer risk assessment	3	For noncancer risk assessment two RBA estimates are needed: (1) RBA for TEQ in corn oil ($ABA_{TEQ,corn\ oil}$/$ABA_{TCDD,corn\ oil}$); and (2) RBA for TEQ in soil ($ABA_{TEQ,soil}$ /$ABA_{TEQ,corn\ oil}$).
RBA_{TEQ} in soil for cancer risk assessment	4	For cancer risk assessment two RBA estimates are needed: (1) estimate of RBA for TEQ in food ($ABA_{TEQ,food}$/ $ABA_{TCDD,food}$); and (2) estimate of the RBA for TEQ in soil ($ABA_{TEQ,soil}$ /$ABA_{TEQ,food}$).
Animal model	5	There is no general consensus on the preferred animal model for estimating RBA for PCDD/F. RBA assays for congener mixtures in soil have been conducted in rats and swine, and these two assay yield different estimates of RBA_{TEQ}.
Dosing regimen	6a	External doses of TEQ should not exert overt systemic toxicity that alters PCDD/F distribution or impairs elimination (metabolism or excretion). External dose should be well below the LD_{50} and preferably, well below to LD_{01}.
	6b	Multiple dose levels of TEQ should be administered to allow and evaluation of the dependence of RBA on dose.
	6c	External doses of TEQ delivered in the test (e.g., soil) and reference material (e.g., corn oil) must result in similar (or overlapping ranges) of internal doses of TEQ. This is needed to prevent different levels of induction of CYP450 and different elimination fractions of TEQ for the test and reference material.
	6d	There is no general consensus as to whether single doses or repeated doses should be administered. Regardless of the dosing schedule, a sufficient cumulative (and non-toxic) external dose must be delivered to allow quantification of the internal dose of the administered congeners that comprise ≥95% of the administered TEQ.
	6e	For assay of RBA of PCDD/F in soils, the administered soil should be the <250 µm fraction
Internal TEQ dose metrics	7	Tissues selected for assay of PCDD/F congeners should provide reliable predictions of the TEQ body burden. There is no general consensus regarding which tissue would satisfy this requirement, and it is likely to vary across animal species. Ideally, if whole body (gastrointestinal tract excluded) is not analyzed for TEQ, selected tissues should include those that collectively contribute ≥50% of total body burden. At a minimum, this should include liver and adipose.
Confidence in RBA Estimates	8	The study design must provide: (1) statistical confidence limits on the estimate (e.g., 95% confidence limits) of the RBA and; (2) an evaluation of reproducibility of RBA estimates when the same test materials are assayed.
Soil characterization	9	Study designs intended to estimate RBA of PCDD/F in soils should include a characterization of the soil that includes a complete analysis of PCDD/F congeners, as well as soil characteristics to including, at a minimum: total solids, pH, total organic carbon, and grain size distribution.