

Determining High-Quality Critical Body Residues for Multiple Species and Chemicals by Applying Improved Experimental Design and Data Interpretation Concepts

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S Supporting Information

ABSTRACT: Ecotoxicological effect data are generally expressed as effective concentrations in the external exposure medium and do thus not account for differences in chemical uptake, bioavailability, and metabolism, which can introduce substantial data variation. The Critical Body Residue (CBR) concept provides clear advantages, because it links effects directly to the internal exposure. Using CBRs instead of external concentrations should therefore reduce variability. For compounds that act via narcosis even a constant CBR has been proposed. Despite the expected uniformity, CBR values for these compounds still show large variability, possibly due to biased and inconsistent experimental testing. In the present study we tested whether variation in CBR data can be substantially reduced when using an improved experimental design and avoiding confounding factors. The aim was to develop and apply a well-defined test protocol for accurately and precisely measuring CBR data, involving improved (passive) dosing, sampling, and processing of organisms.

The chemicals 1,2,4-trichlorobenzene, 1,2,3,4-tetrachlorobenzene, 2,3,4-trichloroaniline, 2,3,5,6-tetrachloroaniline, 4-chloro-3-methylphenol, pentylbenzene, pyrene, and bromophos-methyl were tested on *Lumbricus variegatus* (California blackworm), *Hyalella azteca* (scud), and *Poecilia reticulata* (guppy), which yielded a high-quality database of 348 individual CBR values. Medians of CBR values ranged from 2.1 to 16.1 mmol/kg wet weight (ww) within all combinations of chemicals and species, except for the insecticide bromophos-methyl, for which the median was 1.3 mmol/kg ww. The new database thus covers about one log unit, which is considerably less than in existing databases. Medians differed maximally by a factor of 8.4 between the 7 chemicals but within one species, and by a factor of 2.6 between the three species but for individual chemicals. Accounting for the chemicals' internal distribution to different partitioning domains and relating effects to estimated concentrations in the target compartment (i.e., membrane lipids) was expected to but did not decrease the overall variability, likely because the surrogate partition coefficients for membrane lipid, storage lipid, protein, and carbohydrate that were used as input parameters did not sufficiently represent the actual partitioning processes. The results of this study demonstrate that a well-designed test setup can produce CBR data that are highly uniform beyond chemical and biological diversity.



INTRODUCTION

In the field of ecotoxicology, effect data are usually expressed as concentrations in the external exposure medium (e.g., LC50 in water, soil, or sediment). These 'external effect concentrations' may however display considerable variation among different organisms, due to differences in e.g. uptake kinetics, metabolic capacity, or bioaccumulation potential. Even effect data for a single chemical-organism combination may show scatter as a result of differences in the bioavailability of a compound. For instance, the bioavailability of organic chemicals in water and sediment is strongly affected by the concentration and composition of (dissolved) organic matter.^{1,2} Because variation in effect concentrations hampers accurate risk

assessment, already 25 years ago the so-called Critical Body Residue (CBR) concept was postulated.^{3,4} This concept advocates the determination and use of internal concentrations corresponding to a certain endpoint, e.g., mortality or a sublethal effect, in order to account for the external factors listed above. Internal concentrations represent an intrinsic dose parameter,^{5–7} and past research has demonstrated that the application of CBRs indeed yields reduced data variation

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among species, media,^{7–12} and chemicals, including mixtures.^{12–14}

The CBR concept has been applied most frequently for compounds that act by narcosis. In ecotoxicology, narcosis represents a nonspecific mechanism of action (MoA) for toxicity, by which chemical partitioning into biomembranes causes disruption of membrane structure and/or functioning.¹⁵ Since all organic chemicals have the potential to act via this mechanism, it is often referred to as baseline or minimum toxicity.^{16,17} CBR values of narcotic compounds have been found to cover a rather narrow range between approximately 2 and 8 mmol/kg wet weight.^{4,15,18} For chemicals with an additional, more specific MoA, the CBR concept can also be applied, but the values concerned will always be lower and cover a broader range.^{4,10,18} One of the obvious causes of such larger variation is that the potency of interaction with a specific receptor can vary considerably between different chemicals.^{4,18,19}

Despite the generally observed relatively narrow range in CBR values for narcotic compounds, deviations from the 2–8 mmol/kg range are still frequently observed, as demonstrated by McCarty et al.²⁰ who analyzed the principal publicly available body of CBR data, i.e., the Environmental Residue Effects Database (ERED). These deviations may be caused by (i) biased experimental measurements or inconsiderate experimental design and/or (ii) the fact that CBRs relate to a wet weight basis, although the cell membrane is thought to be the actual target site, where narcosis is triggered. Examples of factors that may contribute to the first cause are the use of metabolizable chemicals, the use of chemicals that have an (unknown) additional MoA or are contaminated with such a chemical, the time span between actual death and sampling of the organism, or exposure parameters (e.g., dose, temperature).⁹ Although a carefully designed test may avoid such bias, cause (ii) represents a more fundamental issue.

Considering the theory on narcosis,¹⁵ it is plausible that the effective concentrations of narcotic chemicals within the cell membrane on a molar basis are rather constant for different chemicals and species. Due to natural variation in the ratio of target (membranes) to nontarget (storage lipids, water, proteins, carbohydrates) compartments among organisms, CBRs can be expected to be somewhat species-dependent. Here, normalization of CBRs to the target compartment should help to reduce the overall variation. However, simple normalization to measured total lipids and/or assumed membrane lipid contents do not often yield a considerable further reduction: the narcotic range on a lipid basis measures 40 to 160 mmol/kg lipid (still a factor of 4).¹⁵ Yet, CBRs for polar and nonpolar compounds, which differ significantly on a wet weight basis,^{21–23} seem to converge when expressed as estimated concentrations in membrane lipids. Most probably, this can be explained by a relatively high affinity of polar compounds for membrane lipids as compared to that of nonpolar compounds.^{22–25} Differences in whole body-based CBR values for a specific chemical in different organisms may be related to differences in the composition of the organisms, i.e., the ratio of polar membrane lipids to nonpolar storage lipids. In addition, other biological compartments such as proteins may act as sorption domains and may thus complicate data interpretation.^{26–29} As such, these compartments should preferably be included in data analysis.

From the above, it may be clear that information on the organisms' composition and a well-considered test design are

essential for the interpretation and understanding of (the variability in) CBR data. So far, however, the above line of reasoning mostly relies on assumptions and estimations. In the present study, we tested the hypothesis that much of the variation in reported CBRs is caused by differences in composition among test organisms, methodological differences between studies, and the influence of confounding factors. To this end, we performed specially designed, high-quality CBR experiments with multiple chemicals and organisms in order to produce a coherent and consistent CBR data set. Special attention was paid to avoiding confounding factors and potential bias. CBR data were interpreted with a mass-balance model describing the internal distribution of the test compounds. Chemical-specific experimental partitioning data for surrogate tissue constituents (i.e., representing membrane lipid, storage lipid, protein, and carbohydrate) and experimentally quantified sizes of these compartments were used as model input parameters. The overall objective of the study was to advance the CBR as a useful and unambiguous metric in risk assessment of chemicals.

■ EXPERIMENTAL DESIGN

To determine high-quality CBR values, a well-considered test setup was developed, aiming to rule out as many potential confounding factors as possible. To produce CBR data for narcotic compounds according to criteria defined by McCarty et al.,²⁰ test substances for the present CBR measurements should (i) not show MoAs other than narcosis, (ii) be relatively inert, (iii) be able to cause lethality within 48 h, (iv) allow dosing in a freely dissolved state at a broad concentration range, and (v) be available in a sufficiently high purity. Based on these criteria, the following eight chemicals were selected: 1,2,4-trichlorobenzene (124-TCB); 1,2,3,4-tetrachlorobenzene (1234-TeCB); 2,3,4-trichloroaniline (234-TCA); 2,3,5,6-tetrachloroaniline (2356-TeCA); 4-chloro-3-methylphenol (4-C-3-MP); pentylbenzene (QBenz); pyrene (Pyr); and bromophosphomethyl (BrM) (see Table S1 for octanol-water partition coefficients, aqueous solubilities, and Verhaar classes³⁰). Note that this set represents both nonpolar and polar narcotic compounds, for which literature CBR data are available in most cases. Although BrM is an organophosphate with an expected specific MoA, experimental data on CBRs in guppy did not demonstrate more than baseline toxicity.³¹ Probably, narcosis exceeded the specific toxicity because of the chemical's relatively high hydrophobicity and affinity for membranes.

Test organisms were selected based on their ability to reside in a glass jar at a disturbed day-night cycle (i.e., with a dark period punctuated by light periods to allow monitoring) at 20 °C, while being withheld food for a 48 h period, without suffering from any adverse effects. Also, the ratio of body weight to volume of the aqueous phase needed to be low enough to prevent depletion of the oxygen pool and of the reservoir of test chemicals in the system. Yet, the organisms should be large enough to enable a reliable weight recording and CBR quantification above detection limits. These criteria led to the selection of *Lumbriculus variegatus* (California blackworm), *Hyalella azteca* (scud), and *Poecilia reticulata* (guppy). These organisms represent aquatic species of different internal compositions and ecologically distinct backgrounds that are frequently used in aquatic and sediment toxicity and bioaccumulation studies.

In the present study we aimed for stable aqueous exposure concentrations throughout the test duration. More specifically,

the test systems were dimensioned such that depletion of the aqueous phase by the exposed animals remained below 10%. Chemicals were either dosed by directly dissolving them in the medium or by passive dosing.³² New passive dosing formats were developed for the specific requirements of the study, so that it was possible to (i) efficiently dose 1–2 L of medium, (ii) avoid additional mixing, (iii) apply dosing systems on a large scale (>100 test systems), and (iv) avoid direct contact between organisms and dosing phase, as this could lead to direct contact uptake. To meet these criteria, recently used silicone rod³³ was applied, as its geometry combines a high surface area with a very high silicone volume. This combination provides a huge buffer capacity for the dosing and good kinetics even at low turbulence levels. Only in the 2 L systems that were applied to accommodate *Poecilia*, dosing kinetics were enhanced by stirring during the test. Because stirring and animal exposure do not combine well, a previously developed setup was employed, involving two conjoint bottles (see Tables S2 and S3 for a visual representation; Jonker, unpublished data), with organisms being exposed in the one and dosing and agitation being performed in the other bottle.

Finally, where feasible, tests were performed at three dosing levels to ensure high lethality within the test duration, while avoiding the occurrence of full lethality at an early stage of the test. Table S2 provides a summary of the different experimental setups.

MATERIALS AND METHODS

Chemicals, Passive Sampling and Dosing Material, and Test Organisms. Details on test chemicals, surrogate materials representing biological compartments, polymer materials used for sampling and dosing of aqueous media, and test animals are provided in the Supporting Information (pages S4–S5).

Preparation of Test Systems. Based on a preliminary test (see Table S4), 124-TCB, 1234-TeCB, QBenz, Pyr, and BrM were selected as substances suitable for a passive dosing approach. Depending on the chemical, silicone rods were loaded following different procedures (see Supporting Information, pages S6–S7). Passively dosed systems were pre-equilibrated for 48 h prior to the test, although the time to steady state was determined to be less than an hour (even in the largest systems) and was largely independent of the chemical's hydrophobicity (see Figure S1). For the remaining compounds, passive dosing was less suitable, because of their unfavorable polymer-water distribution (see Table S4). These chemicals were simply dissolved directly in water intended as exposure medium and equilibrated by stirring for 4 days (see Supporting Information, page S8). To compare the performance of both dosing modes, 124-TCB was added via either method in tests with *Lumbriculus*. A detailed description of the test systems and loading procedures is provided in Tables S2–S3.

Indicative (SPARC-modeled) aqueous solubility values and the chemical activity scale were applied to set the three dosing levels (beside the controls) for each individual test substance. We aimed to cover the higher part of the known chemical activity range for baseline toxicity (i.e., 0.01–0.1)^{34,35} and, when possible, also included one treatment above this range. Generally, geometric dilution series with a factor of 2 or 3 between the levels were used. In some cases, the highest exposure doses were set at (or near) the solubility level. Finally, the selected exposure levels were checked for consistency and

reasonability by comparison with existing toxicity data and some QSARs.¹⁶

CBR Experiments. Three individuals of either *Lumbriculus*, *Hyalella*, or *Poecilia* were added to prepared test vessels, and the time was recorded. The organisms were exposed at 20 (\pm 0.3) °C in oxygen-saturated water (oxygen levels were checked in control systems) and systematically monitored during the 48 h of exposure. The monitoring frequency was adapted to the urgency; moribund animals were closely observed, whereas lively ones were checked at larger time intervals. Immediately upon observation of death, time was recorded, organisms were carefully taken out of their test system by tweezers or, in the case of the more fragile *Lumbriculus*, with a bent piece of metal wire. Dead animals were rinsed with fresh tap water, using a pipet to wash away contaminated adhering water, blotted dry, weighed, and transferred to a vial containing a preweighed volume of acetonitrile (1.5 mL for *Lumbriculus* and *Hyalella* and 20 mL for the larger *Poecilia*). The vials containing the individual organisms were stored at –20 °C until processing. Prior to the introduction of the animals and upon conclusion of the tests, an aliquot of the exposure solution was sampled and gravimetrically transferred to a vial containing hexane. The samples were stored at 4 °C until further processing. From the passive dosing systems, a 10 mg piece of silicone rod was sampled, blotted dry, weighed, transferred to a bottle containing a preweighed volume of acetonitrile, and stored at 4 °C. After 48 h, all remaining tests were concluded and materials, including live animals, were sampled as described above.

Sample Processing. Organisms were extracted in their collection vial through three cycles involving shaking (1800 rpm for 10 min or 150 rpm for 1 h in case of the larger vials containing *Poecilia*) and subsequent sonication (15 min). Next, vials were centrifuged, after which the extracts were diluted. Cleanup was not performed, as it was shown not to be necessary; the undiluted extracts that were produced as described on page S9 of the Supporting Information did not cause any interference (e.g., interfering peaks in the chromatograms or changes in the sensitivity of the detector) during instrumental analysis. Recoveries of the extraction procedure were determined as described in the Supporting Information (page S9) and were at least 99.9%. The water samples were extracted with hexane by shaking at 1800 rpm (10 min), after which the hexane phase was collected. This extraction procedure was then repeated twice with fresh hexane. The three hexane phases were pooled, concentrated using a modified Kuderna-Danish evaporation setup and a gentle nitrogen flow, and finally exchanged to acetonitrile. Silicone samples were extracted with acetonitrile by shaking overnight on an orbital shaker, after which the extracts were diluted.

Prior to analysis, all extracts received appropriate internal standards. Final concentrations in all extracts were corrected for procedural recoveries and blanks.

Characterization of Organisms. In order to convert CBRs to values based on the appropriate target compartment, the composition of the test species was determined in terms of target (i.e., membrane lipid) and nontarget compartments (i.e., water, storage lipid, proteins). The water content of the test species was determined by comparing the weight of unexposed organisms that had gently been blotted dry until no more water was observed to be taken up by the paper towel (i.e., in the same way the animals were dried after the CBR tests), before and after overnight drying at 85 °C. The total lipid content was

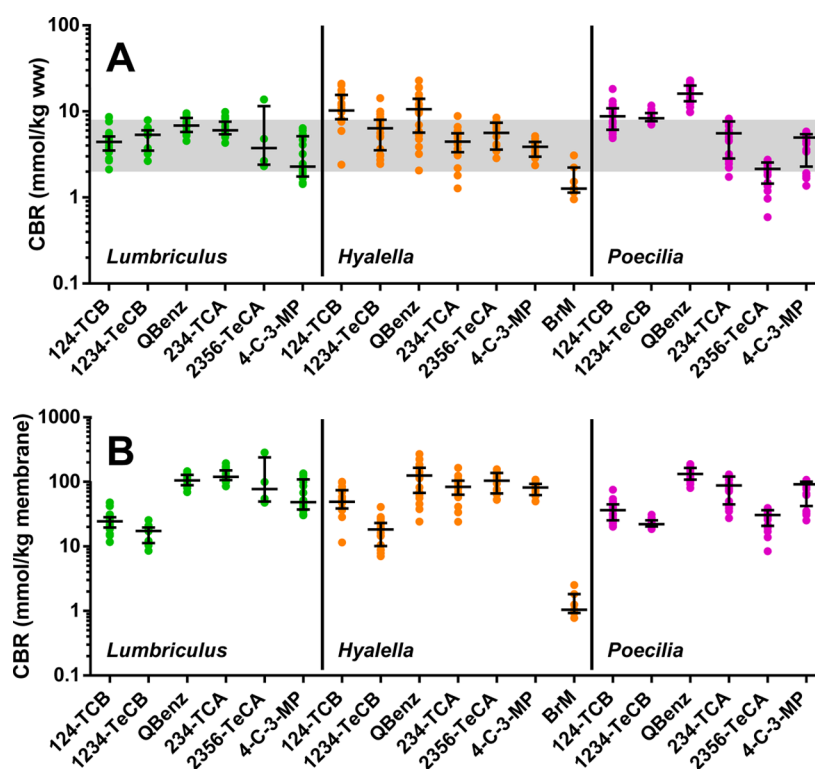


Figure 1. Wet weight-based (graph A) and membrane lipid weight-based (graph B) CBR data for *Lumbriculus* (green markers), *Hyalella* (orange markers), and *Poecilia* (purple markers). The lines with error bars represent the median and the interquartile range. The highlighted gray band in graph A indicates the 2–8 mmol/kg ww interval.

measured by performing a lipid extraction on freeze-dried homogenate, as described by Bligh and Dyer.³⁶ The extracts were separated by Silicagel 60 column fractionation into storage (nonpolar) and membrane (polar) lipids, following the procedure described in ref 37. Homogenates of unexposed organisms were analyzed for proteins according to a procedure adapted from ref 38, which is described in the Supporting Information (pages S9–S10).

Surrogate Lipid-, Protein-, and Carbohydrate-Water Partition Coefficients. Equilibrium partitioning of all chemicals between surrogate biomaterials and water was determined experimentally as described previously.³⁹ 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)-based liposomes, triolein, bovine serum albumin (BSA), glycogen, and chitin were chosen as surrogates for actual biological compartments (i.e., membrane lipids, storage lipids, proteins, and two types of carbohydrates, respectively) and were equilibrated for 4 weeks in aqueous solutions spiked with the chemicals. Polyacrylate-coated SPME fibers served to derive the aqueous concentrations. Experiments were designed so that the mass of the chemical sorbed to the surrogate biological phase was always more than 30% of the total mass in the system.⁴⁰ Polyacrylate-water partition coefficients required for the derivation of aqueous concentrations had either been determined previously²⁵ or were measured as described in ref 41. Newly determined values are listed in Table S5.

Instrumental Analysis. Chemicals were analyzed by GC-ECD (chlorobenzenes and chloroanilines), HPLC/FLU (Pyr), or GC-MS (the remaining chemicals). Details on the analytical procedures are provided in the Supporting Information.

RESULTS AND DISCUSSION

Evaluation of the Applied Test Design. In order to minimize bias, several QA/QC measures were implemented for the experimental determination of CBRs, including analytical QA/QC aspects (discussed in the Supporting Information); testing at a constant temperature (i.e., 20 °C), monitoring oxygen levels, processing and analyzing blanks for all species, analyses of aqueous concentrations of chemicals in all systems before and after the exposures, and continuous monitoring of exposed organisms. Oxygen levels in control systems showed a limited drop from on average 8.5 mg/L to 7.7 mg/L over the course of the experiment with *Poecilia* (the organism with the highest oxygen consumption). Considering that narcosis causes reduced ventilation rates, the reduction in oxygen levels in the chemically dosed systems was expected to be even more limited. This indicates that oxygen depletion was not a confounding factor during the experiments, which is supported by the observation that for all three species the nondosed (control) individuals could not be distinguished from those that were not used in any test in terms of physical appearance and behavior. Chemical analyses of the control organisms additionally showed no or negligible chemical residues (generally below detection limits or else below 1% of the smallest residue detected in exposed organisms), indicating no chemical crossover during exposure, sample processing, and analyses. Analyses of the water extracts taken before and after the exposures further demonstrated that chemical depletion of the systems was also negligible (measured aqueous concentrations are given in Table S6). Although in specific cases the concentration dropped by 27% (lowest dosing level of the relatively volatile pentylbenzene in the small test jars), the average reduction was 1.6 (± 9.2) %, which indicates that the

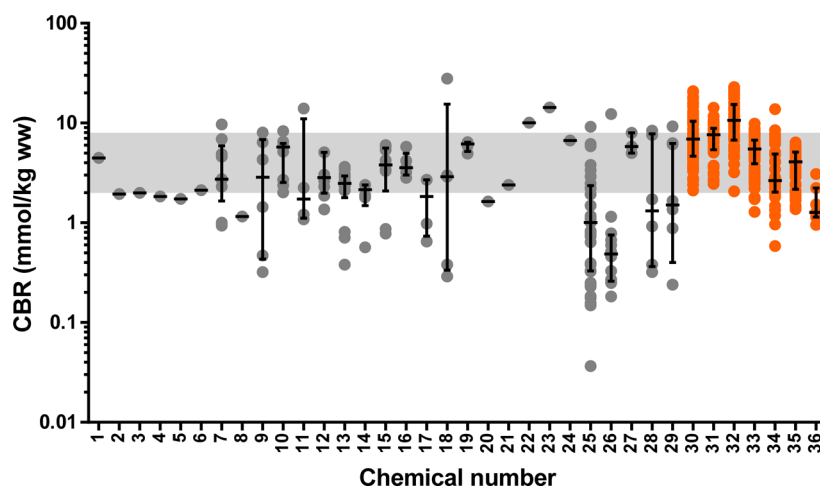


Figure 2. Wet weight-based CBR data taken from the refined database compiled by McCarty et al.²⁰ (gray markers; chemical numbers 1–29) and from this study (orange markers; chemical number 30–36). Orange markers represent individual CBR values. Lines with error bars represent the median and the interquartile range. The highlighted gray band indicates the 2–8 mmol/kg ww interval. Note that the low CBRs for fluoranthene (chemical number 25) may be explained by phototoxicity as discussed in ref 20. Also note that for chemical numbers 1, 2, 3, 4, 5, 6, 8, 20, 21, 22, 23, and 24 only a single value was determined.

organisms were exposed to concentrations that can be considered stable.

Working in shifts allowed a continuous (day and night) monitoring of the organisms. Although during later stages of the exposure this often proved redundant and the monitoring frequency could be reduced to once per hour, particularly during the early stages organisms were even monitored by two persons. This proved necessary as at the higher dosing levels organisms sometimes died quickly. Unfortunately, these efforts could not prevent some individuals to remain in their vessel for a limited length of time after the exact moment of death. This was due to the number of organisms exposed simultaneously in the current setup and the fact that ascertaining death required time and often multiple organisms died at the same time. Still, from the recorded time intervals between the last observed life sign and actual sampling, it was concluded that the unwarranted uptake time was never more than about 10% of the total time to death (TtD). One exception was the highest dosing of 4-C-3-MP in the first round of tests with *Lumbriculus*, where the chemical dosing was so high that mortality occurred within 1 min. As shown in Table S7, CBRs measured for these cases are unrealistically high (up to 70 mmol/kg ww), which will be discussed below. Because of their unreliability, these values were omitted from further analysis. For the series of experiments with the other species, 4-C-3-MP concentrations were lowered. In contrast, based on the lack of effects in other cases, some dosing levels of the other chemicals were increased or omitted, while maintaining a meaningful concentration spacing.

In conclusion, confounding factors were minimized as a result of the QA/QC measures applied in the current test setup for the determination of CBRs. The resulting CBR database should thus reflect minimal experimental bias.

Comparison of CBR Values with Literature Data. In total, the CBR experiments involved the exposure of 569 individual organisms, which yielded 348 actual CBR values, measured for individual organisms (approximately 220 organisms did not die, 39 of which were controls). As shown in Figure 1A, the majority of the CBRs fall within the 2–8 mmol/kg wet weight interval, as proposed in the litera-

ture.^{4,15,18} Based on this interval and a generic lipid percentage of 5%, also a total lipid-based CBR interval has been derived, measuring 40–160 mmol/kg total lipid.¹⁵ The present total lipid-based CBRs are either within or above this interval (see Table S8). Values exceeding the range are probably mainly due to the fact that the actual total lipid content of the species used here deviated from the generic 5% lipid that was used to derive the interval.

Although the medians of the majority of wet weight-based CBRs are either within or close to the 2–8 mmol/kg interval (Table S8), for a few substance/species combinations, values are outside that range. For instance, CBRs of QBenz were consistently higher for *Poecilia*. Considering the wet weight-based values only, there is no apparent explanation for this. However, as stressed previously, wet weight-based values do not directly relate to target tissues and as such are biased by definition; this issue will be further discussed below.

Also, a CBR median below the 2–8 mmol/kg wet weight range was observed in *Hyaella* for the insecticide Bromophos-methyl (1.3 mmol/kg wet weight), which suggests more than baseline toxicity. This “additional” toxicity is not necessarily in conflict with earlier reported CBR values for *Poecilia* that fell within the baseline toxicity range,³¹ since the occurrence of specific toxicity can depend on the species.

Because most literature values are based on wet weight, the variability in the current data set can be compared with that in the literature. A recent paper by McCarty et al.²⁰ presented a rigorous data quality evaluation on the bulk (i.e., over 15,000 entries) of CBR data existing in the literature. Database cleanup by removing low-quality and questionable entries resulted in a data set containing 161 records for 29 neutral organic compounds for algae, invertebrates, and fish species, produced by multiple research groups. This number first of all clearly demonstrates the scarcity of high-quality CBR data. Second, the compiled set showed an overall CBR range spanning about 3 log units.²⁰ Note that the averaged CBRs in the current set span only about 1 order of magnitude (see Figure 2 for a visual comparison of both sets). An obvious explanation for this difference could be that the current set includes data on a smaller number of chemicals and species (seven chemicals and

three species in the present study versus 29 chemicals and 16 species in the refined McCarty et al. set²⁰). However, a large part of the variability in the literature set can already be observed within the data on one chemical in one species.²⁰ Therefore, it is most likely that the difference in data variability relates to the fact that the literature set includes a large number of studies, each having applied distinct experimental methods. Needless to say, this factor was minimized in the present work; the only methodological difference within the present study involved the dosing mode. Averaged CBR values produced through direct dissolution for 124-TCB were only a factor of 1.6 (but significantly; *t*-test, $\alpha = 0.05$) higher than those generated using passive dosing.

A comparison of the aqueous exposure concentrations measured in the direct dissolution and passive dosing systems (Table S6) shows that the concentrations in the former were roughly equal to those in the lowest passive dosing groups. For both dosing modes no depletion of the aqueous phase was observed during the test. Interestingly, animals in the direct dissolution systems died after 5–10 h, whereas eight out of nine in the passively dosed systems survived. Although at present we cannot explain this observation, it should be noted that the differences in CBRs that do result from both dosing modes are minor. Therefore, the present data set does likely allow a more reliable analysis of the true variability in CBR values than would be possible based on data originating from various sources.

Membrane Lipid-Based CBR Values. As mentioned above, the evaluation of wet weight-based CBRs is not fully appropriate, because there is no direct relation between body weight and the volume or mass of the target compartment for narcotic action. For this reason, earlier studies have already advocated that the concentration in the cell membrane may be a better exposure measure for narcosis than a whole body or total lipid-based CBR.^{15,22,23} According to these studies, membrane-based CBRs also address the observation that wet weight-based CBR values for polar chemicals are lower than those for nonpolar chemicals.

This phenomenon is also demonstrated by the current data set (see Table S8): generally, lower wet weight-based CBRs for 234-TCA, 2356-TeCA, and 4-C-3-MP are observed for *Hyalella* and *Poecilia* in particular. Significantly lower CBR values for 234-TCA as compared to those for 124-TCB (about a factor of 6 and 2, respectively) were recently also observed in our lab for the nematode *Caenorhabditis elegans* and *Danio rerio* embryos (Jonker; unpublished results). For *Lumbriculus* though, CBR differences between polar and nonpolar chemicals are not as pronounced. This makes sense, because the fraction membrane lipid of the total lipid is relatively high in *Lumbriculus* (see Table S5). If the lipid fraction is mainly composed of membrane lipids, both polar and nonpolar chemicals will predominantly reside within this fraction, thereby causing death at similar body burdens.

To derive a CBR that gives information about the concentration of a chemical in the target compartment at the time of death, all data were converted into membrane lipid-based concentrations (CBR_{ml}) using a simple mass balance model that considers chemical-specific distribution over membrane and storage lipids, proteins, carbohydrates, and water

$$\text{CBR}_{\text{ml}} = K_{\text{ml-w}} \times C_w =$$

$$\frac{[K_{\text{ml-w}} \times Q_{\text{total}}]}{[(K_{\text{ml-w}} \times M_{\text{ml}}) + (K_{\text{sl-w}} \times M_{\text{sl}}) + (K_{\text{prot-w}} \times M_{\text{prot}}) + (K_{\text{carb-w}} \times M_{\text{res}}) + V_w]}$$

where Q_{total} is the total amount of analyte accumulated by the organism, and M_{ml} , M_{sl} , M_{prot} , and M_{res} are the total masses of membrane and storage lipid, protein, and residue in the organism, respectively. V_w represents the volume of water. $K_{\text{ml-w}}$, $K_{\text{sl-w}}$, $K_{\text{prot-w}}$, and $K_{\text{carb-w}}$ are the partition coefficients of the chemicals between membrane lipids and water, storage lipids and water, proteins and water, and carbohydrates and water, respectively. The sizes of the different compartments in the three organisms were quantified experimentally as described in the Supporting Information. The size of the residual compartment was determined by subtracting the fractional sizes of all other compartments from the total mass. We assumed this residual compartment to consist exclusively of carbohydrates. The required partition coefficients were assumed to be represented by partition coefficients for POPC-based liposomes, triolein, BSA and glycogen, and water, respectively. These values were determined experimentally for the test chemicals (Table S5).

In Figure 1B, CBRs expressed as concentrations in membrane lipids are presented. Remarkably, the mass balance model-based conversion of the experimentally determined wet weight-based CBRs did not reduce any of the variability. On the contrary, relative standard deviations of the derived individual CBR_{ml} values are even higher than those of wet weight-based CBRs (72.5 vs 65.3%). Although for *Hyalella* and *Poecilia* the mass balance-based modeling corrects the above-mentioned differences between wet weight-based CBRs for polar and nonpolar compounds to some degree, the reverse occurs for *Lumbriculus*. This apparent lack of usefulness of the conversion may be explained by the fact that the chemical characteristics of the test compounds, and thus their sorption preferences, were not highly diverse. For BrM however, the CBR_{ml} values in *Hyalella* were markedly set apart by the conversion, stressing this chemical's deviant behavior, which is probably caused by its more specific MoA in *Hyalella*. General explanations for the poor result of the conversion for the other chemicals are (i) the model may be wrong in its assumption that all important partition processes are covered and/or that all compartments have equilibrated and (ii) the applied partition coefficients do not sufficiently represent the actual partitioning processes. For the purpose of demonstrating the latter possibility, we deliberately manipulated the model input partition coefficients for each of the species separately. By doing so, it was possible to decrease the overall relative standard deviation from more than 70 to only 38%. This proved effective in such a way that modification of the biocompartment sizes did not substantially add to the reduction in variability. Considering the agreement between current and reported values for lipid and protein content⁴² (except for the protein content in *Hyalella*, for which the experimentally determined value is considered erroneous; see Table S5), we therefore conclude that at least the compartment sizes reflect reality. For the partition coefficients, however, there are no literature values available that reflect partitioning to actual (nonpurified) biocompartments. Still, given the chemical similarity of the surrogate lipid materials used in this study and actual *in vivo* lipids, we did not expect large differences in their phase properties for chemical partitioning. When altering the partition coefficients to minimize the overall variability, the lipid-water partition coefficients only required a modification of 0.5 log units at most, indicating the expected similarity of the lipids⁴³ (see Table S9 for an indicative set of manipulated coefficients). However, the protein-water partition coefficients had to be

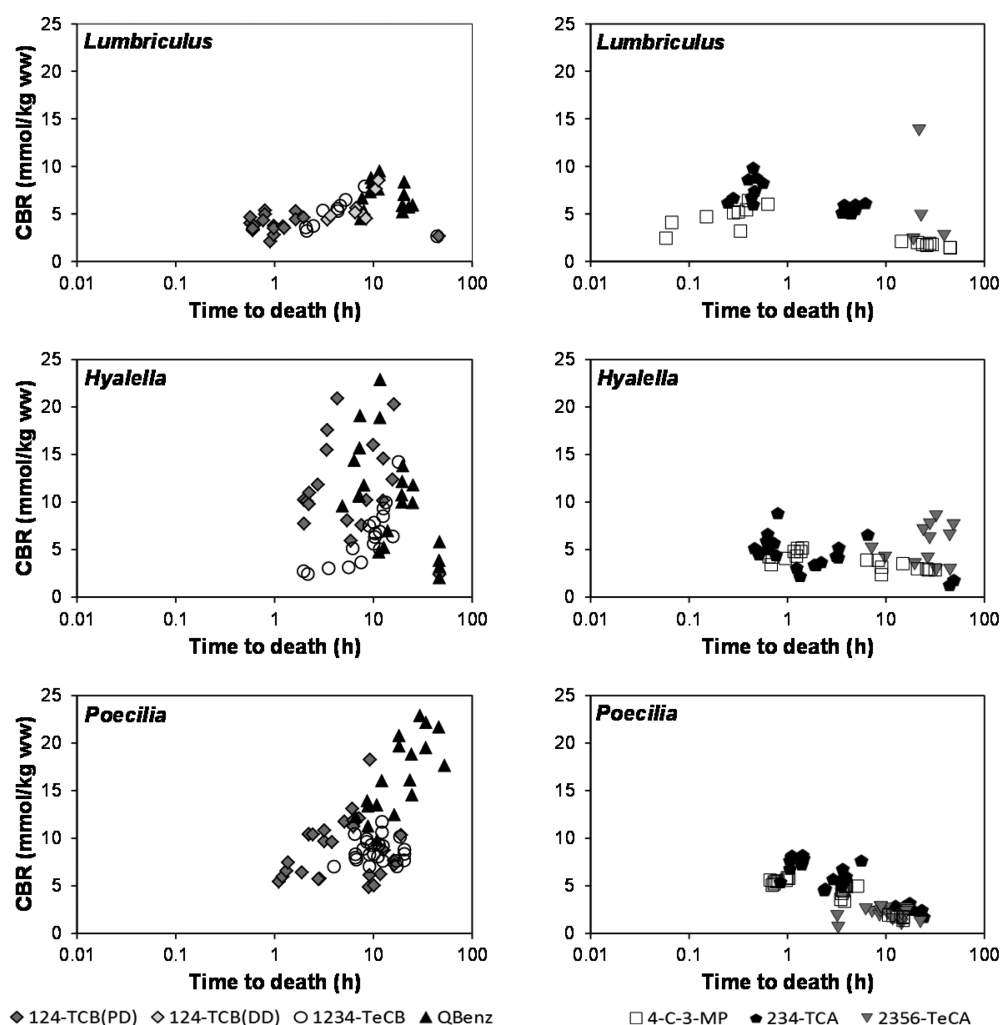


Figure 3. Wet weight-based CBR values plotted against time to death. For clarity, data were split up in a group of nonpolar (left column) and polar (right column) chemicals for all species. This also implies a division of methods, as all nonpolar chemicals were dosed by passive dosing and the polar ones by direct dissolution. The explanation of the markers is provided in the legend. Note that 124-TCB was tested with either passive dosing (PD) and direct dissolution (DD) in *Lumbriculus*.

lowered by up to 1.5 log units in order to minimize CBR_{ml} variability. This agrees with findings by Endo et al.²⁸ that indicated that structural proteins sorb neutral organic chemicals less strong than BSA does. Interestingly, manipulation of K_{carb-w} was not effective at all. Even for *Hyalella*, which may be assumed to contain more of the stronger sorbing carbohydrate chitin (see Table S5), this biocompartment did not influence the internal distribution.

Natural Variation in CBR Values? Based on the variability among CBR values measured for one chemical in a single species (i.e., the variability that cannot be addressed by any currently available mode of correction), it follows that there must be a certain degree of variation that will probably always be present within CBR data sets. Causes for such natural variation may be related to sex-specific and individual physiological differences that affect e.g., composition, ventilation rate, body size, and metabolism. In the present setup, *Hyalella* and *Poecilia* male and female animals were not separated, whereas for *Lumbriculus*, being a hermaphrodite, this aspect will not have affected the results. Nevertheless, for the worms individual CBRs could still differ by up to a factor of 6 for seemingly equal worms in equal test systems, compared to a

factor of 1.7–11.1 that occurred within all distinct species-chemical combinations.

Furthermore, variation may be introduced by a possible time-dependence of the CBR. As demonstrated in Figure 3, in some cases a time trend in the CBR data was observed. Roughly, CBR values for the nonpolar compounds tend to increase with TtD. For CBRs of polar compounds there is no obvious trend, with the possible exception of *Poecilia*, for which CBRs seem to decrease slightly with time. Similar observations have been reported by De Wolf et al.⁴⁴ for CBRs of chlorinated anilines in *Poecilia*. On the other hand, other trends have been reported as well.^{45,46}

This implies a certain ambiguity of the relationship between CBRs and TtD. Ideally, a CBR for narcosis should be a constant value, but the influence of TtD could obscure it due to a number of causes. First, in the case of very high exposure concentrations, organisms will die after a very short exposure time. This may lead to an overshoot phenomenon, where the internal concentration exceeds the CBR (basically derived for longer exposures), simply because the process from the first damage to eventual mortality takes time. This process may underlie the observation of unrealistically high CBR values, such as those measured for the highest dosing group of 4-C-3-

MP in the *Lumbriculus* series (see above), where organisms died almost instantly upon exposure. The overshoot may even have been exaggerated by the short delay between the moment of death and actual sampling, causing a further elevation of the already artificially high CBR values. Second, the MoA may change in time. If a more specific MoA, other than narcosis, would occur after a certain time (e.g., through the action of a metabolite), CBRs would decrease with time. Third, a lack of equilibrium of a chemical's internal distribution between compartments inside the organism may also cause a time-dependency of CBR values. If, for example, the uptake rate of a chemical into membrane lipids is higher than that into the whole body, the concentration in the membrane may already have reached the critical target concentration, while concentrations in other compartments (for example storage lipids) may not yet be at steady state. In this case, whole body CBRs may increase with exposure time.

All these mechanisms may have contributed to some extent to the observed minor time trends, but it is impossible to indicate one specific cause. It seems likely that both individual species characteristics (albeit biological, biochemical, or behavioral), and subtle kinetic effects (that may well be related to the former), simultaneously affect the body burden in the progress of an experiment. As a result, the variability remaining in any CBR data set that has been produced under well-controlled experimental conditions is unavoidable and may, as suggested by the current *individual* values, cover a range of as much as 1.6 log units. In our case, the range within one species exceeded that within one chemical (a factor of 6.5–39.1 against 3.2–23.5, respectively). Perhaps this should be considered “natural” variability caused by the complex interplay of yet elusive processes and should be accepted as such. Nevertheless, it will be interesting to observe whether this particular variability persists in future studies. The current data suggest that the “real” uncertainty is roughly equal for CBRs measured for a single chemical in a single species and for those relating to multiple chemicals in diverse species. Paradoxically, this observation implies that the theoretical concept of a single CBR that is unifying among all narcotic toxicants and species holds, even though CBRs are inevitably operationally defined.

■ ASSOCIATED CONTENT

■ Supporting Information

Listings of chemical properties, schematic, and photographic presentations related to the CBR experiments, passive dosing kinetics, partition coefficients, species composition, aqueous exposure concentrations, and CBR values. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

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