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Baseline Toxic Mixtures of Non-Toxic Chemicals: "Solubility Addition" Increases Exposure for Solid Hydrophobic Chemicals

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Supporting Information

ABSTRACT: This study addresses the question whether hydrophobic organic chemicals exerting no toxicity at their solubility limit (saturation) can form a toxic mixture. Spiking methods generally do not allow testing exactly at saturation without introducing micro-crystals. Passive dosing was thus applied to test the acute toxicity of several high melting point PAHs and their mixtures at the respective saturation levels to aquatic and terrestrial invertebrates. With the aquatic *Daphnia magna*, anthracene, chrysene, and benzo(a)pyrene resulted in no or limited acute toxicity (0−20%), whereas binary and tertiary mixtures of these resulted in significant acute toxicity (70−88%). Toxicity of PAHs and their mixtures could be fitted with one (sum) chemical activity-response curve in accordance with a similar mode of toxic action (i.e., concentration addition). The effective chemical activity (Ea-50) of 0.029 and the effective concentration on a lipid basis ($EC_{lipid,\ eq.}$-50) of 95.7 mM were well within the range for baseline toxicity. Similar mixtures showed less toxicity to the terrestrial *Folsomia candida* due to steady-state body-burdens being below equilibrium partitioning levels. The results of the present study raise questions about the focus of risk assessment schemes and toxicity testing guidelines on individual substances, since apparently non-toxic chemicals might become toxic in a mixture.

INTRODUCTION

The ecotoxicity testing of hydrophobic organic chemicals is technically challenging and requires special attention with regards to introduction of the test chemical(s) into the test medium, maintaining constant exposure concentrations throughout the test and analytically confirming these.[1−3] Due to the low aqueous solubilities of hydrophobic organic chemicals, one immediate question is often whether a chemical is able to exert a given type of toxicity at all when tested at its solubility limit, that is, saturation.

Passive dosing is then a practical way to provide this maximum exposure level, and to maintain it throughout the toxicity test.[4−9] Here, equilibrium partitioning from a saturated polymer donor is used to control the freely dissolved concentration of the chemical at its solubility limit, which is equivalent to testing at exactly its maximum chemical activity (a_{max}). Chemical activity quantifies the energetic level of a compound relative to that of a chosen reference state, with differences in chemical activity driving spontaneous processes including diffusive uptake.[10] In the notation used in this study the pure (subcooled) liquid is the reference state, and chemical activities are defined between 0 and 1. In contrast to a compound in the liquid state (a_{max} = 1), the a_{max} of a solid compound is lower than 1 due to the energetic cost of liberating the molecules from the crystal lattice.[10] Passive dosing from a saturated polymer has recently been applied in toxicity tests with terrestrial and aquatic invertebrates, where the toxicities of 10 polycyclic aromatic hydrocarbons (PAHs) were tested at exactly their respective solubility limits.[7,8] Lethality or immobilization data were plotted against the a_{max} of the PAHs, yielding two data series, which could each be fitted with a single chemical activity-response curve. The resulting effective chemical activities for 50% immobilization (Ea-50s) were 0.036 for the aquatic water flea *Daphnia magna* and 0.058 for the terrestrial springtail *Folsomia candida*. These are within the reported range of chemical activities of 0.01−0.1 observed for the onset of baseline toxicity, that is, narcosis.[10−13] Those PAHs with the highest melting points had a_{max} values that were near to or below 0.01, and no acute toxicity was observed for these compounds even at their saturation level. This absence of toxicity can be explained by a "melting point

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Table 1. Overview of the PAH Exposure Treatments for the *Daphnia magna* Toxicity Experiments from This Study As Well As Smith et al.[8a]

treatment	log K_{OW}	aqueous solubility (μg L^{-1})	$(\sum)a_{max}$	experiment 1	experiment 2	Smith et al (2010)
anthracene	4.85	46	0.011		X	X
pyrene	5.39	137	0.048	X		X
benzo(a)anthracene	6.01	11	0.038	X	X	X
chrysene	6.06	1.9	0.004		X	X
benzo(a)pyrene	6.70	1.6	0.024		X	X
ant+pyr+B(a)A+chry+B(a)P			0.126	X		
ant+B(a)A+chry+B(a)P			0.077	X		
ant+chry+B(a)P			0.039	X	X	
ant+B(a)P			0.035		X	

[a]The log K_{OW} values were calculated for 20°C using SPARC (http://archemcalc.com/sparc, accessed 2012.07.20). The aqueous solubility values are the mean generator-column values from de Maagd et al (1998).[23] The (sum) maximum chemical activities ($(\sum)a_{max}$) were calculated for 20°C according to Yalkowsky et al (1979).[35] PAH names in the mixtures are abbreviated as anthracene: ant; pyrene: pyr; benz(a)anthracene: B(a)A; chrysene: chry and benzo(a)pyrene: B(a)P.

cut-off" in toxicity that occurs when chemicals form crystals before reaching a toxic level.[12] This leads to the question, whether these apparently non-toxic compounds will also remain non-toxic when part of a mixture. This is highly relevant, since both toxicity testing guidelines and the risk assessment of chemicals mainly focus on the effects of single compounds,[14] whereas in their natural environment organisms are in fact exposed to complex mixtures of chemicals.[15−20]

The solubility of mixtures of hydrophobic organic chemicals forms the physicochemical basis of the present study. Here, it is crucial to distinguish between mixtures of substances that form a liquid mixture, and mixtures of solid chemicals that remain in the solid state when mixing them.[21] The difference is that compounds forming a liquid mixture tend to partition into each other thereby suppressing each other's solubility, whereas the solubilities of solids are approximately additive.[21] This implies that upon dissolution of a mixture of solid chemicals, the total concentrations in the exposure medium can increase substantially when going from single chemicals to mixtures. This was recognized by Sugatt et al,[22] who found that for saturated solutions derived from a mixture of solids the toxicity was equal to, or exceeded that of, a corresponding saturated solution of its most toxic component, and this tended to increase with the complexity of the mixture.

The present study goes one step further, by investigating whether chemicals that display no acute toxicity at their solubility limit when tested individually, can make up a toxic mixture. The general working hypothesis is that "non-toxic chemicals can become toxic in a mixture". The specific working hypothesis is that "high melting point, and thus low maximum chemical activity, PAHs that are individually non-toxic can start to exert acute toxicity in a mixture when reaching a sum of chemical activities of 0.01 to 0.1". Toxicity studies with mixtures of high melting PAHs were thus conducted with *D. magna* and *F. candida*. In both cases, passive dosing was applied to provide exposure of each mixture component at the solubility level. The present article mainly focuses on the experiments with *D. magna*, with the full details of the *F. candida* experiment described in the Supporting Information (SI).

MATERIALS AND METHODS

Chemicals and Materials. Autosampler vials (10 and 20 mL) with Teflon lined screw caps were bought from Mikrolab (Aarhus, Denmark). Medical grade polydimethylsiloxane (PDMS) silicone was made using the MDX4-4210 kit from Dow Corning supplied by the Institute of Anaplastology (Velten, Germany). The following PAHs were used: anthracene (99%, Acros, Belgium), pyrene (>99% Fluka, Germany), benz(a)anthracene (99%, Aldrich, Germany), chrysene (99% Cerilliant, TX) and benzo(a)pyrene (99%, Cerilliant). For cleaning, lint-free lens tissue was used (Bie & Berntsen A/S, Denmark). Methanol (HPLC grade) was used for extraction and analysis (Merck, Darmstadt, Germany). Milli-Q water was used (Super Q treated, Millipore, MA).

Casting and Loading of the Polydimethylsiloxane (PDMS) Silicone. To make the passive dosing vials, the PDMS silicone prepolymer and catalyst were mixed and 500 mg (±1%) cast into the base of each autosampler vial.[7,8] These were left overnight at 4 °C to allow any bubbles to escape, and then placed in an oven at 110 °C for 48 h to cure. The cured PDMS silicone was rinsed three times with ethanol, to remove oligomers and other impurities. Ethanol adhering to the PDMS silicone was removed by rinsing three times with Milli-Q water and the surfaces dried using lint free tissue.

The PAH single compound and mixture exposures for the two *D. magna* experiments are shown in Table 1, with two types of mixtures being prepared:

(i) Mixtures comprised of anthracene, chrysene and benzo-(a)pyrene, which individually have no or very limited acute toxicity to *D. magna*.[8] Their toxicity was measured both as single substances and in parallel as dual and triple mixtures (Experiments 1 and 2, Table 1).

(ii) A mixture of benz(a)anthracene plus the individually non-toxic anthracene, chrysene and benzo(a)pyrene (Experiment 1, Table 1), to see whether the latter contribute to the previously determined intermediate acute toxicity of benz(a)anthracene.[8]

Details of the single PAHs and mixtures used in the *F. candida* experiment are given in the SI (Table S1).

Separate methanol suspensions were made up for individual PAHs and their mixtures, and the PDMS silicone was loaded with PAHs directly in the passive dosing vials by partitioning from a 2 mL methanol suspension for a minimum period of 72 h at 20 °C.[7,8] After loading, the methanol suspension was poured off and the surfaces thoroughly wiped to remove adhering PAH crystals. Finally, the PDMS silicone was rinsed three times with 1 mL of Milli-Q water, each time for at least 1 h, to remove any adhering methanol and then wiped dry.

Two controls were included in each experiment: autosampler vials (n = 5) without PDMS silicone were used to confirm full survival under the test conditions, whereas passive dosing vials that were loaded with pure methanol (n = 5, no PAH) were used to account for effects of the PDMS silicone and the loading procedure.

PAH Toxicity to *Daphnia magna*. *Toxicity Testing.* Single clone *D. magna* (Clone K6, Pond, Kiel (Antwerp), Belgium) cultures of the same age were held in 1 L glass beakers with aerated and biofiltered tap water and each holding 20−25 individuals. These were maintained at 20 ± 1 °C with a photoperiod of 14 h light/10 h dark. The medium was renewed three times a week, and the *D. magna* fed a mixture of *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* in a 3/1 ratio (4×10^5 cells mL^{-1}).

After loading 20-mL passive dosing vials with PAH(s) (n = 5 for each treatment, see Table 1), each vial was filled with 10 mL standardized OECD water prepared according to Annex 2 in OECD Guideline 203 ($CaCl_2 \cdot 2H_2O$, 2 mM; $MgSO_4 \cdot 7H_2O$, 500 μM; $NaHCO_3$, 771 μM; KCl, 77.1 μM; water hardness, 250 mg $CaCO_3$; pH 7.8). The vials were vigorously shaken and left for 24 h to ensure equilibrium partitioning was reached with the overlying water.[8] Toxicity tests were started by adding *D. magna* neonates between 0 and 24 h old at a density of one organism mL^{-1} (i.e., 10 individuals per vial), and maintaining these at 20 ± 1 °C with a photoperiod of 14h light/10h dark. Immobilisation of individuals was measured at 2, 4, 24, and 48 h by counting those animals that were not able to swim within 15 s after gentle agitation of the test container. Graphpad Prism 5 (San Diego, CA) was used to fit the *D. magna* toxicity data with a sigmoidal exposure−response curve with variable slope.

Details of the toxicity test with *F. candida* are given in the SI.

Exposure Confirmation. At test completion, the exposure medium was poured off, all surfaces cleaned with lint-free tissue and the vials rinsed three times with <1 mL volumes of Milli-Q water. One mL of Milli-Q was added to each passive dosing vial and these left to stand in the dark at 20 °C for at least 48 h before measuring the equilibrated aqueous concentrations of PAHs. These represent the freely dissolved PAH concentrations in equilibrium with the PDMS silicone at test completion, and 500 μL of equilibrated water were mixed with 500 μL of methanol for analysis.

HPLC Analysis. PAH analysis was by HPLC with fluorescence detection (Agilent 1100 HPLC equipped with a G1321A FLD operated at Ex: 260 nm and Em. 350, 420, 440, and 500 nm). Injection was of 30 μL sample at 28 °C, and the PAHs separated on a CP-EcoSpher 4 PAH column (Varian Inc., Palo Alto, CA), using methanol and water as the mobile phase at a flow rate of 0.5 mL min^{-1}.[8] PAH concentrations in the samples were quantified using a nine-point external standard calibration curve. Signal integration was performed using the HP Chemstation software (B.03.01, Agilent Technologies, Palo Alto, CA).

RESULTS AND DISCUSSION

Passive Dosing Quality Control. In the control vials without silicone, only a single *D. magna* individual in one of the replicates from experiment 1 did not survive. All individuals in the passive dosing vial controls from both experiments 1 and 2 survived after 48 h exposure. This demonstrates the suitability of the passive dosing vial format, as well as the loading and cleaning procedures, for aquatic toxicity tests with small invertebrates such as *D. magna*.

Reproducibility of the measured immobilization between experiments performed on separate occasions and with different *D. magna* cultures was good. Benz(a)anthracene toxicity has been measured in experiments 1 and 2 from this study as well as in Smith et al,[8] and the percent immobilization was similar at 82 (Relative standard deviation (RSD) 13%), 98 (RSD 5%) and 86 (RSD 10%), respectively. In Figure 1, the *D. magna* immobilization after 48 h exposure to PAHs with limited toxicity, as well as to mixtures of these, is shown for experiments 1, 2, and Smith et al.[8] Again, between different experiments reproducibility of the observed toxicity is good, both for single PAHs and their mixtures. Therefore, passive dosing allows for the reproducible determination of the acute toxicity to *D. magna* for individual hydrophobic compounds and their mixtures. In part, this is due to the improved exposure control with passive dosing compared to spiking for example.

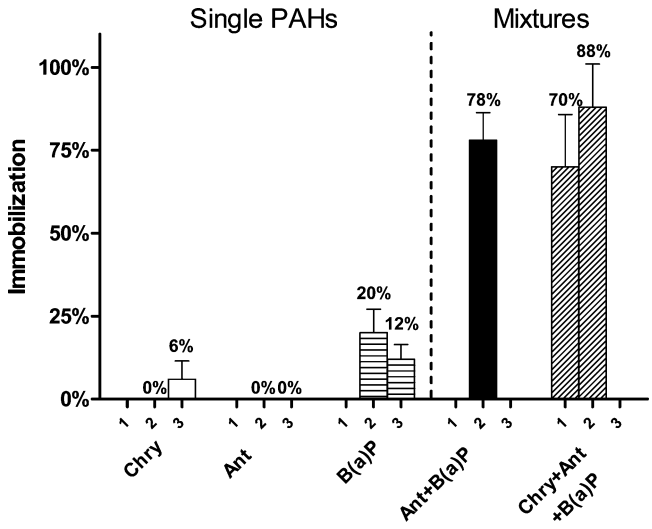

Figure 1. Immobilization of *Daphnia magna* after 48 h exposure to PAHs with no or limited acute toxicity, and mixtures comprised solely of these (1: experiment 1, 2: experiment 2, and 3: Smith et al.[8]). The mean percentage immobilization is shown above the corresponding bar, and where a treatment was not tested in an experiment this has been left blank. Error bars represent the standard deviation of the five replicates. PAH names are abbreviated as chrysene: chry; anthracene: ant and benzo(a)pyrene: B(a)P.

Solubility of Single PAHs and Their Mixtures. SI Figure S1 shows the exposure confirmation results for the two *D. magna* experiments. Final equilibrium concentrations for all PAHs were close to their respective literature aqueous solubilities,[23] confirming the animals were exposed at (or very near to) the saturation level. Benzo(a)pyrene concentrations showed a slightly higher variability, with final measured concentrations ranging between 0.9 and 2.4 $\mu g\ L^{-1}$. The concentrations which were slightly elevated above aqueous solubility were probably due to small amounts of sorbing biomaterial such as organic material excreted by the *D. magna* during the assay remaining in the vials despite the cleaning procedure. However, since passive dosing is based on partitioning between the saturated PDMS silicone and water, freely dissolved exposure concentrations will still have been at benzo(a)pyrene aqueous solubility.

For all PAHs, the measured final equilibrium concentrations of the different PAHs were similar either when tested as single compounds or in a mixture (SI Figure S1). This confirms that

the solubility of the PAHs in the mixtures did not influence one another, and thus that the solubility was additive. The measured final concentrations for the single and mixture PAH treatments are summed in Figure 2, where it can be seen that the aqueous

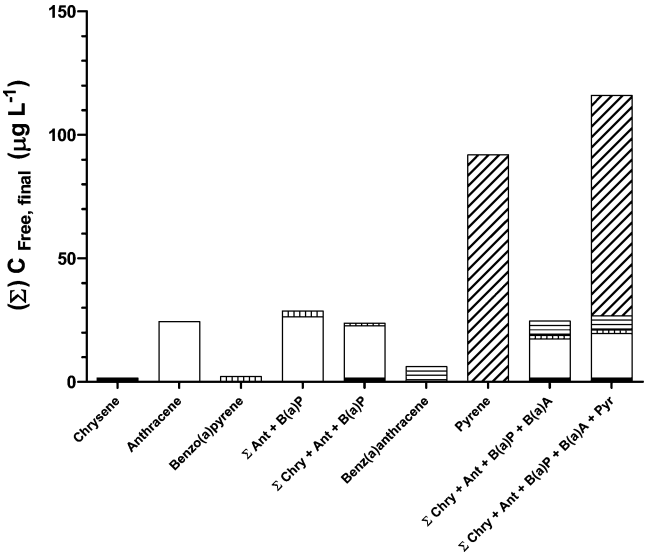

Figure 2. (Sum) final freely dissolved concentrations ($(\sum)C_{free,\ final}$) of the single PAHs and their mixtures as measured during the exposure confirmation step. Mean measured values of $C_{free,\ final}$ from experiments 1 and 2 have been used, except for the chrysene concentrations which were below method detection limits and where the values were taken from de Maagd et al.[23] PAH names in the mixtures are abbreviated as chrysene: chry; anthracene: ant; benzo(a)pyrene: B(a)P; benz(a)-anthracene: B(a)A and pyrene: pyr.

solubilities of individual PAHs in the mixture treatments are indeed additive. Therefore, total freely dissolved concentrations in the exposure medium increased when going from single chemicals to increasingly complex mixtures of up to five PAHs, analogously to that found by Banerjee et al.[21]

Toxicity of Mixtures Comprised Solely of Non- or Limited Toxic PAHs. Figure 1 shows the results of the *D. magna* toxicity tests after 48 h exposure to anthracene, chrysene and benzo(a)pyrene, as well as to mixtures of these. The data come from the two experiments performed in this study, as well as for single PAHs from Smith et al.[8] At aqueous solubility, anthracene resulted in no immobilization in two different experiments, whereas chrysene resulted in only a limited immobilization of 0% (RSD 0%, experiment 2 from this study) and 6% (RSD 91%, from Smith et al).[8] Benzo(a)pyrene resulted in a slightly higher immobilization of 20% (RSD 35%, experiment 2 from this study) and 12% (RSD 37%, Smith et al).[8]

However, when anthracene (no immobilization) and benzo-(a)pyrene (12 and 20% immobilization) were combined, the acute toxicity of the mixture markedly increased to 78% (RSD 11%). This provides the first compelling indication that two compounds with limited acute toxicity at their respective aqueous solubilities, can in fact result in a very strong effect when combined. Further addition of chrysene to a mixture of anthracene and benzo(a)pyrene resulted in a further increase in immobilization (Figure 1). In experiment 2, immobilization was 78% (RSD 11%) as a result of exposure to the binary mixture of anthracene and benzo(a)pyrene, whereas exposure to a mixture additionally containing chrysene resulted in an increase in immobilization to 88% (RSD 15%). Therefore the data from these experiments confirm the general working hypothesis that "non-toxic chemicals can become toxic in a mixture".

Toxicity of Mixtures of Partly Toxic and Non-toxic PAHs. The ability of partly and non-toxic PAHs to contribute to the toxicity of a mixture is also shown by comparing the toxicity of benz(a)anthracene at aqueous solubility to that of a mixture additionally containing anthracene, chrysene and benzo(a)pyrene. For benz(a)anthracene alone, 82% (RSD 13%) immobilization was observed in experiment 1 (Figure 3). However, in a mixture additionally containing anthracene, chrysene and benzo(a)pyrene immobilization was increased to 100% (RSD 0%) (Figure 3). Although this experiment does not elucidate which of the three PAHs dominated this increase in toxicity, it nevertheless shows that non- or limited toxic PAHs additionally contribute to acute toxicity. Adding pyrene to a mixture comprising the above four PAHs similarly resulted in full immobilization (Figure 3), as of course would be expected.

Acute Toxicity As a Function of Chemical Activity. In Figure 3A, the immobilization after 48 h exposure to the single PAHs and their mixtures from experiments 1 and 2 are plotted against the respective (sum) maximum chemical activities ($(\sum)a_{max}$). For comparison, the sigmoidal activity-response curve from fitting the measured immobilization data for 10 PAHs at a_{max} (i.e., aqueous solubility) from Smith et al.[8] is shown. Measured immobilization upon exposure to the PAH mixtures (and indeed the single PAHs) of experiments 1 and 2 fitted well to this activity-response curve, with the onset of acute toxicity occurring within the previously reported range in chemical activities of 0.01−0.1.[10−13] The anthracene/benzo(a)pyrene and chrysene/anthracene/benzo(a)pyrene mixtures had $\sum a_{max}$ values of 0.035 and 0.039, respectively. These fall within this 0.01−0.1 range, supporting the specific working hypothesis that "high melting point PAHs that are individually non-toxic can start to exert acute toxicity in a mixture when reaching a sum of chemical activities of 0.01−0.1". Fitting an activity-response curve to the *D. magna* immobilization data after 48 h exposure to the single PAHs and their mixtures from this study (Figure 3A) resulted in an effective chemical activity for 50% immobilization (Ea-50) of 0.029 (r^2 = 0.94). This is similar to the Ea-50s from Smith et al.[8] of 0.019 (r^2 = 0.98; naphthalene activity-response), 0.036 (r^2 = 0.99; 10 single PAHs at saturation) and 0.090 (r^2 = 0.96; phenanthrene activity-response). The importance of exposure duration for toxicity is evident from a comparison of immobilization after exposure for 24 h (SI Figure S2) or 48 h (Figure 3A). Internal exposure concentrations and target concentrations will increase with time (toxicokinetics), and the toxic action will also require time (toxicodynamics). However, because of the toxic mode of action (acute and narcosis), toxicodynamics are fast and reversible, and therefore, toxicokinetics will largely explain the observed effects.[24]

Acute Toxicity As a Function of Equilibrium Lipid Concentrations. In Figure 3B, the a_{max} values for each PAH have been converted into equilibrium partitioning concentrations in lipids ($(\sum)C_{lipid,eq.}$; mmol L lipid^{-1}). This was done by dividing the a_{max} values for each PAH (see Table 1) by the respective chemical activity coefficient in lipid as determined by Mayer et al,[25] and for the case of the mixtures summing the $C_{lipid,eq.}$ values. Although for some PAHs the actual concentrations in the lipids might be below equilibrium partitioning levels due to bioconcentration kinetics and metabolism, the onset of acute toxicity nevertheless occurred within a narrow

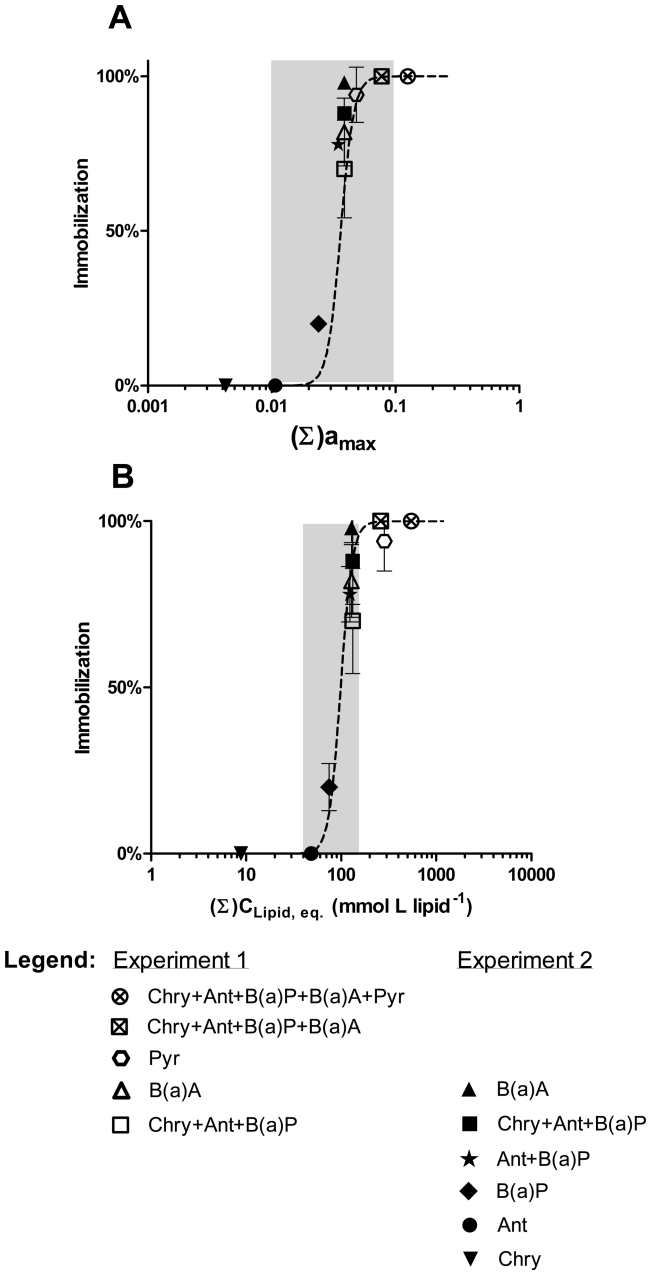

Figure 3. Percent immobilization of *Daphnia magna* after 48 h exposure to single PAHs and their mixtures plotted against (A) (sum) maximum chemical activity ($(\sum)a_{max}$) and (B) calculated (sum) equilibrium lipid concentrations ($(\sum)C_{lipid,\,eq.}$). The gray areas show the chemical activity range (0.01−0.1, A) and the membrane burden range (40−160 mmol L lipid^{-1}, B) for the onset of baseline toxicity.[10−13,26] The dashed line in A shows the activity-response curve from Smith et al.[8] In B, the dashed line shows the equivalent exposure-response curve when the immobilization data from Smith et al.[8] are plotted against $C_{lipid,\,eq.}$ values calculated from the respective a_{max} values (see text for details). Error bars represent the standard deviation of five replicates and are sometimes smaller than the symbols. PAH names are abbreviated as chrysene: chry; anthracene: ant; benzo(a)pyrene: B(a)P; benz(a)anthracene: B(a)A and pyrene: pyr.

range, and furthermore within the reported critical membrane burden range for baseline toxicity of 40−160 mmol kg lipid^{-1} (assuming a 1:1 conversion of L to kg lipid).[26] Thus, the observed toxicities seem to be in general agreement with baseline toxicity as being the mode of action. Fitting a concentration−response curve to the 48 h immobilization data of the single PAHs and mixtures from this study (Figure 3B) resulted in a lipid-based $EC_{lipid,eq.}$-50 of 95.7 mmol L^{-1} lipid (r^2 = 0.93). Again, this is similar to the $EC_{lipid.eq.}$-50 of 98.9 mmol L^{-1} lipid (r^2 = 0.99) after 48 h exposure to 10 single PAHs at saturation obtained by plotting the immobilization results versus $C_{lipid,eq.}$ calculated using the data from Smith et al.[8]

Using activity coefficients to calculate the equilibrium lipid concentration has the advantage that it directly translates the chemical activity set by the PDMS loading procedure into a lipid concentration. Another approach for calculating the equilibrium concentrations in the lipids is by applying liposome to water partition ratios. This approach has the advantage that liposomes are probably a better model for the membrane lipids serving as the site of toxic action for baseline toxicity, but of course requires accurate partition ratios and aqueous solubility data. Equilibrium partition ratios between POPC-liposomes and water have been determined for PAHs at 20 °C.[27] These were multiplied by the respective PAH aqueous solubilities[23] to give the equilibrium lipid concentrations, with these shown in SI Figure S3. Although similar to the equilibrium lipid concentrations calculated using activity coefficients, these were consistently higher by a factor of 1.3−2.2 and this might perhaps be partly due to the choice of specific phospholipid bilayers. Once again, the onset of acute toxicity for single PAHs and their mixtures occurred within the 40−160 mmol kg lipid^{-1} critical membrane burden range, albeit closer to the upper limit (SI Figure S3).

Contributions of the Individual PAHs. The contributions of the individual PAHs to $(\sum)a_{max}$ or $(\sum)C_{lipid,eq.}$ are shown in Figure 4A and B, respectively. Their contributions are similar when expressed on either a chemical activity or lipid basis, as follows from the narrow factor of 2.8 in the range of lipid activity coefficients determined for these PAHs.[25] Furthermore, a comparison of Figures 2 and 4 shows that those PAHs with the highest aqueous solubilities, and therefore highest freely dissolved concentrations, do not make a proportional contribution to either $\sum a_{max}$ and $\sum C_{lipid,eq.}$. For example, in the binary mixture of anthracene and benzo(a)pyrene, their respective contributions to $\sum C_{free,final}$ are 92 and 8%. However, their respective contributions to $\sum C_{lipid,eq.}$ are 39 and 61%. This of course reflects the different compound hydrophobicities, driving partitioning into the lipids.

Acute Toxicity of Low a_{max} PAHs and Their Mixtures to *Folsomia candida*. All individuals in the control passive dosing vials were alive at day 7. SI Figure S4 shows the exposure confirmation results with the final equilibrium concentrations for all PAHs lying close to their respective literature aqueous solubilities.[23] Therefore, the animals were exposed at (or very near to) the saturation level. Furthermore, the final equilibrium concentrations of each PAH in the different treatments were all similar, confirming that the solubility of the PAHs in the mixtures did not influence one another, and thus that the solubility was additive. SI Figure S5 shows the percent immobilization of *F. candida* after 7 days exposure to single PAHs and their mixtures at $(\sum)a_{max}$ using a passive dosing setup for terrestrial invertebrates.[7,28] Mean *F. candida* immobilization percentages when exposed to chrysene, anthracene and benzo(a)pyrene were 2, 9, and 6%, respectively (SI Figure S5). Therefore, exposure to those PAHs with the lowest a_{max} values did not result in appreciable toxicity, similar to what was found for *D. magna*. However, in contrast to *D.*

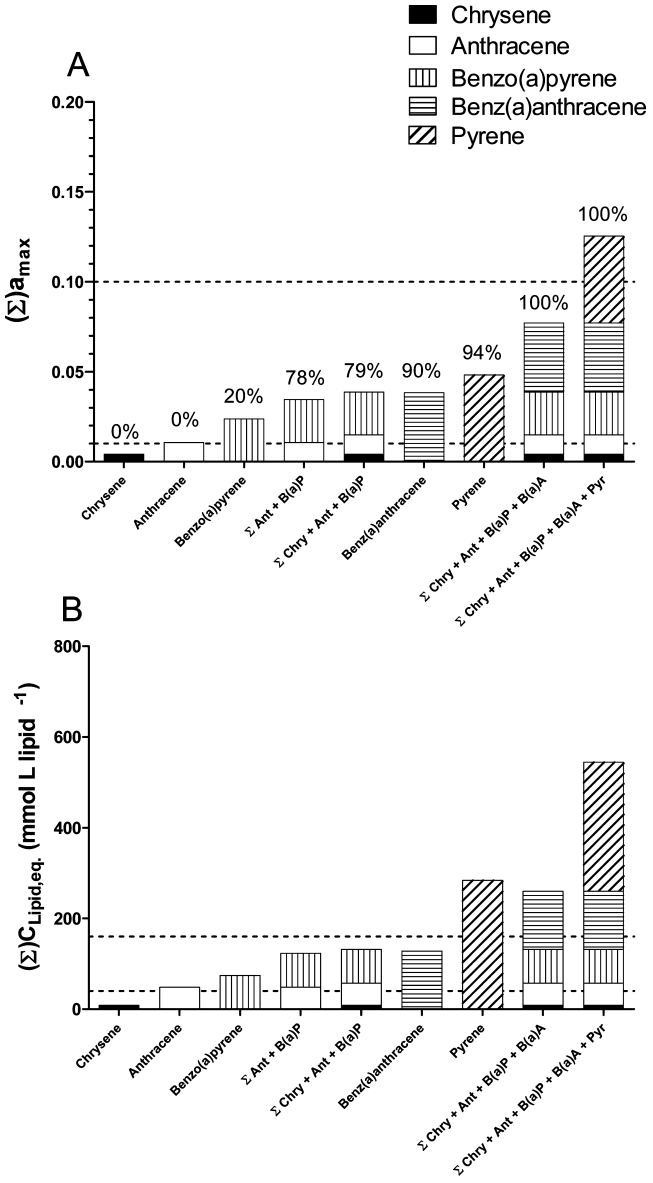

Figure 4. (Sum) maximum chemical activities ($(\sum)a_{max}$) (A) and calculated (sum) equilibrium lipid concentrations ($(\sum)C_{lipid,\,eq.}$) (B) of the single PAHs and their mixtures. The dashed lines show the chemical activity range (0.01−0.1, A) and the membrane burden range (40−160 mmol L lipid^{-1}, B) for the onset of acute toxicity.[10−13,26] The percentages above the columns in (A) are the (mean) immobilization from experiments 1 and/or 2. PAH names in the mixtures are abbreviated as chrysene: chry; anthracene: ant; benzo(a)pyrene: B(a)P; benz(a)anthracene: B(a)A and pyrene: pyr.

magna, no toxicity of benz(a)anthracene (0% immobilization) and a much reduced toxicity of pyrene (23% immobilization) were observed with *F. candida* (SI Figure S5). Furthermore, all mixtures without pyrene also resulted in low immobilization (range 0−12%, SI Figure S5), this despite the values of $\sum a_{max}$ being in the 0.01−0.1 range expected for the onset of acute toxicity.[10−13] In the meantime, bioconcentration and toxicity experiments with *F. candida* have revealed that the observed non-toxicity of several of the higher molecular weight PAHs is partly explained by lower steady-state rather than higher equilibrium partitioning concentrations in *F. candida* being reached after 7 days, perhaps due to detoxification and excretion.[28] However, a closer inspection of the data indicates that even PAHs which individually have a minimal acute toxicity, can contribute to the toxicity when part of a mixture. Pyrene alone resulted in 23% immobilization. However, when pyrene was combined with chrysene, anthracene, benzo(a)-pyrene and benz(a)anthrance (0−9% range in immobilization when tested individually at saturation), the immobilization increased to 77% (SI Figure S5).

Application of Chemical Activity within Concentration Addition. The suitability of $(\sum)a_{max}$ as a metric for describing the exposure to PAH mixtures is evident from the observation that toxicities of several individual PAHs and their mixtures could be fitted to just one activity-response curve (Figure 3A). This is in agreement with the well-established concept of concentration addition for describing the toxicity of mixtures of chemicals with a similar mode of action.[29,30] Furthermore, the onset of toxicity observed here is in agreement with baseline toxicity initiating within a rather narrow range of chemical activities.[10−13] Note that the conventional approach to describe and predict concentration addition using toxic units could not be applied in the present study. This is because toxic units cannot be determined for compounds that are individually non-toxic, and thus their contribution to the total toxicity cannot be taken into account.

Wider Implications. Over the past decades, major effort has been devoted to determining the toxicity of individual congeners, isomers, or enantiomers rather than of complex mixtures and products. In some cases this has meant that the process of separating these mixtures into the individual components has precluded observations of toxicity. For example, in the 1970s commercial PCB mixtures were found to be acutely toxic at low concentrations.[31] However, as individual PCB congeners were identified, and these became increasingly pure, it became more and more difficult to experimentally observe this acute toxicity.

The observation that hydrophobic organic compounds not exerting acute toxicity at saturation can still contribute to the total toxicity of a mixture is rather fundamental. Although mixture toxicity is considered in specific cases such as workplace exposure or pesticide residues in food and feed,[32] most regulatory frameworks for chemicals still focus on the testing of single substances (e.g., the European Union's REACH legislation),[14] and here such chemicals will be categorized as being non-toxic from acute toxicity tests. However, when exposure is to mixtures containing these chemicals (as is the case in the environment), these might in fact contribute to the overall toxicity. Additionally, a number of efforts aim at using QSAR approaches to derive cut-offs for acute toxicity to reduce testing efforts in order to minimize animal numbers and testing costs.[33] Currently, the classification of a chemical as being toxic or non-toxic is based on the testing results from individual substances, but the findings of this study raise questions concerning this approach. How can one classify a compound that individually displays no acute toxicity even at saturation, but contributes to toxicity when part of a mixture? Nevertheless, as is also clear from the toxicity results from the *F. candida* experiment, whether a hydrophobic compound contributes to mixture toxicity also depends on factors additional to its intrinsic toxicity, such as the uptake kinetics and metabolism. Future experimental efforts should therefore be aimed at understanding these aspects more fully.

This study focused on solid hydrophobic organic chemicals with high melting points, which by themselves resulted in no or limited toxicity. When mixtures of such compounds come into

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contact with water, the individual components dissolve independently and the solubilities are additive. This leads to an increased exposure and to mixture toxicity for *D. magna*, which clearly answers the question posed by Mayer and Reichenberg[12] as to whether apparently non-toxic solid chemicals can form toxic mixtures. The situation is fundamentally different for liquid mixtures, where individual compounds partition into each other thereby suppressing each other's solubility.[21] For liquid mixtures of liquid substances, the findings of the present study thus do not apply due to the lack of additive solubilities. For liquid mixtures of solid substances, such as Aroclor mixtures of PCBs and tars containing PAHs, the situation is slightly more complicated. They have certainly the potential for increased mixture exposure and toxicity relative to the individual components, but Raoult's law will set an upper limit for mixture exposure and toxicity. Finally, it should be mentioned that although this study focused on acute toxicity, the implications are also relevant for other types of toxicity. The lack of a specific type of toxicity for a single compound, does not per se imply a lack of toxicity when part of a mixture. As an example, when endocrine disrupting compounds present at concentrations below the no-effect level are mixed, significant endocrine activity can result. [34]

■ ASSOCIATED CONTENT

Supporting Information

Figures show exposure confirmation for the *Daphnia magna* experiments, *D. magna* immobilization after 24 h plotted against (sum) maximum chemical activity, *D. magna* immobilization after 48 h plotted against equilibrium lipid concentrations calculated using aqueous solubilities and lipid to water partition ratios, exposure confirmation for the *Folsomia candida* experiment and *F. candida* immobilization after 7 days plotted against (sum) maximum chemical activity. Table S1 gives the PAH exposure treatments for the *F. candida* experiment. Finally, the full details of the *F. candida* experiment are described. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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