Emergent synergistic lysosomal toxicity of chemical mixtures in molluscan blood cells (hemocytes)

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Abstract

The problem of effective assessment of risk posed by complex mixtures of toxic chemicals in the environment is a major challenge for government regulators and industry. The biological effect of the individual contaminants, where these are known, can be measured; but the problem lies in relating toxicity to the multiple constituents of contaminant cocktails. The objective of this study was to test the hypothesis that diverse contaminant mixtures may cause a greater toxicity than the sum of their individual parts, due to synergistic interactions between contaminants with different intracellular targets. Lysosomal membrane stability in hemocytes from marine mussels was used for *in vitro* toxicity tests; and was coupled with analysis using the isobole method and a linear additive statistical model. The findings from both methods have shown significant emergent synergistic interactions between environmentally relevant chemicals (i.e., polycyclic aromatic hydrocarbons, pesticides, biocides and a surfactant) when exposed to isolated hemocytes as a mixture of 3 & 7 constituents. The results support the complexity-based hypothesis that emergent toxicity occurs with increasing contaminant diversity, and raises questions about the validity of estimating toxicity of contaminant mixtures based on the additive toxicity of single components. Further experimentation is required to investigate the potential for interactive effects in mixtures with more constituents (e.g., 50 –100) at more environmentally realistic concentrations in order to test other regions of the model, namely, very low concentrations and high diversity. Estimated toxicant diversity coupled with tests for lysosomal damage may provide a potential tool for determining the toxicity of estuarine sediments, dredge spoil or contaminated soil.

Two line capsule of paper:

Synergistic interactions have been observed in mixtures of toxic chemicals and relatively non-toxic chemicals increase the toxicity of the mixture. Toxicity increases with chemical diversity.

Key words: *complex pollutant mixtures; effect isobole; molluscan hemocytes; lysosomal membrane stability; neutral red retention test; synergistic interactions*

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Introduction

In the past, the problem of chemical contamination in the environment has generally been addressed in terms of chemical characterisation of micropollutants such as polycyclic aromatic hydrocarbons, organochlorines, pesticides (Cassee et al., 1998; European Commission, 2011; LeBlanc & Olmstead, 2004; Readman, 1996; Readman et al., 1986, 1992a & b, 1993a & b; Smith et al., 2013; Tolosa & Readman, 1996; Tolosa et al., 1996, 1997). However, chemical analysis only provides limited windows into a very complex mixture often believed to contain anything from 1000 to >100,000 compounds. Such analyses are very expensive and provide no direct information on harmful effects. However, effective measurement of direct toxicity *in situ* is now possible and has been applied increasingly to earthworms, fish and shellfish (Sforzini et al., 2015; Koehler et al., 1992; Lowe et al., 1992; Moore, 1988). The major difficulty has been to relate toxicity in the real environment to the chemicals believed to be present in soils, sediments, effluents and dredge spoils. At present Toxicity Identification and Evaluation (TIE) method probably offers the best option (Mount & Anderson-Carnahan, 1988). First introduced by the US Environmental Protection Agency, TIE uses various procedures to fractionate the toxins within a sample. Bioassays and high level fractionation are used to determine causative agents and quantitative high resolution GC–MS or LC–MS analysis is then used to investigate the fractions producing the greatest toxicity. However, in many environmental situations there is limited knowledge of which toxic chemicals are actually present, as well as their physical chemical speciation and bioavailability, and this can impose a level of uncertainty on attempts to predict the toxic effects on the biota and potential human health impact (e.g., through consumption of seafoods).

Assessing the harmful impact of mixtures in the environment and food is a major concern to regulators (McCarty & Borgert, 2006; Bringholf et al., 2007; Cedergreen, 2014; Kienzler et al., 2016; Kortenkamp et al., 2009; Sarigiannis & Hansen, 2012; Tallarida, 2012, 2016; Tang et al., 2014). Attempts have been made to relate measured harmful endpoints (e.g., pathology, mortality) to data on the toxicity of individual constituents of the mixture (Doi, 1994). However, success has been limited in this respect and the hypothesis that the "toxicity of a complex mixture is simply the summation of the toxicity of its individual constituents" is now treated with some scepticism, since in this model there is no accounting for emergent interactive effects (Sahai, 1997; Fig. 1). For instance, there are numerous instances of synergistic interactions, even in simple mixtures, of drugs used in medical therapeutics (Di Dodato & Sharom, 1997; Kanazawa et al., 1997; Piras, et al., 1997; Tallarida, 2012; Valenti et al., 1997). There is also evidence for this type of emergent effect

in estrogenic effect of mixed pollutants (Ashby et al., 1996; Kortenkamp & Altenburger, 1998), and in the synergistic interactive effects of non-toxic sucrose polyester, a zero-calorie cooking food additive and the polycyclic aromatic hydrocarbon anthracene (Moore et al., 1997).

Nevertheless, confounding factors include the lack of information about which chemicals are actually present as mentioned above, as well as their concentrations and toxicities (Smith et al., 2013). Furthermore, in a complex mixture situation where the chemicals are often associated with particle surfaces or lipid-rich coatings of particles, the probability of catalytic reactions occurring to generate new compounds will be increased, since reactions such as oxidative changes will readily take place in a two dimensional environment (i.e., surfaces) where the chemicals are highly concentrated (Fig. 1; Li et al., 2017).

In essence, this situation presents a major problem and challenge for ecotoxicology and environmental toxicology, and one that is also widely recognised in mammalian and human pharmacology and toxicology; and due of the enormous diversity of chemicals, it is very difficult to develop generalised rules that will determine the toxicity. Consequently, understanding the toxicity of chemically diverse mixtures is one of the major challenges for the future in toxicology (Cassee et al., 1998; Cedergreen, 2014; European Commission, 2011; Kienzler et al., 2016; Kortenkamp et al., 2009; LeBlanc & Olmstead, 2004; Kortenkamp et al., 2009; McCarty & Borgert, 2006; Sarigiannis & Hansen, 2012; Smith et al., 2013; Tallarida, 2012, 2016).

An alternative approach to the problem of predicting the toxicity of pollutant cocktails (complex mixtures) involves treating the probable harmful impact as supracritical cascades of self-propagating chemical interactions, leading to a burst of toxic molecular diversity which results in cell and tissue damage. By trying to ascertain the laws that govern the emergence of toxic interactions in the complexity of contaminated environments (Kauffman, 1993), we have to consider the types of chemical and biochemical interactions that can occur within a highly diverse molecular environment. For instance, such a situation must have prevailed in the early prebiotic history of our planet and yet it was from this diverse molecular mixture, also containing many toxic chemicals that life originated. Living organisms are highly organised molecular and supramolecular aggregations where order and structure have emerged as a direct result of this very molecular diversity (i.e., self-organised criticality), but in which, destructive toxic cascades (**supracriticality**, see Fig. 1) are prevented by protective homeostatic regulation (Bak & Chen, 1991; Kauffman, 1993). However, vestiges of the prebiotic condition are probably still represented in the universal

use by cells of limited toxic cascades in intracellular signalling processes (e.g., free Ca^{2+}, oxyradicals and nitric oxide; Yermolaieva et al., 2000).

Consider then, an environment containing a diversity of toxic chemicals (e.g., a contaminated sediment or soil) and the consequences of this on the indigenous animals and plants. At some critical diversity and contaminant concentration, the protective homeostatic processes within the cells will be overwhelmed and the cells will become supracritical (e.g., cascades of reactive free radicals) leading to cell injury and death. Hence, toxic cascades will occur at a high concentration of total contaminants where the molecular diversity is very low. What is not known is whether in a highly diverse toxic mixture, the total concentration can be very much lower (Fig. 1). Essentially, the question that is posed here is as follows: does the diversity of pollutant molecules and multiplicity of modes of action increase toxicity or are the effects generally additive?

Consequently, the hypothesis being tested is that complex mixtures of contaminant chemicals will result in a cascade of toxicity (supracriticality) if the molecular diversity rises above a critical threshold (i.e., a phase transition). Examples of analogous behaviour have been demonstrated in autocatalytic systems and proposed for the behaviour of bacterial ecosystems (Kauffman, 1993). This hypothetical model is readily testable in relation to environmental toxicity (Fig. 1).

If this hypothesis provides a satisfactory explanation for mixture toxicity, then the total concentration of pollutant chemicals in a mixture is such that it would be relatively non-toxic, or have low toxicity, for any single compound (i.e., subcritical behaviour). However, when the chemicals are combined in a mixture, they will interact in a complex manner with cellular processes to produce toxicity (i.e., supracritical behaviour), if their molecular diversity exceeds the threshold of the critical phase-transition boundary. This would be an example of emergent behaviour.

Unfortunately, additive effects at single test concentrations cannot be used reliably to test for interactive effects, and dose responses are a necessary requirement, as demonstrated by Berenbaum (1989) and Kortenkamp and Altenburger (1998). Additionally, non-additive emergent interactions can readily be identified by using the method of effect isoboles, which is reliant on the concept of concentration additivity proposed by Loewe and Muischnek (1926), and used by Kortenkamp and Altenburger (1998) to demonstrate emergent estrogenic effects. Tallarida (2012 & 2016) has recently reviewed the use of the isobole method in relation to the interactions of pharmaceuticals.

The phagocytic blood cells (hemocytes) of marine mussels (*Mytilus galloprovincialis*), a common commercially and ecologically important animal, were used as the main experimental tool in this study. Mussels are used globally as sentinels for envionmental monitoring and impact assessment, hence, they provide an appropriate model for this investigation (Cheung et al., 1998; Krishnakumar et al., 1994; Moore, 1988; Widdows et al., 1992). Their blood cells are immunocytes, and hence, are a key part of the cellular or innate immune system of the mussel, and there are many functional parallels with phagocytic mammalian white blood cells. They are also known to be the target for chemical pollutant impact, which relates directly to important pathological consequences such as suppression of immune function (Galloway & Depledge, 2001; Moore et al., 2009). *In vitro* tests with live cells can be performed rapidly and in large numbers (Loizou, 2016); and experimental exposures of molluscan blood cells (hemocytes) to mixtures will be tested for evidence of additive, synergistic and antagonistic interactions. Toxic cellular reactions induced by the various experimental treatments will be measured using a lysosomal membrane stability method as an indicator of cell injury involving damage to intracellular membranes (Lowe et al., 1992; Moore et al., 1996, 2009). Lysosomal membrane stability was chosen as it is an integrated biomarker of cellular health/dysfunction, which is functionally related to protein turnover (degradation component), endocytosis, autophagy, oxidative stress and correlated with DNA damage caused by benzo[a]pyrene (Moore et al., 2006; Sforzini et al., 2018).

Finally, the primary objective of this investigation will be to test the hypothetical model described in Figure 1 relating sublethal pollutant toxicity in complex mixtures to the molecular diversity of pollutant species; and the experimental results will be used to establish the subcritical-supracritical boundary for various concentrations and combinations of chemicals. Environmental contaminants that will be tested include polycyclic aromatic hydrocarbons, pesticides, a biocide and a surfactant (Readman, 1996; Tolosa et al., 1996a & b). These chemicals have been chosen on the basis of their well documented toxicity and their continued presence in the environment in various parts of the world (Cassee et al., 1998; European Commission, 2011; LeBlanc & Olmstead, 2004; Patel et al., 2016; Readman, 1996; Readman et al., 1986, 1992a & b, 1993a & b; Sapozhnikova et al., 2013; Smith et al., 2013; Tolosa & Readman, 1996; Tolosa et al., 1996, 1997). The concentrations of test chemicals used in this study were higher than would probably be encountered in the natural environment; however, the aim of the investigation was to demonstrate “proof of principle”.

Materials and methods

Animal husbandry

Blue mussels (*Mytilus galloprovincialis*, 40-50 mm shell length) were collected from Freathy Beach (Whitsand Bay, Cornwall; Grid ref: SX 39390 52066); and held for 24 hours without food in a seawater aquarium system at 15 ± 1^{o}C and 34 psu salinity with natural daylight prior to harvesting the blood cells (hemocytes).

Lysosomal membrane stability (neutral red retention – NRR test)

Lysosomal stability was assessed in the hemocytes or blood cells of mussels using neutral red as described by Lowe (1995) and Moore et al. (2008). Briefly, approximately 50 µl of haemolymph was removed from the posterior adductor muscle of mussels (n = 20 for each set of test concentrations) and added to 50 µl ml of physiological saline (0.02 M HEPES, 0.4 M NaCl, 0.1 M $MgSO_4$, 0.01 M KCl, 0.01 M $CaCl_2$, pH 7.3). 40 µl of cell suspension was aliquoted onto a microscope slide and left in a dark moisture chamber at 15^{o}C for 15 minutes to allow the cells to adhere, following which the cells were incubated in the test treatment solutions (see below). A stock solution of 100 mM neutral red in DMSO (28.9 mg of neutral red in 1 ml of DMSO) was prepared and stored in a refrigerator prior to use. However, the solution will solidify in the refrigerator and must be raised to room temperature for dilution in physiological saline to the working strength solution. The saline containing neutral red comprised 10 µl of stock neutral red in DMSO in 5ml of mussel physiological saline. 40 µl of neutral red saline solution was added to the slides and left for 15 min in a dark moist environmental chamber at 15^{o}C to allow the neutral red to enter the cells and accumulate in the lysosomes. The slides were maintained under these conditions for the duration of the test, with slides only being removed briefly for microscopical examination before being returned. The cells were examined microscopically after 15, 30, 60, 90, 120, 150 and 180 minutes. The test was terminated after 180 minutes, since the neutral red itself becomes a toxic xenobiotic stressor. The end point of the test was when > 50% of the cells, based on a visual determination, exhibited lysosomal leakage of neutral red dye into the cytoplasm or showed significant abnormalities such as lysosomal enlargement (Lowe et al 1992, Moore et al., 2009).

Experimental treatments

The chemicals tested were phenanthrene, anthracene, lindane, malathion, irgarol-1051, *cis*-permethrin and sodium dodecylbenzene sulphonate (LAS). Dimethyl sulphoxide (DMSO) was used as an initial solvent to prepare the 100 mM stock solutions for the test compounds, with the exception of LAS which was water miscible. Exposures were conducted at 10, 50, and 100 µM in physiological saline for 30 minutes at 15^{o}C. Various test mixtures (Mix 2 – phenanthrene + anthracene; Mix 3 - phenanthrene + anthracene + LAS; and Mix 7 – all

seven test compounds) were made up to each of these concentrations with all of the constituents being an equal proportion of the final dose. The controls were actually vehicle controls with the equivalent concentration of DMSO (0.1 % v/v) in physiological saline in all cases. DMSO at the concentration used is non-toxic to mussels; and the NRR values for the vehicle controls were within the normal range (Banni et al., 2017; Bellas et al., 2005, 2006).

Microscope slides with attached hemocytes (see section above) were then shaken to remove the excess haemolymph and the slides placed into 50 ml Coplin jars containing the test chemicals dissolved in physiological saline. Slides were incubated in the Coplin jars, in the absence of light for a further 30 minutes, at a constant temperature of 15^{o}C. Slides were then removed from the coplin jar, drained and 40µl of the neutral red working solution was added (see section above). All slides were coded to prevent operator bias and only decoded after the analyses of all samples were complete. The results were expressed as lysosomal toxicity (i.e., 100% - NRR [*lysosomal membrane stability*] as a % of control ± 95% CL).

Isobole method

50% toxicity values were determined for the individual compounds and the mixtures and the sum of concentration additivity for the 50% isobole calculated from the generic formula $\mathbf{d_a/D_a + d_b/D_b \ldots\ldots}$ where d_a and d_b are the doses/concentrations of A and B in a mixture that produces a specified effect (50% toxicity) and D_a and D_b are the doses/concentrations of the single compounds, which on their own elicit the same effect as the mixture (Kortenkamp & Altenburger, 1998). 95% confidence bands were generated for the determination of the confidence limits (± 95%) for the 50% isoeffective concentrations and; and a pooled variance estimate was used to determine the estimated 95% confidence limits for the additivity concentrations (Cohen, 1988; Kortenkamp & Altenburger, 1998). 84% confidence limits were employed on the graphical plots rather than 95% confidence intervals, it then being true (for large n, as here) that non-overlapping intervals correspond to a significant difference in a 5% level test (Buzatto et al., 2015).

Linear-additive statistical method

A conventional statistical modelling approach was also employed, to examine robustness of the conclusions to choice of predictive model. Rather than the threshold-based approach of supracritical cascades this postulates a simple linear additive structure for the dose-response models. Computations are performed on the raw NRR data, rather than as expressed by a percentage of controls, to preserve the statistical independence in formal inference from standard linear models and to allow visualisation of NRR levels under control conditions (higher NRR denotes lower toxicity). Observed NRR means and confidence

intervals for the 9 mixture experiments (3 mixtures at 3 concentrations, n = 20 replicates each) are contrasted with predictions from an appropriate linear combination of NRR estimates from the separate regressions for each of the 7 compounds. Confidence intervals for these predictors were based on standard errors computed under the usual rules for variance of a linear combination of independent random variables. Formal testing of a difference between a predicted and observed mean relied on standard normality assumptions, justifiable here by the central limit theorem since the two statistics are each means over a large number of observations. For this model, under the null hypothesis of no effect of toxicant diversity, only of toxicant concentration, predicted and observed means will not differ. These test results are conveniently visualised on means plots by employing 84% rather than 95% confidence intervals, it then being true (for large n, as here) that non-overlapping intervals correspond to a significant difference in a 5% level test (Buzatto et al., 2015).

Non-parametric Kruskal-Wallis tests were also used on the lysosomal toxicity data, to provide additional robustness to test conclusions.

Chemicals

All chemicals were obtained from Sigma-Aldrich. Anthracene and phenanthrene were $\geq$ 99% pure; LAS (dodecylbenzene sulphonate) was Pharmaceutical Secondary Standard - Certified Reference Material; pesticides and herbicides were analytical standard grade; DMSO ($\geq$ 99.9%); and neutral red powder (N4638) was graded as suitable for cell culture. All other reagents used were of ANALAR grade.

Results

Lysosomal toxicity

Effects of the test chemicals and the 3 mixtures on % lysosomal toxicity (100% - NRR [*lysosomal membrane stability*] as a % of control ± 95% CL) were measured at three concentrations (10, 50 & 100 µM; Fig. 2). Lysosomal toxicity was also plotted as a series of dose responses for all of the test conditions (Fig. 2A - H), as these were required to determine the 50% effect isoboles.

Dose/concentration responses for the individual test chemicals showed that LAS had the greatest effect on lysosomal toxicity (Fig. 2C). Phenanthrene and anthracene also had a significant effect on the lysosomal toxicity ($p < 0.05$, n = 20; Kruskal-Wallis test; Fig. 2A, B). Malathion showed slight toxicity, but only at 10 µM; while lindane, irgarol-1051, and *cis*-

permethrin caused no significant effect on lysosomal retention ($p > 0.05$, $n = 20$; Kruskal-Wallis test; Fig. 2D - G). Controls maintained high retention times throughout all the exposures and no significant differences could be found ($p > 0.05$; Kruskal-Wallis test). The mixtures of test compounds caused a significant change in lysosomal toxicity for the mixture of phenanthrene and anthracene (Mix 2) at 100 µM ($p < 0.05$, $n = 20$; Kruskal-Wallis test; Fig. 2 H). However, significant increases in lysosomal toxicity were observed at 10, 50 and 100 µM for the mixture of phenanthrene, anthracene and LAS (Mix 3), as well as in the mixture of all 7 test chemicals (Mix 7; $p < 0.05$, $n = 20$; Kruskal-Wallis test; Fig. 2H). When the dose responses for the individual compounds were compared with the mixtures, the mixture with 7 chemicals (Mix 7) was the most toxic (Fig. 2H), and the mixture with 3 components (Mix 3) was comparable to the dose response for LAS (Fig. 2C, H).

Application of isobole method

Isoeffective concentration values were determined for the individual compounds and the mixtures; and the sum of concentration additivity for the 50% isobole was calculated for each mixture using the generic formula described by Kortenkamp & Altenburger (1998) (Fig. 3). Anthracene did not quite achieve 50% lysosomal toxicity anthracene, and was extrapolated slightly beyond the 100 µM concentration (Fig. 2B). Some of the test compounds (i.e., malathion, *cis*-permethrin, irgarol 1051 and lindane) did not achieve 50% lysosomal toxicity, and also, were not significantly different from the control (Fig. 2D - G). Consequently, these compounds could not be included in the 50% concentration additivity calculations. 95% confidence bands were generated for the determination of the confidence limits (± 95%) for the 50% isoeffective concentrations; and a pooled variance estimate was used to determine the estimated 95% confidence limits for the additivity concentrations (Cohen, 1988; Kortenkamp & Altenburger, 1998).

The isobole method demonstrated that there was an additive toxic effect with a mixture of phenanthrene and anthracene (Mix 2) at the 50% effect isobole ($P < 0.05$, $n = 20$; Fig. 3A, B). When LAS was added to phenanthrene and anthracene (Mix 3), there was a significant synergistic interactive effect for the observed isoeffective concentration at the 50% effect isobole (Fig. 3A, B; Kortenkamp and Altenburger, 1998). With a mixture of all seven compounds (Mix 7), there was a significant synergistic interaction for the observed isoeffective concentration at the 50 % effect isobole ($P < 0.05$, $n = 20$; Fig. 3A, B).

Application of linear additive model

The linear additive statistical model allows comparison of observed with predicted effects on lysosomal membrane stability in the absence of any effect of toxicant diversity (Fig. 5). Major

declines in NRR (i.e., greater toxicity) were seen for Mixtures 3 & 7 that cannot be explained solely by toxicant concentration under this linear additive model (Fig. 4); and these findings were indicative of synergistic interactions (Fig. 3A, B). These results have to be treated with caution as the dose responses for phenanthrene, anthracene and LAS were not strictly linear. However, the results from the linear additive model are in very good agreement with the findings from the use if the isobole method.

Modelling

The enhanced toxic effects were evident with the most diverse toxic mixture (Mix 3 & 7), when the data for the mixtures were plotted as % lysosomal toxicity (100% – NRR as % of control) against the diversity (i.e., number of test compounds) of the toxic mixture (Fig. 5). Results shown in Figure 6 tentatively indicated that there may be a phase shift in relative toxicity with the test mixtures with 3 & 7 components.

Finally, the lysosomal toxicity data was used to test the concentration & diversity model proposed in the hypothesis. The results are presented in the log concentration versus log chemical diversity matrix shown in Figure 6 that indicates that the hypothesis being tested is probably supported by the data. These findings are in agreement with the results of the 50% effect isobole method, and the linear additive statistical model, that synergistic interactions are in fact occurring in the more complex mixtures.

Discussion

Living cells as the basic units of life operate below or near to the subcritical-supracritical boundary, sometimes referred to as “the edge of chaos” (Kaufmann, 1993; Fig. 1). If cells were supracritical then the introduction of any foreign molecule (i.e., xenobiotic chemical) will probably unleash a potentially harmful and reactive molecular cascade (Kauffman, 1993). Such a reactive cascade will propagate, since each new molecule can potentially interact with another biological molecule, such as membrane lipids, proteins and DNA and potentially initiate a further cascade (i.e., a chain reaction). Therefore, there is a high probability that many of these cascading toxic molecules will perturb the homeostatic regulation of cellular processes (i.e., sublethal toxicity) and lead to cell injury, pathology and cell death. Essentially, supracritical conditions within cells will be lethally destructive. However, cells have evolved a number of protective processes in order to protect themselves. These protective systems include sequestration within the interior microenvironment of a membrane-bound vesicle (e.g., the lysosome), membrane pumps to remove novel molecules from the cell (e.g., multidrug-resistance system - MDR), detoxifying

379 enzymes to metabolise toxic molecules (e.g., cytochromes P-450 [CYP superfamily] and
380 esterases) and antioxidant enzymes to protect against free radicals (e.g., superoxide
381 dismutase, catalase and glutathione peroxidase), as well as scavenging molecules that bind
382 to toxic reactive molecules (Minier & Moore, 1996a, b; Sies, 1997).
383
384 Lysosomes are central to innate or cellular immune function, and normal turnover of cellular
385 proteins and other biomolecules (Moore et al., 2004, 2006, 2007). Lysosomes are well
386 established targets for many environmental xenobiotic chemicals that are also known to
387 accumulate in lysosomes (i.e., metal ions and organic chemicals with many modes of toxic
388 action; Moore et al., 2004; Rashid et al., 1991). Furthermore, the lysosomal vacuolar system
389 has an important cellular protective function and, when lysosomal storage capacity is
390 overloaded, the lysosomes display characteristic low membrane stability reactions to toxic
391 injury (Minier & Moore, 1996a, b; Moore, 1985, 1986, 1990; Moore et al., 1996, 2004, 2006,
392 2007).
393
394 Lysosomal integrity is an effective measure of integrated physiological function and,
395 therefore, is more functionally relevant than many other biomarker tests that only measure a
396 change in the level or function of a particular protein (Lowe et al., 1992; Moore et al., 2004,
397 2006a; Regoli, 1998; Ringwood et al., 1992; Sforzini et al., 2015, 2017). The lysosomal
398 system also has the propensity for accumulating many xenobiotic chemicals including
399 polycyclic aromatic hydrocarbons (de Duve, 1974; Minier & Moore, 1996a, b; Moore et al.,
400 1996, 2004, 2006a; Rashid et al., 1991). Only some of the compounds tested were
401 lysosomotropic: these were anthracene and phenanthrene (Moore et al., 2006). Of the other
402 chemicals, LAS is a detergent (surfactant) and disrupts cell membranes, while lindane, *cis*-
403 permethrin and malathion are neurotoxic pesticides and irgarol 1051 is a herbicide (Patel et
404 al., 2016; Sapozhnikova et al., 2013). The effects of the pesticides and irgarol 1051 have
405 been tested in several species of mussel and found to have relatively low toxicity (Bellas,
406 2006; Bringholf et al., 2007; Khessiba et al, 2005; Lehtonen & Leiniö, 2003).
407
408 Four of the compounds tested in several species of mussel (i.e., malathion, lindane, *cis*-
409 permethrin and irgarol 1051) had either very low or else no lysosomal toxicity and this is
410 supported by other studies (Bellas, 2006; Bringholf et al., 2007; Khessiba et al, 2005;
411 Lehtonen & Leiniö, 2003; Fig. 2). It was, therefore, surprising that these relatively low toxicity
412 chemicals, when combined with the two PAHs and LAS, apparently contributed to the
413 enhanced lysosomal toxicity in the hemocytes (Smith et al., 2013; Figs. 2H, 3A). However,
414 this type of effect has been observed previously in mice exposed to a mixture of estrogenic

methoxychlor and non-estrogenic dieldrin (Ashby et al., 1997; Kortenkamp & Altenburger, 1998; Smith et al., 2013).

When the results from the isobole and linear additive method for interactions in the mixtures (Mix 2, Mix 3 & Mix 7) were applied to the concentration/diversity model, there was a clear demarcation between those test conditions showing evidence for a synergistic interaction and those with no interaction (Fig. 6). The 50% isoeffective concentrations for the three mixtures, with transposed axes from Figure 3B, were used to generate the subcritical-supracritical boundary curve (Fig. 6). Consequently, the hypothesis is apparently supported by the data that increasing diversity in a mixture of contaminants contributes to emergent toxicity (Kauffman, 1993).

There is some indication that a phase shift is occurring at the level of the mixture with 3 compounds leading to emergent synergistic toxicity as indicated in Figure 5; and this is supported by the evidence for increased variance for the isoeffective concentration of Mixture 3 (Fig. 3A, B). Although it is probable that in other combinations of chemical compounds, this type of phase transition will occur at a different level of chemical diversity; it is reasonable to argue that a phase transition will generally indicate that emergent behaviour is happening (Kauffman, 1993; Kortenkamp et al., 2009). Nevertheless, the consequences of synergy between contaminants in the natural environment may be greater than those observed in this investigation, under relatively simple conditions over a short time period. Hence, in more chronic conditions it is possible that the interaction between contaminants may be of much greater concern for animal health status (Doi, 1994).

Ideally we would have wished to explore the effects of a larger number of combinations of these chemicals, however, this was not logistically possible within the scope of the investigation. Further experimentation is required for investigating the potential for interactive effects in mixtures with more constituents (e.g., 50–100). Such investigation will provide data sets for testing regions of the current model with very low environmentally realistic concentrations and very high diversity (Fig. 6; Cedergreen, 2014).

Conclusions

The fact that the results support the complexity-based hypothesis that there is emergent toxicity with increasing contaminant diversity should perhaps urge a cautionary attitude to disposal of toxic mixtures until the hypothesis is disproved. However, if further support for the hypothesis is forthcoming, then serious questions arise as to the validity of estimating potential emergent toxicity of complex mixtures based on the additive toxicity of single

components. Regulation of discharges based on toxicity rather than chemical composition will probably provide a more practical solution to this problem, where the toxicity of the main components has been determined by bioassays or ecotoxicity tests (Cassee et al., 1998; Cedergreen, 2014; European Commission, 2011; Kienzler et al., 2016; Kortenkamp et al., 2009; LeBlanc & Olmstead, 2004; McCarty & Borgert, 2006; Sarigiannis & Hansen, 2012; Smith et al., 2013; Tallarida, 2012, 2016; Tang et al., 2014).

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Figures

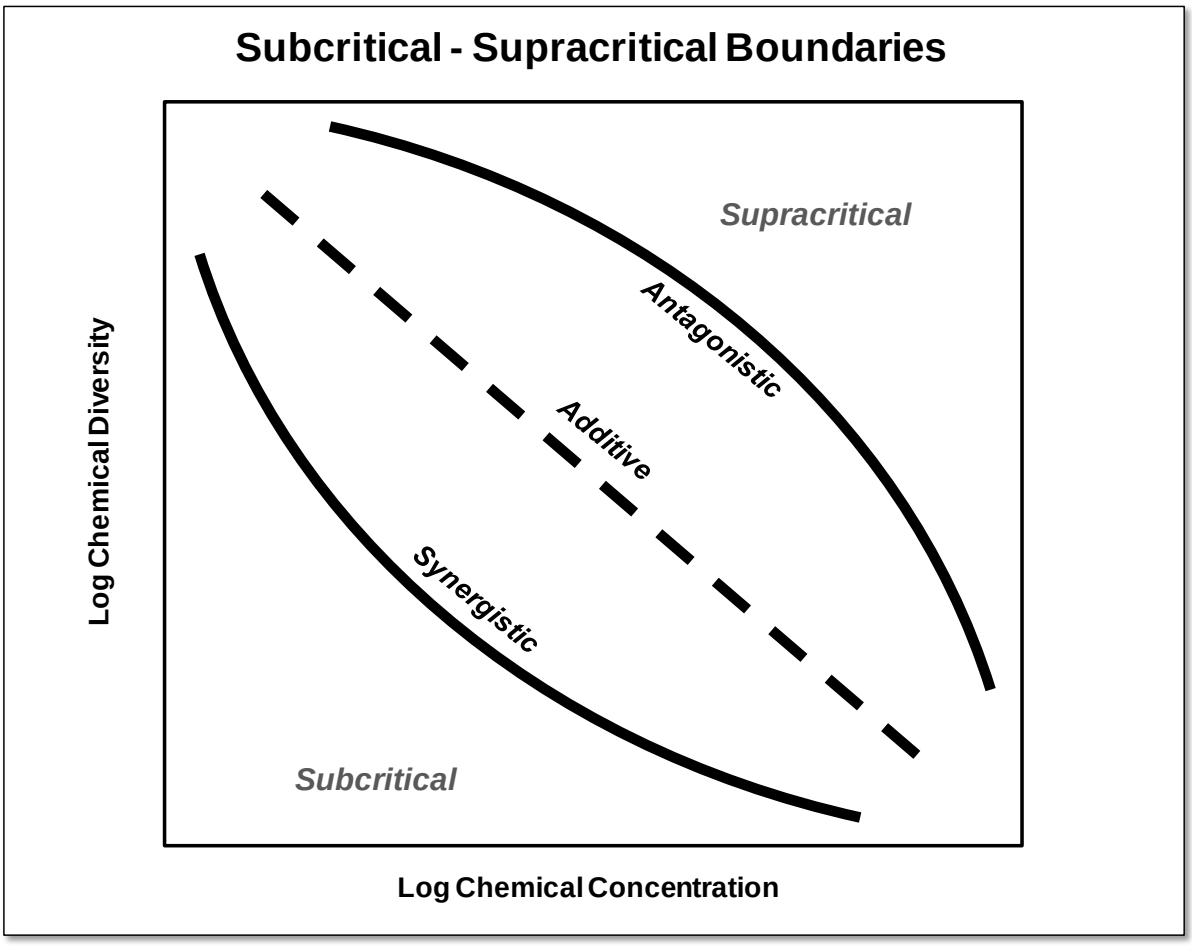
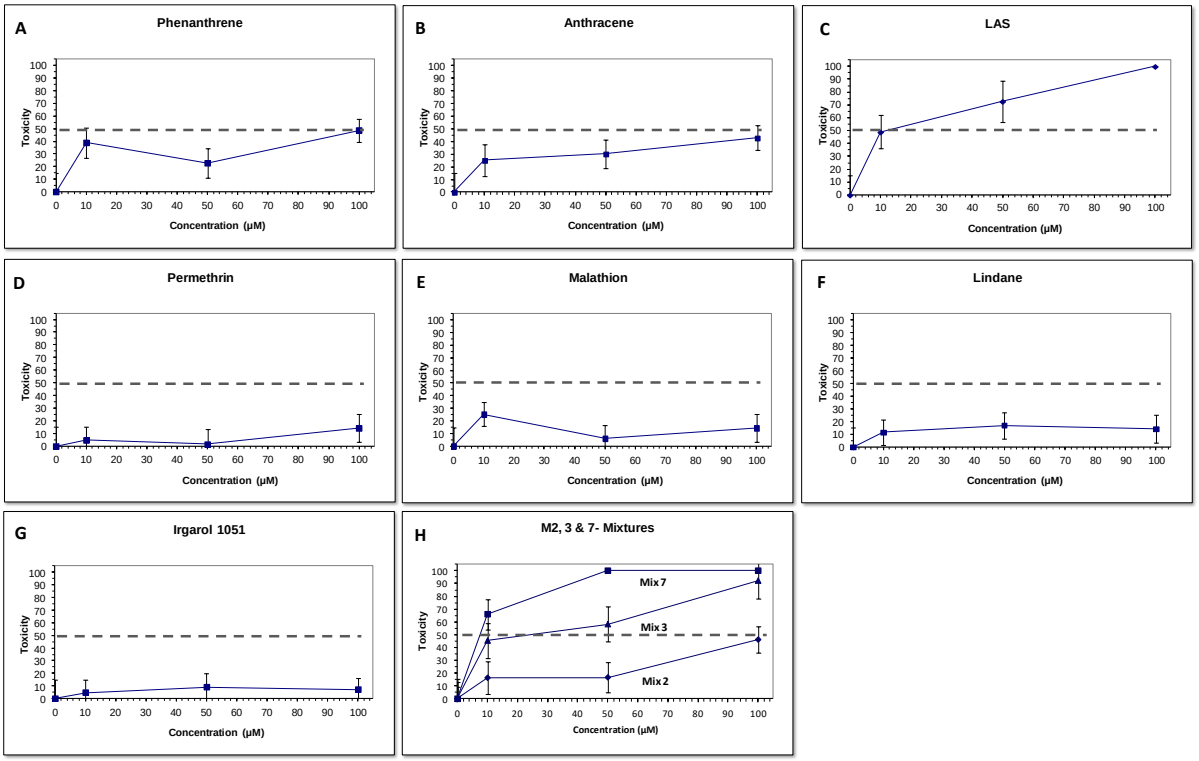


Fig. 1. Toxicity as a cellular supracritical reaction based on complexity theory (Kauffman, 1993). Using logarithmic scales, the molecular diversity of pollutant chemical species is plotted against the total concentration of pollutants in a complex mixture. A variety of modes of action is implicit in the diversity (see Kauffman, 1993). Idealised subcritical-supracritical boundaries are shown for alternative models where the effects are either synergistic, additive or antagonistic.



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669 **Fig. 2.** The % lysosomal toxicity derived from the NRR time (as % of control) plotted against
670 toxicant diversity (Mean ± 95% CL). Individual dose/concentration responses are
671 shown in A-G with the dose responses of the 3 mixture groups shown in H. The 50%
672 effect isoboles are shown as grey dashed lines. The dose response data for
673 lysosomal toxicity was tested using the Kruskal-Wallis non-parametric test.

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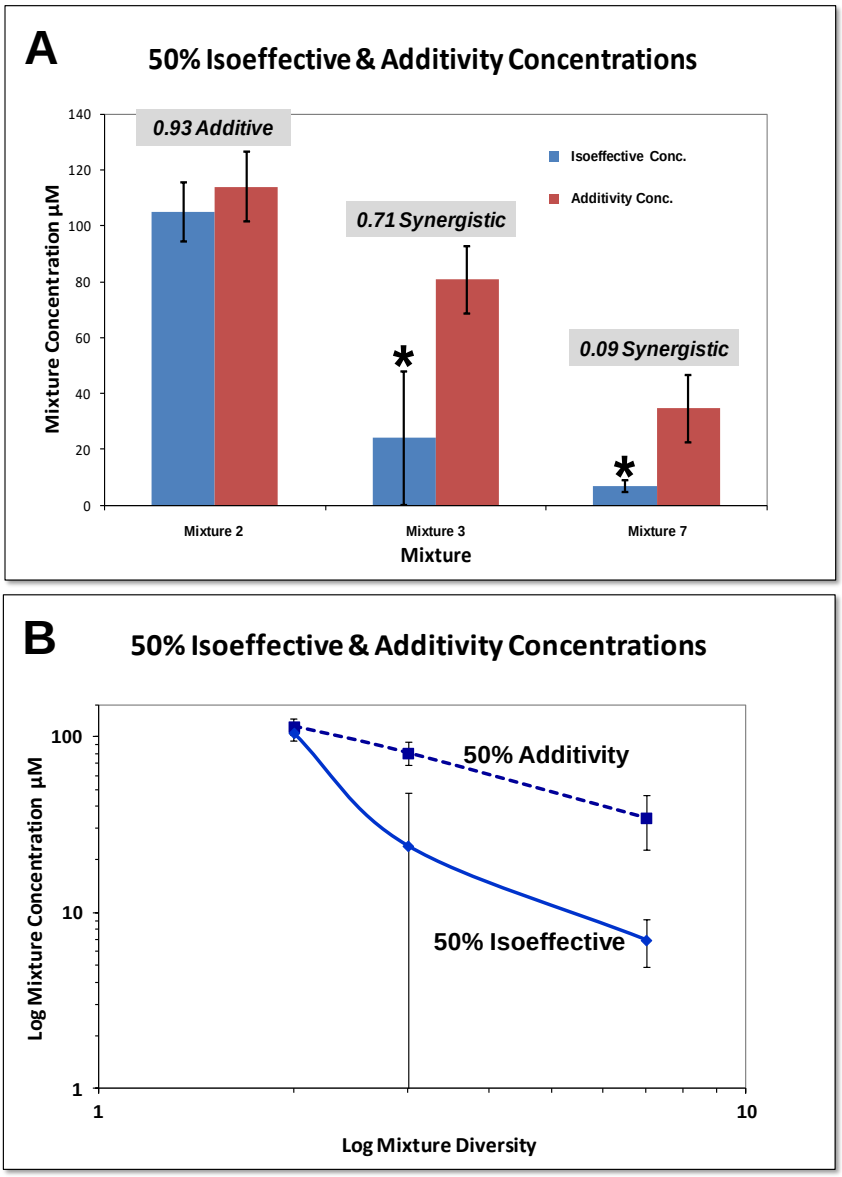


Fig. 3. A - Results of the 50% effect isobole method for identification of toxic lysosomal interactions. 50% isoeffective and additivity concentrations were determined for the three mixtures as explained in the Results section. The sums of concentration additivity (shaded boxes) were calculated from the generic formula $\mathbf{d_a/D_a + d_b/D_b +}$ **etc……** where d_a and d_b are the doses/concentrations of A and B in a mixture that produces a specified effect (50% toxicity) and D_a and D_b are the doses/concentrations of the single compounds, which on their own elicit the same effect as the mixture (Kortenkamp & Altenburger, 1998). Additivity is indicated by a sum concentration additivity of 1.0, synergy by a sum concentration additivity of <1.0, and antagonism by a sum concentration additivity of >1.0. Isoeffective concentrations determined from dose response graphs for individual compounds and mixtures based on the 50% lysosomal toxicity (100% – NRR value as % of control). **B** - The same data is also shown as a Log_{10} scale graphical plot for both axes, as these are used in Figure 6 with transposed axes. Significant differences were indicated by non-overlapping 84% CIs (i.e., employing 84% rather than 95% confidence intervals [Buzatto, et al., 2015], it then being true for large n [n = 20], that non-overlapping intervals correspond to a significant difference in a 5% level test). ***** - indicates significant difference ($P \leq 0.05$).

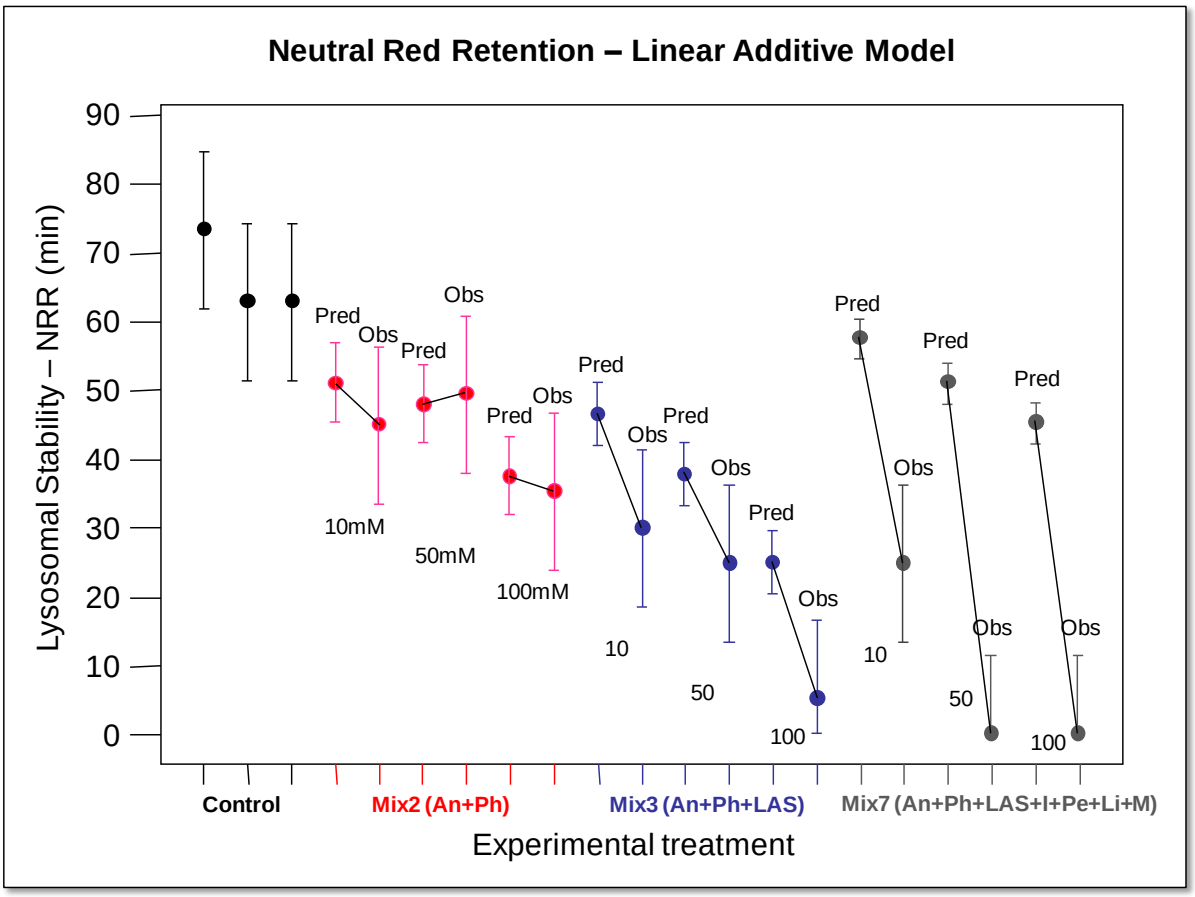
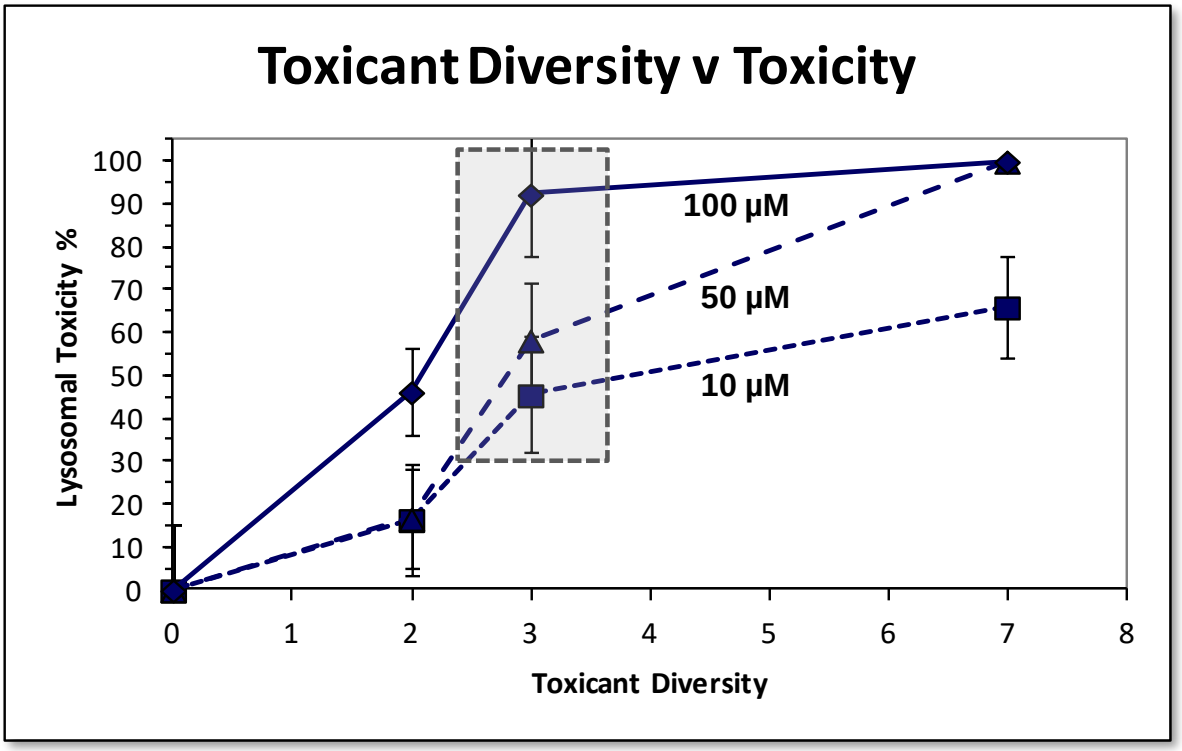


Fig. 4. Observed (Obs) NRR values for the three mixtures and controls at three concentrations, and predicted (Pred) estimates from separate components (linear additive model), plus 84% confidence intervals for both (non-overlapping intervals imply significant differences). An – anthracene, Ph – phenanthrene, LAS - dodecylbenzene sulphonate sulphonate, I – irgarol 1051, Pe – *cis*-permethrin, Li – lindane, M – malathion. Significant differences were indicated by non-overlapping 84% CIs (i.e., employing 84% rather than 95% confidence intervals [Buzatto, et al., 2015], it then being true for large n [n = 20], that non-overlapping intervals correspond to a significant difference in a 5% level test**).**



711
712
713 **Fig. 5.** Lysosomal toxicity (%) calculated from the NRR time (as % of control) plotted against
714 mixture diversity (Mean ± 95% CL). The possible phase transition observed with the
715 3-component mixture (Mix 3) is outlined by the shaded box.
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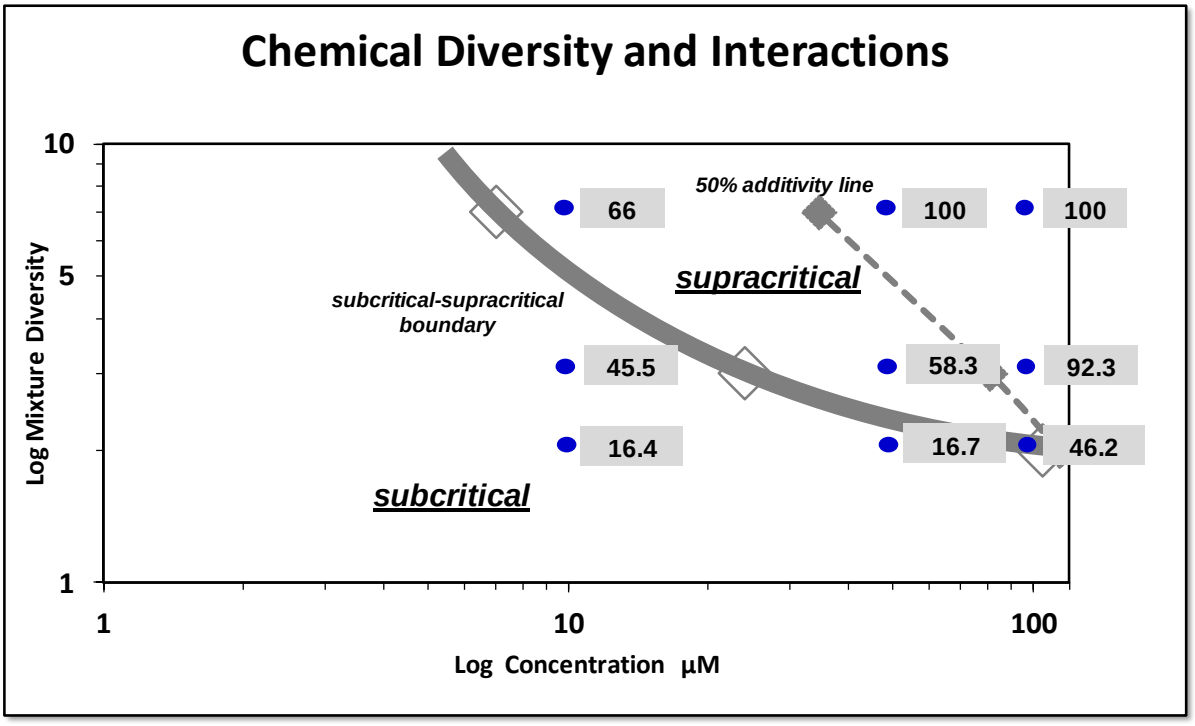

Fig. 6. Evidence for synergistic toxicity as a consequence of increasing diversity of harmful chemicals. Test matrix (circular symbols) for chemical molar concentrations versus chemical diversity (log_{10} scales) with the % lysosomal toxicity (shaded boxes) shown for the corresponding treatment. The conjectured curve for the subcriticality /supracriticality boundary (broad grey line; see Figure 1) employs the 50% isoeffective concentrations (open grey diamond shapes) for the 3 mixtures (transposed from Fig. 3B; Kortenkamp & Altenburger, 1998). The 50% additivity concentrations are shown as a dashed line (transposed axes from Fig. 3B).