A Review of the Ecotoxicity of Mixtures, Approaches to, and Recommendations for, their Management

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A Review of the Ecotoxicity of Mixtures, Approaches to, and Recommendations for, their Management

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1 INTRODUCTION

There is a marked dichotomy in the manner in which the toxicity of mixtures is dealt with. There is the 'bottom-up approach' where the mode of action or site of action of the chemicals in the mixture are clarified in order to determine which model will predict the toxicity of the mixture. Then there is the 'top-down approach' in which the type of interaction between the chemicals is not relevant – rather the total toxicity of the mixture is measured. The strengths and weaknesses of these two approaches to dealing with the toxicity of mixtures will be examined. A review of the ecotoxicology of mixtures will also be presented. The various management strategies implemented by regulatory agencies for dealing with mixtures will be discussed. Finally, recommendations on how to deal with mixtures will be made.

2 TERMINOLOGY

The plethora of terms used to describe the toxicity of mixtures is confusing and has hindered the development of the study of the toxicity of mixtures. There are:

> 'different words describing phenomena that seem to be the same but are often employed in totally different ways by different authors' (Calabresse, 1991)

Some examples of the terms used are presented in Table 1.

Table 1. Examples of the varied terms used to describe different types of joint action (modified from Marking, 1985).

Synergism	Additive	Antagonism
Greater than additive	Expected action	Less than additive
Supra-additive	Simple addition	Infra-additive
Potentiation	Additive action	Competitive addition
Positive summation	Summation	Competitive antagonism
Joint action	Joint action	Joint action
Interaction	Interaction	Interaction

Calamari and Alabaster (1980), Marking (1985), and Calabrese (1991) argue that there are only three basic classes of joint action. These are additivity, synergism and antagonism and they are defined as:

Additivity (additive joint action) is when the toxicity of the mixture is equal to that expected from the summing of the known toxicities of the individual chemicals present in the mixture (ie. 1 + 1 = 2). Please note that there are two types of additivity (concentration or response additivity). The differences between the two are explained in the following section.

Synergism is when the toxicity of the mixture is greater than expected if the mixture was additive (ie. 1 + 1 > 2).

Antagonism is when the toxicity of the mixture is less than that expected if the mixture was additive (ie. 1 + 1 < 2).

3 TYPES OF TOXICANT JOINT ACTIONS

Hewlett and Plackett (1952) expanded on the scheme developed by Bliss (1939) on the possible types of interactions that can occur between chemical components of mixtures (Table 2).

Table 2. The four possible types of joint action for mixtures developed by Plackett and Hewlett (1952).

	Similar Joint Action	Dissimilar Joint Action
Non-Interactive	Simple similar (concentration addition)	Independent (response addition)
Interactive	Complex similar	Dependent

3.1 SIMPLE SIMILAR JOINT ACTION

In this type of joint action the chemicals have the same site of action and the chemicals do not affect the biological activity of the other chemicals in the mixture, in other words they do not interact. This type of joint action is also called concentration addition because they can substitute for each other and the combined effect is equal to the sum of the concentrations of each chemical expressed as a fraction of their own individual toxicity. The toxicity of a mixture with concentration addition is determined using the formula:

$$\text{Total Toxicity} = (C_a/T_a) + (C_b/T_b) + \ldots. (C_n/T_n) \quad (1)$$

where the subscript indicates the chemical in the mixture, C is the aqueous concentration of the each chemical in the mixture and T is a measure of the toxicity for each chemical (eg. EC50 or LC50). It is essential that the endpoint measured (ie. lethality, immobilisation, reproductive impairment) is the same for each chemical in the mixture but the measure of toxicity does not have to be. Thus, T could be EC50 for chemicals a to d, but be an EC10 for chemical e.

3.2 INDEPENDENT JOINT ACTION

In this type of joint action the chemicals have different sites of action but the chemicals do not affect the biological activity of the other chemicals in the mixture, in other words they do not interact. This occurs when each component in the mixture acts on a different physiological or biochemical system but contributes to a common response (Anderson and Weber, 1975). This type of joint action is often referred to as response addition because the total toxicity of a mixture is equal to the sum of the biological responses (BR) elicited by each chemical individually. The biological response is determined by referring to the concentration–response relationship for each chemical. Response addition can be expressed mathematically as

$$\text{Total Toxicity} = BR_1 + BR_2 \ldots\ldots BR_\infty \quad (2)$$

Thus, for example, a mixture consists of 'x' mg/L of compound 1 and 'y' mg/L of compound 2 which cause a 50% and 20% toxic effect respectively, then the total toxicity of the mixture would be a 70% effect.

The toxicity that would occur if the joint action was concentration additive or response additive can be quite different. This difference is discussed later in the paper.

3.3 COMPLEX SIMILAR AND DEPENDENT JOIN ACTIONS

For these types of joint action at least one chemical in the mixture affects the biological activity of at least one other chemical in the mixture. The biological activity can be modified by affecting the rates of absorption, metabolism, or elimination. The difference between complex similar and dependent joint actions is that the former has the same site of action whereas, the latter have different sites of action. Because the chemicals affect each other's biological activity it is difficult to predict the toxicity of such mixtures. As these mixtures do not have additive joint action they tend to have either synergistic or antagonistic toxicity.

4 EXPERIMENTAL METHODS FOR DETERMINING THE TOXICITY OF ARTIFICIALLY CREATED MIXTURES

Artificially created mixtures used in laboratory based experiments are either equitoxic or non-equitoxic. Equitoxic mixtures are where each component in the mixture is present at the same fraction (F) of their own individual toxicity.

$$F = C/T \quad (3)$$

where C is the aqueous concentration of the chemical and T is the concentration at which a selected toxic effect occurs (eg. LC50 or EC50) for the chemical acting by itself. The fraction can be of any magnitude as long as all chemicals in the mixture have the same value. Thus, for example, F could be ½ or 1/10. The sum (S) of the F values for an equitoxic mixture is calculated by

$$S = F_a + F_b \ldots . F_n \quad (4)$$

where the subscript denotes each particular chemical in the equitoxic mixture.

Non-equitoxic mixtures are mixtures in which the components are not present at the same F value. For example, in a mixture of three chemicals F might be 1/2, 1/5 and 1/25.

The experimental designs of the tests used to determine the toxicity of these different types of mixtures are different and will be discussed below.

4.1 TESTS FOR EQUITOXIC MIXTURES

For such tests a stock equitoxic mixture is made so that S has a predetermined value (eg. 5 or 2). The toxicity tests are conducted using the same methods as if a single toxicant was being tested, except that the toxicity and the concentration of the test solutions are both expressed in terms of S rather than mg/L. The stock solution is then diluted typically to create five treatments two of which will have S values greater than 1, one will have a S of 1, and two treatments will have S values less than 1. A S-response relationship is then

developed and used to calculate the value of S that causes the selected toxic effect (eg. 50% mortality or 50% affected).

If at the selected toxic effect S equals 1 then the mixture is concentration additive. If S is less than or greater than 1 then the mixture is synergistic or antagonistic respectively.

4.2 TESTS FOR NON-EQUITOXIC MIXTURES

The simplest non-equitoxic mixture contains just two components (binary) and the vast majority of the tests conducted on these mixtures have examined these binary mixtures. Therefore, the following will discuss binary mixtures by using the example of a mixture of chemicals 'a' and 'b' (Table 3). The most common experimental design used for such tests are full factorial designs (Groten *et al.*, 2001). The first step is to determine the toxicity (eg. LC50) of each chemical individually. The concentrations that correspond to the selected toxic effect are denoted as 1 toxic unit (TU). Two mixtures are then made that cover the case where there is 1 TU of chemical 'a' and none of the chemical 'b' and where there is 1 TU of chemical 'b' and none of chemical 'a'. Then TU values for each chemical that are spaced between 0 and 1 are determined (eg. 0.33 and 0.66 TU for 'a' and 'b', Table 3). A number of solutions are then created in which the first TU value of the first chemical is constant but the TU value for the other chemical varies (eg. mixture 2, Table 3) and the toxicity determined. Then solutions in which the second TU value of the first chemical is constant but the TU value for the other chemical varies are created and their toxicity determined (mixture 3, Table 3). This process is repeated whereby the TU of the second chemical are held constant and the TU of the first vary (mixtures 4 and 5, Table 3). The TU values of the non-equitoxic mixtures that cause the selected toxic effect (eg. LC50) are then plotted on an isobologram.

Table 3. Example compositions of solutions that would need to be created and have their toxicity determined in order to quantify the toxicity of non-equitoxic binary mixtures of chemicals 'a' and 'b'.

Mixture	**TU of Chemical 'a'**	**TU of Chemical 'b'**
1	1	0
2	0.66	0.1, 0.5, 1.0, 2.0, 5.0
3	0.33	0.1, 0.5, 1.0, 2.0, 5.0
4	0.1, 0.5, 1.0, 2.0, 5.0	0.33
5	0.1, 0.5, 1.0, 2.0, 5.0	0.66
6	0	1

An isobologram (Figure 1) is a two dimensional graph with the concentrations (units are TU) of chemical 'a' and 'b' as the axes. In the isobologram one or several lines (isoboles) are shown that connect different combinations of the chemicals that cause the same toxic effect. A diagonal isobole linking the values on the y and x axes with values of 1 TU is the line of concentration addition (Figure 1). If the values that cause the selected toxic effect lie above and to the right of the additivity line then the mixture is antagonistic. If the values that cause the selected toxic effect lie below and to the left of the additivity line then the mixture is synergistic.

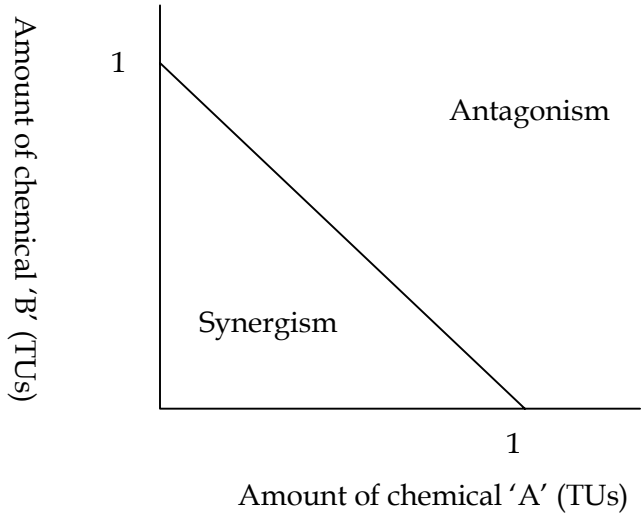

Figure 1. An example of an isobologram showing the isobole for additivity and the regions that represent synergism (to the left of the additivity isobole) and antagonism (to the right of the additivity isobole).

The beauty of this approach is that it provides much more information on the toxicity of mixtures than when equitoxic mixtures are examined. The result of a toxicity test on an equitoxic mixture would be a single point on a isobologram. By examining non-equitoxic mixtures a much fuller account of the toxicity of the mixture is obtained. In addition, this approach is more environmentally realistic, as in the environment it is highly unlikely that chemicals in a mixture will occur in an equitoxic form.

A more modern way to present toxicity data for non-equitoxic mixtures is response surfaces. These are essentially a three dimensional display of the data that is in an isobologram. Non-linear regression analysis and similar statistical techniques (Groten *et al.*, 2001) can be used to describe the response surface and therefore enable one to calculate the toxicity of any given combination of concentrations of the two chemicals.

Isobolograms and response surfaces work very well for binary mixtures but can not adequately deal with mixtures that contain more components. For such mixtures a range of new experimental designs are used including fractional factorial designs, ray designs and concentration-effect surface analysis (Groten *et al.*, 2001). The resulting data are typically analysed using multi-variate techniques including cluster analysis, principal component analysis and projection to latent structures (Groten *et al.*, 2001).

The investigation of non-equitoxic mixtures has revealed that for some mixtures the type of joint action (ie. additive, synergistic, antagonistic) depends on the ratio of the chemicals in the mixture (Figure 2). This complicates the situation considerably.

5 REVIEW OF THE TOXICITY OF MIXTURES

Very little research on the toxicity of mixtures of chemicals to terrestrial organisms has been published. What little information is available is insufficient to allow any reliable conclusions to be drawn. Therefore, this review will deal exclusively with the toxicity of mixtures to aquatic organisms. While soil and water are quite different and will affect the

behaviour and bioavailability of chemicals differently there is no reason to assume that the response of terrestrial and aquatic organisms to mixtures of toxicants will differ.

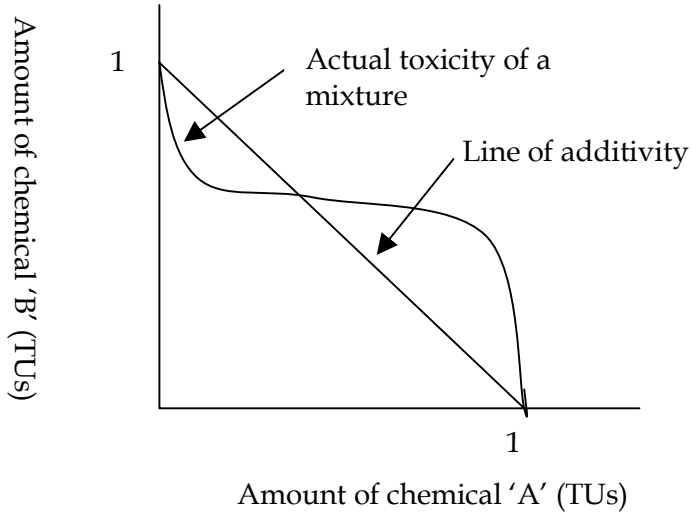

Figure 2. An example isobologram showing the variation in joint action that can occur with the ratio of the chemicals in the mixture.

One of the first major reviews of the toxicity of mixtures was conducted by the European Inland Fisheries Advisory Commission (EIFAC) (1980). They examined the toxicity of 76 mixtures to fish. They were mainly equitoxic, binary mixtures commonly found in sewage and industrial effluents. They found that the toxicity ranged from 0.4 to 26 times additive toxicity, that 87% of the values had toxicity between 0.5 and 1.5 times additive toxicity and that the median toxicity was 0.95 times additive toxicity. From this they concluded that the joint action of mixtures of common pollutants was likely to be additive. More controversially, they concluded that concentrations of chemicals below 0.2 TU did not contribute to the toxicity of the mixture. Support for this came from Lloyd (1982) who found that when the TU for phenol was greater than 0.3 binary mixtures were additive, for TU values between 0.3 and 0.1 the mixtures were less than additive, and where the TU was less than 0.1 the mixtures were antagonistic.

This view has been challenged by a series of papers originating from The Netherlands (eg. Könemann, 1981; Deneer *et al*., 1988; Hermens *et al*., 1984; 1985). These and related papers showed that equitoxic mixtures containing 8, 9, 11, 24, 33 and 50 organic chemicals were concentration additive. In the case of the mixture containing 50 chemicals each chemical was present at 1/50th of their LC50. Similarly, Broderius and Kahl (1985) found mixtures of 23 chemicals were additive. These finding clearly contradict the conclusion made by EIFAC (1980) and indicate that even very small concentrations of chemicals can and do contribute to the toxicity of mixtures. However, it is not clear whether there is a concentration below which the chemicals do not contribute to mixture toxicity or whether there is no limit and the presence of chemicals always contributes to mixture toxicity. However, Vighi and Calamari (1996) stated that:

> "for classes of organic substances with a common and well defined QSAR there is apparently no lower concentration at which individual toxicants do not contribute their full toxic potential"

According to Hewlett and Plackett (1952) for chemicals to exert a simple similar joint action (ie. have concentration addition) they must have the same site of action and not interact with the biological activity of the other chemicals. However, the problem of how to determine the site or mode of action of chemicals was a limitation to the ability to predict the toxicity of mixtures. This was at least partially resolved by Könemann, (1981) who proposed that chemicals that belonged to the same high quality relationship between the logarithms of toxicity and the octanol-water partition coefficient (called quantitative structure-activity relationships, QSARs) had the same mode of action and would therefore have concentration additive toxicity. This work was expanded on and experimentally supported by work of Hermens and co-workers (Deneer *et al*., 1988; Hermens *et al*., 1984; 1985) and Broderius and co-workers (Broderius and Kahl, 1985; Broderius, 1991; Broderius *et al*., 1995). While these developments help to predict whether mixtures of chemicals will have concentration additive toxicity, QSARs are not available for many groups of chemicals. Nonetheless, Verhaar *et al.* (1995) have proposed an approach that uses QSARs to improve the understanding of the effects of mixtures of chemicals with diverse physicochemical properties and/or modes of action. Another potential use of QSARs is to model the toxicity of mixtures. Warne *et al*. (1989) developed QSARs able to model ($r^2 = 0.85$) the toxicity of 18 mixtures containing chemicals with different modes of action, that had additive and synergistic toxicity. Xu and Nirmalakhandan (1998) have also used QSARs to predict the toxicity of mixtures.

Similarly, Roberts and Marshall, (1995) found that the toxicity of mixtures of the same type of surfactants (eg. non-ionic, anionic and cationic) were concentration additive. Warne and Schifko (1999) found that the toxicity of 21 of 23 commercial formulations of laundry detergents examined were additive despite containing chemicals with different modes of action.

According to Wang (1987) who reviewed the acute toxicity of binary and complex metal mixtures there is no consistent pattern and nor can the toxicity be readily predicted. Despite this, 21 out of 37 mixtures examined were additive or antagonistic. A similar conclusion was reached for the chronic toxicity mixtures of metals by ECETOC (2001). This unpredictability was felt to be due to the metals being examined (ie. having different modes of action), test conditions and the test species (ECETOC, 2001). For example, Braek *et al*. (1976) found that a binary mixture of copper and zinc was synergistic or antagonistic depending on the species of algae being tested. Other researchers have reported antagonism, additivity, slight synergism, and synergism for mixtures of metals. ECETOC (2001) therefore recommended that to assume 'additivity is probably the most balanced choice, unless there is clear evidence in the literature that mixtures of the metals under examination behave differently'.

The toxicity of mixtures of pesticides has been extensively investigated due to their high economic value and extensive use. Deneer (2000) reviewed the acute toxicity of 202 pesticide mixtures to aquatic organisms. He found that the toxicity of approximately 90% of the data differed from concentration addition by a factor of less than two. Unfortunately, this fact is not that useful, because the generally accepted point at which additivity stops and antagonism or synergism starts is a difference of 1.5. Thus, all that can be said without re-analysing Deneer's data is that at least 10% of the pesticide mixtures were either antagonistic or synergistic. Interestingly, many of the mixtures that did not exhibit concentration additivity contained chemicals with the same mode of action. This finding contradicts the model for joint action postulated by Hewlett and

Plackett (1952) and the findings of Broderius and co-workers (Broderius and Kahl, 1985; Broderius, 1991; Broderius *et al*., 1995). As such, these results warrant further investigation.

The two largest studies on the chronic toxicity of binary pesticide mixtures were by Faust *et al*. (1994) and Altenburger *et al*. (1996). Faust *et al.* (1994) found that for 66% of the pesticide mixtures they examined with different modes of action the toxicity to an algae was concentration additive. Altenburger *et al*. (1996) found for the 137 pesticide mixtures they examined that the best overall fit to the toxicity was concentration addition. Thus, it appears that the majority of pesticide mixtures have additive acute and chronic toxicity, however between 10 to 40% of these mixtures are either antagonistic or synergistic.

The largest review of mixture toxicity data conducted to date (Ross, 1996; Ross and Warne, 1997) examined the toxicity of 973 predominantly binary, tertiary and quaternary mixtures. This analysis revealed that the median toxicity of the mixtures was essentially concentration additive, that between 75 and 80% of the mixtures were concentration additive, and 20-25% were antagonistic or synergistic. This proportion of mixtures that exhibit antagonism or synergism is very similar to those obtained by Faust *et al*. (1994) (34%) and Deneer (2000) (>10%) and Warne and Hawker (1995) (23%). This relative constancy of this proportion obtained by the three studies supports the concept that approximately 10-30% of mixtures are antagonistic or synergistic. In addition both the work of Warne and Hawker (1995) and Ross and Warne (1997) revealed that there was approximately an equal percentage of antagonistic and synergistic mixtures. Thus, based on the above data approximately 70-80% of mixtures have additive toxicity, 10-15% have antagonistic toxicity and 10-15% have synergistic toxicity.

While roughly 20-30% of mixtures are antagonistic or synergistic the magnitude of the deviation from toxic additivity is generally not large. The analysis by Ross (1996) and Ross and Warne (1997) indicated that 5% of the mixtures had toxicity that differed from concentration addition by a factor greater than 2.5, and 1% of mixtures had toxicity values that differed by more than a factor of 5.

The above findings are in contrast to the conclusion reached by Broderius *et al*., (1995) who stated that:

> "because our data set represents diverse chemicals from the inventory of discrete US industrial chemicals, it is concluded from previous research and the results presented that, when numerous industrial chemicals of a similar mode of action are present in a mixture, one would expect them to be strictly (concentration) additive in their joint toxicity"

In the vast majority of aquatic ecotoxicology mixture research the toxicity of the mixtures is compared only to the toxicity predicted by concentration addition. However, as discussed earlier, there is another type of addition – response addition. Backhaus *et al*. (2000) examined how well the concentration and response addition models predicted the toxicity of mixtures composed of chemicals with dissimilar modes of action. They found that concentration addition underestimated the EC50 values (ie. overestimated toxicity) of the mixtures by a factor of less than three. They therefore concluded that if the type of joint action occurring in the mixture is not known that it is better to assume concentration addition occurs. This is because this model will accurately predict concentration addition

and will provide a conservative estimate of the toxicity of mixtures with response addition.

5.1 THE FUNNEL HYPOTHESIS: A UNIFYING HYPOTHESIS OF MIXTURE TOXICITY

In 1991 Warne observed that simple mixtures (containing less than five compounds) tended to have highly variable toxicities (anything from highly antagonistic to highly synergistic) but complex mixtures (greater than ten compounds) generally had additive toxicity. This was also observed by McCarty and Mackay (1993). An explanation for this trend was proposed by Warne and Hawker (1995).

The mode of action of chemicals can play a significant role in determining the type of interaction that occurs. However, according to the base-line toxicity concept these rules do not apply at all concentrations. All chemicals regardless of whether they have a specific mode of action also exert a non-specific toxic effect. However, there is a threshold concentration below which the specific mode of action does not occur and above which it does. At the concentrations at which acute toxicity usually occurs, the toxicity will be predominantly due to the specific mode of action. However, as the concentration decreases the relative contribution to the total toxicity by the specific mode of action decreases while that for the non-specific remains the same (ECETOC, 2001). Warne and Hawker (1995) used this concept and the critical volume hypothesis of narcotic toxicity (eg. Abernethy *et al*., 1988) to develop the Funnel Hypothesis which is a unifying hypothesis.

This hypothesis states that as the number of components in mixtures increases there is an increased tendency for the toxicity to be additive (Figure 3). Conversely, as the number of components decreases the tendency is for the toxicity of mixtures to increasingly deviate from additivity (Figure 3). Warne and Hawker (1995) collected toxicity data for 104 equitoxic mixtures composed of 182 chemicals. These data included seven test species including bacteria, crustacea, amphibia and fish, covered a variety of measures and endpoints of toxicity and included both acute and chronic values. They found that these data conformed to the hypothesis (Figure 4).

Similarly, Ross (1996) and Ross and Warne (1997) found that toxicity data for 973 mixtures, that included organic-organic, metal-metal, and metal-organic mixtures, conformed to the predictions of the hypothesis. Further, they found that the funnel hypothesis held true for each of these different types of mixtures.

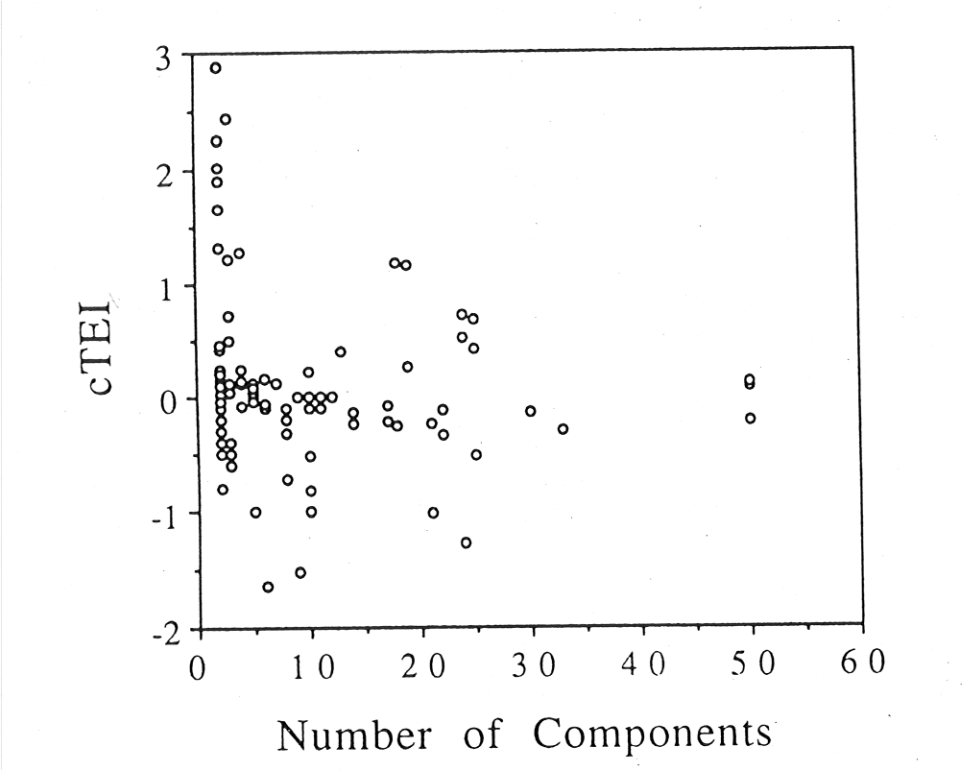

Figure 3. The variation in toxicity of mixtures with the number of components in the mixture predicted by the Funnel Hypothesis (from Warne and Hawker, 1995).

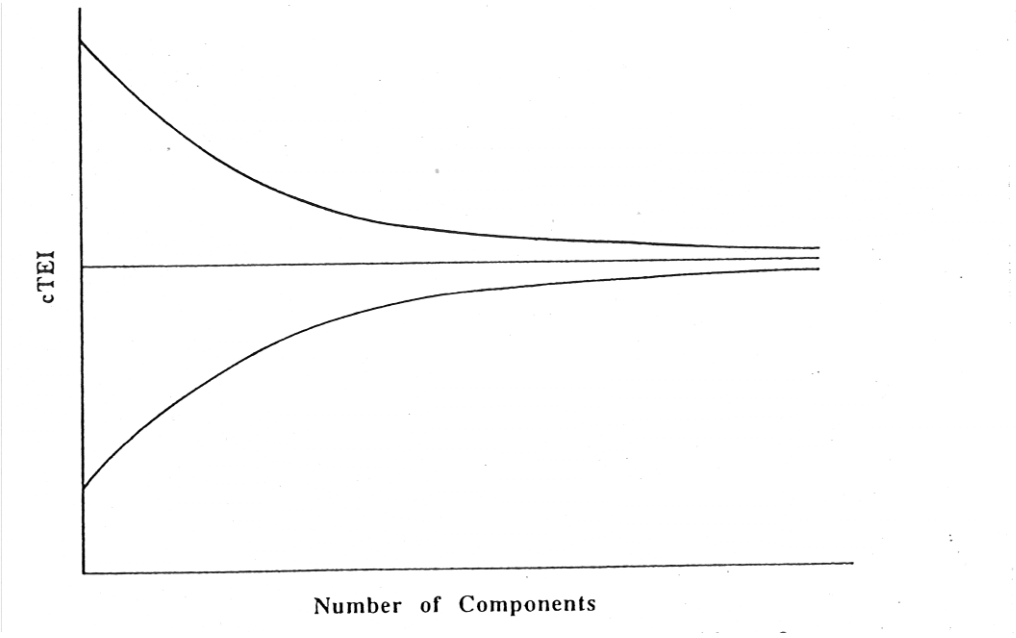

Figure 4. The observed variation in toxicity of mixtures with the number of components (from Warne and Hawker, 1995)

A recent review of the aquatic toxicity of mixtures stated of the Funnel Hypothesis that:

> "importantly, this thought process transcends modes of action"

and that:

> "the literature is quite large that supports this hypothesis (ECETOC, 2001)."

A limitation of this hypothesis is that at present, it only applies to equitoxic mixtures whereas in all likelihood, mixtures in the environment will be non-equitoxic. The relevance of this hypothesis to non-equitoxic mixtures is unknown.

6 USING TOXICITY TESTS TO DETERMINE THE TOXICITY OF EFFLUENTS AND AMBIENT WATER

The traditional approaches to determining the toxicity of mixtures rely on knowing all the chemicals in the mixtures and their mode of action (bottom-up approach). When dealing with mixtures created in laboratories this information may be known. However, when dealing with effluents and ambient waters invariably not all chemicals in the mixture are known or can not be identified. Thus traditional methods can not be reliably used in the real world.

As a result of this two new methods have been developed. These are direct toxicity assessment (DTA), which is also called whole effluent toxicity (WET) testing in the USA, and toxicity identification and evaluation (TIE). These are top-down approaches. For the purposes of this review the term direct toxicity assessment will be used to describe DTA and WET processes which are just different names for the same process. DTA and TIE were developed to resolve different problems in dealing with mixtures. DTA was developed to determine the total toxicity of the effluent or ambient water irrespective of the chemicals present whereas TIE was developed in order to identify the chemical or chemicals in effluents and ambient waters that cause the observed toxicity. These methods will each be discussed below.

6.1 DIRECT TOXICITY ASSESSMENT

This is a fancy name for conducting toxicity tests on complex mixtures, effluents and ambient waters that contain mixtures of chemicals. The prime aim of DTA according to Grothe and Johnson (1996) is to ensure that waste water releases into aquatic ecosystems do no harm. As such they are similar to the aim of toxicity tests for single chemicals, except that DTA assesses the combined toxicity of a number of chemicals of unknown identity and concentration and types of joint action. As such these tests are far more environmentally realistic than single chemical toxicity tests. The clear advantage of this approach to using information about the toxicity and mode of action of individual chemicals to predict mixture toxicity (the bottom-up approach) is illustrated in a Dutch study (Tonkes *et al*., 1995). This showed that in only 7 of the 15 mixtures/effluents examined could the toxicity of the mixture be explained using individual chemical data.

The Australian and New Zealand Water Quality Guidelines (WQGs) (ANZECC & ARMCANZ, 2000) state that additional advantages of DTA are that they: provide a direct measure of toxicity and hence bioavailability; are reliable predictors of impacts on communities (eg. de Vlaming and Norberg-King, 1999), they can provide early warning of changes in toxicity; and that compared to other forms of biological assessment they are relatively rapid and cost-effective. As DTA is a function of bioavailability it has a distinct advantage over the 'bottom-up approach' which relies on measured concentrations of the chemical. So even when the chemicals in a mixture can all be identified and quantified,

and their modes of action are known, the observed toxic effect may not match the predicted effect because the total concentrations used in the assessment did not reflect the bioavailable concentration of the chemicals. The major limitation of DTA is that it does not identify the chemical or chemicals in the mixture that cause the observed toxicity. Such information can be of great importance if the toxicity is to be addressed regardless of whether it is by modifying existing systems or improving technology. DTA is not a one-step solution to the discharge of effluents, rather it is just one of a number of processes. Toxicity identification and evaluation (TIE) procedures are one way of identifying the toxic components of effluents and mixtures and this will be discussed in the following section.

Because of the above strengths DTA is now widely used in Austria, Australia, Canada, Denmark, Ireland, New Zealand, Sweden, UK, and the USA amongst others (ANZECC & ARMCANZ, 2000). An excellent review of the current status of DTA in Australia and New Zealand is provided in the Australian and New Zealand WQGs (ANZECC & ARMCANZ, 2000) and is expanded on in Van Dam and Chapman (2001). A couple of examples of where it has been used successfully are the Mount Lyell Remediation Research and Demonstration Program in Tasmania, the Australian Newsprint Mill's program (NSW) and the Sydney Water Corporation Hawkesbury-Nepean River STPs Toxicity Assessment Program (ANZECC & ARMCANZ, 2000).

DTA assessments fall into three broad categories; standardised tests; site-specific tests and *in situ* tests. Standardised DTA is where the test procedures and organism are highly standardised. The USEPA and Environment Canada for example, have developed an array of standardised procedures. The advantages of standardised DTA tests are that experimental variation is minimised and it allows comparison of the results from one effluent/site to another.

Site-specific DTA uses test organisms that are local to the impacted area for testing and local water from upstream of the discharge point or from a clean reference site. The experimental conditions may also be modified from the standard in order to reflect the exposure scenarios (eg. frequency and duration) likely to be experienced at the site. Such tests will obviously give a far more relevant assessment of the toxicity at the site in question but the ability to make comparisons between sites is limited. The use of local water can also introduce complexing factors into the experiments.

In situ DTA is the same as site-specific with the exception that the tests are conducted not in the laboratory but in the water of the site being investigated. These are the most environmentally realistic DTA tests but they suffer due to variability that is inherent in the environment.

The decision of which type of DTA tests to use depends on the objectives of the investigation. Similarly, the choice of which species to use in the site-specific and *in-situ* DTA tests depends on the existing fauna, ease of collection or culturing, whether they are robust to handling and can be used reliably in toxicity tests.

The results of DTA assessments can be used to establish licences to discharge effluents. A common approach to deriving licences for individual chemicals is to calculate predicted no effect concentrations (PNECs). These are obtained by dividing the lowest available toxicity value for the chemical of concern and dividing it by an application factor (also

called assessment or safety factors). The minimum data required using this approach is a fish, a crustacean and an alga toxicity datum (OECD, 1995). Alternatively, one of a variety of statistical extrapolation techniques (ie. Stephan *et al*., 1985; Wagner and Løkke, 1991; Aldenberg and Slob, 1993; Shao, 2000) can be used. Typically, these statistical approaches require at least toxicity data for five species that belong to at least four taxonomic groups (Wagner and Løkke, 1991; Aldenberg and Slob, 1993; Warne, 2001). It is therefore, more likely that DTA assessments will be used to derive water licences using the application method than the statistical methods (Pedersen and Petersen, 1996).

Pedersen and Petersen (1996) conducted a study in which they examined the variability of species sensitivity to complex mixtures. This revealed that smaller application factors were needed to account for this variability for complex mixtures than for individual compounds (Table 4). They then derived a set of application factors to be used in deriving PNEC values for complex mixtures.

No such modifications are required in order for the statistical methods to derive PNECs using DTA data. They are derived using exactly the same process as for individual chemicals.

Table 4. Application factors (AFs) recommended by the OECD (1995) in order to determine PNECs for individual compounds and the application factors to be used to calculate PNECs based on chronic toxicity data for complex mixtures (Pedersen and Petersen, 1996).

Toxicity data available	OECD AFs for individual chemicals	AFs for complex mixtures
Lowest EC50 for acute data	1000	200
Lowest acute EC50 for algae, crustacean and fish	100	20
Lowest acute EC50 for five species from at least four taxonomic groups	-	10
Lowest NOEC for chronic algae, crustacean and fish	10	5

6.2 TOXICITY IDENTIFICATION AND EVALUATION

This method was developed in order to identify the chemical or chemicals in samples that cause toxicity. This is necessary, as once the cause of toxicity is known then more targeted remediation or treatment processes can be implemented.

There are a number of TIE systems but the most widely used is that developed by the USEPA and hence this will be discussed. The TIE methods arose from the push by regulatory agencies to reduce the toxicity of effluents being discharged (Doi, 1998). One of the earliest publications on TIE methods was by Walsh and Garnas (1983). The approach was developed further by Burkhard and Ankley (1987) and expanded and systematised by the USEPA (1991a, b and c) and has since been widely adapted by many developed nations including Australia. Prior to the use of TIE in Australia research and development work was conducted in order to develop the TIE procedures for local species. TIE methods have now been developed for the following Australian aquatic species: the

freshwater crustacean *Ceriodaphnia* cf. *dubia* (Manning *et al*., 1993; Pablo *et al*., 1997; Bailey *et al*., 2000a, 2000b); the freshwater algae *Pseudokirchneriella subcapitata* (Pablo *et al*., 2002); the marine invertebrate (sea-urchin) *Heliocidaris tuberculata* (Doyle *pers.comm*.; Krassoi, *pers. comm*.) and terrestrial plants (Pablo *et al*., 2002). Within Australia TIE procedures have predominantly been used for regulatory purposes. The NSW EPA has used TIE to investigate the sources of invertebrate toxicity in South Creek (Pablo *et al*., 1997) and the causes of phytotoxicity in market gardens along South Creek (Pablo *et al*., 2002). TIE has also been extensively used by Sinclair Knight Merz in their toxicity assessments of all the freshwater sewage outfalls of Sydney Water Corporation (Bailey *et al*., 2000a, 2000b).

The Australian and New Zealand Guidelines for Fresh and Marine Water Quality (ANZECC & ARMCANZ, 2000) recommend the use of TIE in combination with DTA when assessing mixtures and/or a site specific investigation is being conducted (ANZECC & ARMCANZ, 2000; Van Dam and Chapman, 2001).

6.2.1 TIE procedures

TIE investigations are typically conducted in three sequential phases, called Phase I, II and III. Phase I is where the type of chemicals causing the toxicity are identified, Phase II is where the chemicals are chemically identified and quantified, and Phase III is where tests are conducted to confirm the results of Phases I and II. The steps involved in each phase are summarised below. Readers are recommended to read the USEPA (1991b and c) TIE guidance document for more details.

In Phase I the toxicity of the original sample to the test species is determined. Once this is done, aliquots of the sample are chemically and physically treated in order to remove different types of chemicals (Table 5).

Table 5. Examples of some of the chemical and physical treatments of samples that occur during Phase I and the types of chemicals that are removed by each treatment.

Treatment	Types of chemicals removed
Aeration	Volatiles
C-18	Non-polar organics
Filtration	Particulates
Cation-exchange resin	Anions
EDTA	Cations
Sodium thiosulfate	Chlorine

After each treatment the toxicity of the treated sample has its toxicity determined. If there is no decrease in toxicity then the chemicals removed by that treatment did not contribute to the toxicity. However, if the toxicity does decrease then the chemicals removed by the treatment contributed to the toxicity of the sample.

In Phase II various chemical analyses are conducted depending on the type of chemicals identified in Phase I. This can be relatively straightforward for metals, or it can be complex in the case of non-polar organics. For the latter, it is often necessary to separate the components in the treated sample. This can be done using a high performance liquid chromatograph and collecting fractions at different time intervals or passing the sample through a C-18 solid phase extractor and then serially eluting chemicals with solvents of different polarity. The fractions generated by these processes are then tested to determine

which contains the toxicant. Only then would analysis by methods such as gas chromatography combined with mass spectrophotometry be conducted.

In Phase III a variety of different techniques can be used to confirm whether the chemicals identified in Phase II are the causes of the toxicity in the original sample. These techniques include correlation, species sensitivity, spiking, mass balance, matching of symptoms, and deletion of chemicals. Further information on these techniques is provided in USEPA (1989) or Doi (1998).

While TIE can be very successful it does have a number of limitations. Firstly, it is best suited to use with acute toxicity tests as longer chronic toxicity tests would lead to the potential problem of temporal changes in the composition and toxicity of the sample. Nonetheless, the USEPA (1991b) has developed TIE procedures utilising chronic toxicity tests. To the author's knowledge chronic toxicity tests have not been used for TIEs in Australia. This means that chemicals that might cause chronic toxicity may not be identified. A second limitation, is the inability to determine all the chemicals that contribute to toxicity. The method can really only identify those chemicals that contribute significantly to the toxicity (ie. typically toxic effects greater than 20%). It is quite well known from work by Könemann (1981), Deneer *et al*. (1988) and Van Leeuwen (1991) that chemicals at concentrations as low as 0.02 of their NOEC (where a NOEC typically causes a 20-30% effect) values contributed to toxicity. However, in the majority of TIE investigations the toxicity is accounted for by three or less chemicals (Doi, 1998). This highlights the insensitivity of TIE procedures. A third limitation is that there are not Phase II methods for polar organics, anionic compounds and high molecular weight compounds such as polymers (Doi, 1998). This means that if any of these compounds cause toxicity in the original sample they will not be identified. However, as appropriate analytical techniques for these compounds are developed they can be incorporated into TIE.

7 INCORPORATING THE TOXICITY OF MIXTURES INTO ENVIRONMENTAL QUALITY GUIDELINES AND HAZARD AND RISK ASSESSMENTS

Because most chemicals occur in the environment as mixtures and it is known that the toxicity of mixtures is different to that of the individual components it is logical to attempt to incorporate the toxicity of mixtures into environmental quality guidelines (ie. water, soil, sediment and air quality guidelines) and into hazard and risk assessments.

The vast majority of hazard and risk assessments do not directly address the toxicity of the mixtures that are encountered, rather they conduct the analyses on each of the individual chemicals. However, there is no reason why the toxicity of the mixtures can not be addressed in at least a conservative manner (ie. assuming concentration additivity). There is no excuse for not considering the toxicity of mixtures in hazard and risk assessments. The adoption of such an approach for human health issues would not be accepted, and nor should it be for the environment. To do so is to knowingly underestimate the hazard or risk and therefore under-protect the environment.

A series of articles on the issue of incorporating the toxicity of mixtures into environmental quality guidelines (EQGs) were published in 1996 (Vighi and Calamari, 1996; Van Leeuwen *et al*., 1996; Grimme *et al*., 1996) all concluded that the toxicity of mixtures should be incorporated into the derivation of guidelines and recommended the

concentration addition model. They recommended a number of possible methods including:

- Assuming that non-reactive organic chemicals have the same mode of action and therefore the toxicity of mixtures of these chemicals is concentration additive. As so many of these chemicals occur together this would have the effect of the permissible aqueous concentrations 'approaching zero' (Van Leuuwen *et al*., 1996).

- Deriving guidelines for individual chemicals and then applying an arbitrary safety factor. This is essentially what the Dutch did to derive their negligible risk values. Some have argued (Van Leeuwen *et al*., 1996) that the Dutch by dividing the acceptable risk levels by a factor of 100 to derive their negligible risk values, have taken into account the toxicity of mixtures in their water quality guidelines. The basis of this factor of 100 was that water in the Rhine at any time contains at least 100 chemicals (VROM, 1994) and therefore if additivity is assumed the WQG for each chemical should be divided by 100 in order to protect the environment. However, this argument is misleading as the water quality guidelines currently implemented in The Netherlands are the acceptable risk levels, rather than the negligible risk levels which do account for the toxicity of mixtures. Thus, the WQGs of The Netherlands do not account for mixture toxicity.

- Deriving guidelines for groups of chemicals that have the same mode of action and that could reasonably be expected to occur together. The method recommended to derive these was the toxic equivalent factor (TEF) or the toxic equivalents (TEQ) method. This method has been most widely used to derive EQGs for dioxins. Each member of the group of related chemicals is allocated a TEF based on their toxicity relative to that of the most toxic member, which is given a value of one. The total toxicity of the mixture is calculated as the sum of the concentration of each member (C_i) multiplied by its TEF (TEF_i) using the following equation

$$\text{Total toxicity of mixture} = \sum(C_i \times TEF_i) \qquad (5)$$

This system works well in human toxicology as there is only one test species to determine the TEF values for. But in ecotoxicology, where there are multiple species it becomes more problematic to determine the most toxic member and assign relative toxicities. Because of this difficulty, to the author's knowledge, WQGs have only been derived using this method to protect organisms from secondary poisoning. This method assumes concentration addition and it also assumes that all the chemicals used to determine the TEF for the mixture will always be present. The method also does not provide any guidance on how to deal with mixtures of the chemicals dealt with in the TEF manner and other individual chemicals or other groups of chemicals.

It is not simple to incorporate the toxicity of mixtures when deriving environmental quality guidelines. The principal reason for this is that such guidelines are generally derived on a national basis and it is therefore not possible to consider all the permutations that could occur at specific sites. More specific reasons are:

- Temporal changes to the chemistry of mixtures – due to chemical reactions, volatilisation, degradation, differing partitioning behaviour and environmental

persistence etc. There can be changes to the actual chemicals present in the mixture or simply a change to the relative concentration of the chemicals in the mixture.

- The compounds that occur at any given site are not and will not necessarily be known.

- The concentrations of the chemicals in the mixtures that occur at any given site are not and will not necessarily be known.

- Temporal changes to the chemistry of mixtures due to changes in the processes that create the effluents (eg. a factory uses a different chemical reaction to produce the same product or produces a different product, a user changes the herbicide they use)

- Temporal changes to the chemistry due to changes in seasonal usage patterns of certain chemicals (eg. spraying of herbicides and pesticides)

The only practical option at this stage, is to derive WQGs for individual chemicals and then to put in place a system for dealing with mixtures in the implementation phase of the guidelines. Most countries including the USA, The Netherlands, South Africa and Denmark have done the former but not the latter. A process that did both the former and latter was developed and recommended by Warne (1998) and was subsequently adopted in the Australian and New Zealand Guidelines for Fresh and Marine Water Quality (ANZECC & ARMCANZ, 2000).

The Australian and New Zealand water quality guidelines (ANZECC & ARMCANZ, 2000) recommend the following in dealing with mixtures:

1. if the mixture is simple (ie. up to 5 components if the toxicity is additive) estimate the total toxicity of the mixture (TTM) using the following equation

   $$TTM = \sum (C_i/WQG_i) \quad (6)$$

   where C_i is the concentration on the 'i'th compound in the mixture and WQG_i is the water quality guideline for that component. If TTM equals or is greater than one then the mixture has equalled or exceeded the water quality guidelines respectively. If however, the TTM is less than one then the guidelines have not been exceeded.

   If the guidelines have been exceeded then further action is triggered. This can take the form of managing the site, remediation or direct toxicity assessment. An explanation of direct toxicity assessment is provided in the following section. An example of determining the toxicity of a mixture using the above method is provided in Table 6. A limitation of this method is that if there is not a WQG for a chemical then the toxic contribution of that chemical can not be included.

Table 6. The concentrations of benzene, toluene, ethyl-benzene and ortho-xylene that have the same mode of action and their water quality guidelines and whether or not the WQGs have been exceeded when assessed on an individual basis and as a mixture.

Chemical	Concentration (mg/L)	WQG (mg/L)	Proportion of WQG	Exceed WQG?
Benzene	310	500	0.62	No
Toluene	150	180	0.83	No
Ethyl benzene	11	80	0.14	No
Ortho-xylene	156	350	0.45	No
Mixture of the four chemicals above	See above	1*	2.04	Yes

* Refer to equation 6 for why the WQG for the mixture has a value of 1.

Another example of the implementation of this method is where WQGs have been derived for a number of structurally related chemicals but chemical analytical methods generally are not able to differentiate between the chemicals. An example of this situation for ortho-, meta- and para-xylene is provided in Table 7. In such a case a conservative assumption is made that the lowest WQG applies for the mixture (ie. the WQG for para-xylene applies to the mixture). The proportion of the WQG is then calculated (ie. total concentration of xylenes/WQG for mixture) in order to determine if the WQG for the mixture has been exceeded.

Table 7. The total concentration of xylenes (meta-, ortho-, and para-) and their water quality guidelines and whether or not the WQGs have been exceeded when assessed on an individual basis and as a mixture.

Chemical	Concentration (µg/L)	WQG (µg/L)	Proportion of WQG	Exceed WQG?
meta-xylene	nm[1]	350	nc[2]	nc
ortho-xylene	nm	-	nc	nc
para-xylene	nm	200	nc	nc
Total xylenes	250	200	1.25	Yes

[1] not measurable. [2] not calculable

2. if the mixture is complex (ie. > 5 components or uncertain mixture effects), then it is recommended that the investigation proceed to direct toxicity assessment.

The advantage of this scheme over those proposed by Vighi and Calamari (1996), Van Leeuwen *et al*. (1996) and Grimme *et al*. (1996) is that it does not require the complete chemical characterisation of the sample, which is seldom possible. It is the firm believe of the author that the default position should be that the toxicity of all mixtures (even complex ones) is assumed to be concentration additive until the proponents can prove otherwise using existing information from the literature or by conducting mixture experiments or direct toxicity assessments.

This method also has assumed that the concentration addition form of joint action is the most appropriate method to predict the toxicity of mixtures. The response addition model was not used because it requires the concentration-response relationship for each component in the mixture and this information is generally not provided in the literature (Warne, 1998). It could be argued by some, that this assumption is overly conservative.

However, the data provided by Ross and Warne (1997), Faust *et al*. (1994), Deneer (2000), Warne and Hawker (1995) clearly showed that approximately 10-15%, 70-80% and 10-15% of mixtures have antagonistic, additive and synergistic toxicity respectively. Thus, assuming additivity only overestimates the toxicity of 10-15% of mixtures (ie. antagonistic mixtures). It correctly estimates the toxicity of 70-80% of mixtures and under-estimates the toxicity of 10-15% of mixtures. Thus, the assumption of concentration addition is realistic, it is not overly conservative.

Caux and Kent (2001) stated that:

> "single substance approaches are not relevant and guideline derivation for complex mixtures will need to be based on new concepts and measurements. For example, to supplement the use of chemical and physical guidelines, the use of biological guidelines (biocriteria) can be made".

While they were referring to water quality guidelines, this could equally apply to guidelines for any other medium.

The new Australian and New Zealand Guidelines (ANZECC & ARMCANZ, 2000) have moved significantly in this direction and readers are referred to this document. For example, for 'high conservation value' ecosystems there are a number of different bases for determining the guidelines that apply. The possible guidelines are: the 80% percentile of the background concentration or the single chemical guidelines that protect 99% of species. However, whatever is applied no measurable change in the form and functioning of the ecosystem is permitted. The best way to measure if ecological change is occurring is by biomonitoring and extensive guidance on this is provided in the Australian and New Zealand Guidelines (ANZECC & ARMCANZ, 2000) and by Humphrey *et al.* (2001). While these developments are a start in the direction indicated by Caux and Kent (2001) considerably more progress will most likely be made in the near future.

While the above discussion has focussed on the incorporation of mixture toxicity into water quality guidelines the same methods could equally easily be incorporated into sediment, soil or air quality guidelines providing there is sufficient appropriate toxicity data available.

8 RECOMMENDATIONS

It is argued that the results of mixture toxicity testing using aquatic organisms and the conclusions drawn from these studies are directly applicable to terrestrial organisms. Therefore, it is argued that the following recommendations should be used for all environmental quality guidelines (ie. water, soil, sediment and air) until there is sufficient data for the other compartments of the environment that the validity of these recommendations can be checked and modified if appropriate.

It is recommended that environmental quality guidelines be derived for individual chemicals rather than mixtures. Further, it is recommended that a system based largely on the Australian and New Zealand Guidelines for Fresh and Marine Water Quality (ANZECC & ARMCANZ, 2000) but containing an additional feature is used to deal with mixtures in contaminated sites. The recommended system is:

1. where mixtures that contain chemicals with unknown modes of action or different modes of action it is assumed that the toxicity is mildly synergistic (ie. all mixtures are 2.5 times more toxic than concentration addition). This is recommended, as only 5% of mixtures are more synergistic than 2.5 times additivity. In other words this assumption will provide adequate protection from 95% of mixtures. The total toxicity of the mixture (TTM) is determined using equation 6 and compared to the cut-off value to determine if the WQG for the mixture has been exceeded. The cut-off value is determined as 1 ÷ the number of times the mixture is more toxic than concentration addition. As it is assumed that the toxicity is 2.5 times concentration addition the cut-off is 0.4 (ie. 1 ÷ 2.5). Thus if the TTM is greater than 0.4 then the WQG for that mixture has been exceeded. However, if the mixture can be proved not to be synergistic using literature or direct toxicity assessment (point 3), then the cut-off value should be recalculated in order to determine whether the mixture meets the WQG. This can be done by comparing the TTM to a new cut-off value calculated using the above method and the toxicity of the mixture from the literature or toxicity tests.

2. where mixtures contain chemicals with the same mode of action it is assumed that the toxicity is concentration additive. The vast majority of the literature shows that mixtures of chemicals with the same mode of action have concentration additive toxicity irrespective of the number of components in the mixture. Thus if the total toxicity of the mixture (equation 6) is greater than 1 then the WQG for that mixture has been exceeded. However, if the mixture can be shown (using literature or toxicity tests) not to have additive toxicity then whether the mixture exceeds the WQG is determined using the procedure outlined in the previous point.

3. conducting mixture toxicity experiments (bottom-up approach) or direct toxicity assessment (DTA) and, where appropriate, toxicity identification and evaluation procedures for complex mixtures. Direct toxicity assessment has a number of advantages over the bottom-up approach and is therefore recommended over the latter approach. It is preferable to conduct DTA on at least five species that belong to at least four taxonomic groups (ie. conforming to the minimum data requirements of the statistical distribution methods used to derived WQGs). However, the very minimum number of species to be tested is three – which must consist of a vertebrate (usually a fish), an invertebrate (usually a crustacean) and a plant. An equivalent suite of organisms would be required for terrestrial situations.

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