Aquatic Toxicity of Mixtures

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SUMMARY

The inherent toxicity of a substance to aquatic organisms is typically determined via single species laboratory tests. Results from these tests are used to determine a Predicted No Effect Concentration (PNEC) for ecosystems per substance. Since organisms in the environment are exposed simultaneously to a wide array of substances, it is important to understand the potential effects of mixtures to aquatic organisms. The effects of mixtures can be generally categorised as additive, greater than additive (synergism) and less than additive (antagonism). Effects that correspond to the addition of toxicities for each mixture component are considered additive.

Via acute toxicity tests, mixtures of substances that are chemically related or have the same mode of action are generally found to be additive. However, some "groups" of substances when tested in relatively simple mixtures do not behave in a readily predictable manner (e.g. metals and some pesticides). Even so there are only a few examples in the literature of synergism where the effects are more than three times greater than those predicted from additivity of acute toxic effects. When large numbers of substances are present in mixtures at low concentrations relative to their individual acute toxicities, additivity of acute toxic effects is closely followed. This holds true even when the substances are not related chemically, or exhibit different modes of action when acting as acute toxicants alone. This phenomenon for organic substances has been called "baseline toxicity", or narcosis.

The same theory applies to chronic toxicity tests. That is, if organic mixture components are at concentrations below the level exerting chronic toxicity, then additivity can be expected - thereby supporting the concept of baseline toxicity. Mixture components with the same mode of action can be expected also to act additively. However, it is not possible to make generalisations about the chronic toxicity of mixtures containing metals. Such mixtures can give responses across the entire range of interactions from antagonism to synergism. This may be due largely to different modes of action and differences in metal speciation in mixtures compared to single toxicant tests.

While data from model ecosystems, field studies and effluent studies are generally limited and difficult to interpret from the standpoint of the toxic effects of mixtures of substances, the evidence tends to support the basic concept of additivity, particularly when the role bioavailability can play in reducing toxic effects under environmental conditions is taken into account. Predicted mixture effects based on body residues from organisms exposed in the field indicate that additivity of substances below their PNECs (baseline toxicity approach) is sufficiently conservative for protection of aquatic resources.

1. INTRODUCTION

Single-species laboratory aquatic toxicity tests are used widely in the assessment of the potential impact of substances in the aquatic environment. In these laboratory tests, toxicity is normally determined for single substances. The toxicity observed in these studies may be used to calculate a Predicted No Effect Concentration (PNEC) for the individual substance. In the environment however, organisms will be exposed simultaneously to a variety of substances[1] . This report discusses how the potential effect of these mixtures of substances in the environment might be addressed.

Exposure to more than one substance at the same time may:

- elicit toxic effects which would be expected if the toxicities of the individual substances were simply added together (concentration addition);
- elicit toxic effects less than would be expected if the toxicities of the individual substances were simply added together (less than additive; antagonism); or
- elicit toxic effects greater than would be expected if the toxicities of the individual substances were simply added together (more than additive; synergism).

The challenge for environmental toxicologists and regulators is to assess to what extent consideration of the toxic effects of mixtures of substances is needed, and how this might be achieved to protect adequately the environment. This report reviews the current state of knowledge and considers some possible approaches. The report does not address the testing of preparations in the context of mixtures of substances prepared for sale as products.

Different models describing the toxicity of mixtures of individual substances have been described and thoroughly investigated for the aquatic environment. Key substance attributes appear to be:

- dosed concentration (acute to subchronic);
- the mode of action of each substance;
- whether these modes of action are interacting or not.

To address these issues, ECETOC formed a Task Force with the following Terms of Reference:

- Review the data on acute and chronic mixture toxicity from the primary literature as well as internal company databases;
- critically evaluate the available data concerning the potential for mixture toxicity in chronic exposures for representative substance classes;
- if necessary, recommend a research programme to address outstanding issues in the area of aquatic mixture toxicity.

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1 Exposure to mixtures in the environment will be the result of the integration of differing partitioning and loss processes for individual substances, which results in different temporal and spatial distributions and concentrations of these individual substances in the environment. Therefore, this report focuses on environmental mixtures rather than product formulations or other 'initial' mixtures which are not present as such in the environment.

2. BACKGROUND

Consideration of the toxic effects of mixtures of substances is not new. Development of a terminology and definition of different potential outcomes, dependent on the properties of a binary mixture, occurred prior to 1960. However, relatively few experimental data have been generated on the toxicity of mixtures, compared to the wealth of data on individual substances. This is due primarily to the complexity of experimental designs and the large number of tests required for exhaustive examination of even fairly simple mixtures. Given these and other practical constraints, the field of environmental risk assessment has evolved to deal primarily with single substances, with occasional consideration of interactions with other factors that may alter their bioavailability, such as humic acids or suspended solids. However, the potential impact of mixtures of individual substances in the environment is recognised as an unresolved issue.

2.1 Mixture Toxicity: Terminology and Classification

The development of terminology and classification of the toxicity of mixtures by Bliss (1939) has been elaborated by Plackett and Hewlett (1952). The different models of joint action are briefly described below, with a comparison of the terms applied in Table 1.

Table 1: The four types of joint action for mixtures

	Similar joint action	**Dissimilar joint action**
Non-interactive	**A. Simple similar** (concentration addition) (simple addition)	**B. Independent** (response addition)
Interactive	**C. Complex similar** (more than additive [synergistic]) or (less than additive [antagonistic])	**D. Dependent** (more than additive [synergistic]) or (less than additive [antagonistic])

Where:

interactive	=	one substance influences biological activity of the other substances
non-interactive	=	no one substance influences the biological activity of the other substances
similar joint action	=	same site of primary toxic action
dissimilar joint action	=	different site of primary toxic action
synergistic	=	toxic effect more than additive for two or more substances
antagonistic	=	less toxicity observed than for the sum of the individual toxicities

Note

Different authors have used different criteria to determine whether the experimentally-derived results meet the criteria of additivity. Some criteria are based on complex statistical

manipulations, others are *a priori* determinations (e.g. if values are within + 50% of the calculated endpoint). For further discussion of the statistical treatment and its implications, the reader is referred to Christensen and Chen (1985), Nirmalakhandan *et al* (1994), and Pounds and Kodell (1991). The Task Force refers to the various authors' statistical or decision making framework in all sections throughout the report, not only those in the discussions in this section.

2.1.1 Types of joint actions

Four types of joint action with respect to quantal responses (A,B,C and D) have been defined for binary mixtures (i.e. by classifying each member of a group of organisms similarly treated as having responded or not) (Plackett and Hewlett, 1952).

- When the substances in a mixture have similar joint action and when interaction between them is absent, the toxicities of the substances in the mixture are considered simple similar (type A in Table 1).
- When interaction between the substances is absent, but the substances have dissimilar sites of action, the toxicity is considered independent, also termed response addition (type B in Table 1).
- The joint action of the two above can be either complex similar (type C in Table 1) or dependent (type D in Table 1).
- If any aspect of the toxicity observed is less than the sum of that of the two individual substances, this is termed antagonism.
- Where only one of a pair of substances is toxic and the other enhances its toxicity this is termed potentiation.

Plackett and Hewlett (1952) have developed mathematical models to describe the resulting dose-response curves for mixtures. Christensen and Chen (1985) updated and contrasted several mathematical approaches.

When two compounds are interactive (one influences the biological activity of the other), as in types C and D, numerous outcomes are possible which may be difficult to predict. For example, one substance may modify the absorption, distribution, metabolism, and/or elimination of another substance(s). Thus, induction of detoxification enzymes or other changes brought about by the presence of one substance may result in the more rapid or complete biotransformation of another substance, leading to reduced levels in the organism and therefore reduced toxicity due to that substance. On the other hand, one substance may increase the penetration of another to the target receptor (organ/tissue) within the organism, leading to an apparent increase in toxicity. Furthermore, a mixture may result in physical or chemical interactions which alter the identity of one or more initial substances, such that their individual toxicities are not expressed or are neutralised, e.g. HCl and NaOH, which react to form NaCl and H_2O.

As an additional confounding factor, some researchers consider bioavailability to be an interactive factor. Bioavailability is considered in more detail in Section 5.2.

Because of the difficulty in predicting and modelling substance interactions, most of the theoretical and experimental research carried out in this area has focused on concentration

addition (type A), and to a lesser extent on response addition (type B) - the two types of joint action which are non-interactive. As the review of the literature shows (Sections 3 and 4), this focus turns out to be appropriate for the vast majority of cases and for both acute and chronic laboratory exposures.

In the following discussion and throughout the rest of this report, we will use the concept "additivity" (= concentration addition) as a reference point. For multiple chemical mixtures (n > 2), the terms "less than additive" and "more than additive" will be used if the mixture is not simply additive. Within a complex mixture, the mechanism(s) by which less or more than additivity is elicited is often not known, such that the strict definitions of synergism or antagonism would not hold. We will also refer primarily to the Toxic Units (TU) approach as described below.

2.1.2 Models of mixture toxicity, with emphasis on TU approach

In the literature, several different theoretical models describing mixture toxicity based on concentration addition can be found. For example, the toxicity of a mixture can be expressed by:

- Toxic Unit (TU) (Brown, 1968; Sprague, 1970),
- Additivity Index (AI) (Marking, 1977),
- Mixture Toxicity Index (MTI) (Könemann, 1981).

Other models take response addition into account (e.g. Finney, 1971).

The toxic unit (TU) concept is based on the endpoint of an acute or chronic toxicity test. For acute toxicity the $L(E)C_{50}$[1] is commonly used, for chronic toxicity the NOEC, LOEC, EC_{10} or EC_{20} of the substances in a mixture is used. The concentration of a substance is expressed as a proportion of the response. For example, the concentration of each individual component of a mixture is divided by its LC_{50} to derive the TU of that component (Brown, 1968, for an example of TU using 48-h LC_{50} to rainbow trout). The TUs of the mixture are summed (Brown, 1968) however there is no statistical or theoretical basis for arriving at the value at which a significant deviation from the expected toxicity occurs.

- If the sum of toxic units equals 1.0, and the response is what would be expected when TU equals 1.0 for any single compound the toxicity is assumed to be simple additive (equals concentration addition).
- A more-than-additive toxicity is indicated if the sum of toxic units is less than 1.0 but the response is close to that expected at TU equals 1.0.
- If the sum of the TUs in a mixture that produces a response expected at TU equals 1.0 exceeds 1.0, the toxicity of the mixture is indicated as less-than-additivity or antagonistic if less than half of the organisms respond, depending on the strength of the effect seen.

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1 Some investigators have included information from the dose response curve (DRC) into account rather than replying solely on point-based joint action assessments. An example from the field of drug interaction is Poch, 1993.

Since the assessment of whether on not a mixture of chemicals acts by concentration addition is likely to require some empirical measurement, it is prone to experimental error. Such errors are associated with the measurement or prediction of the individual chemicals in the mixture as well as with the determination of the toxicity of the mixture itself. The uncertainty associated with these errors can make it difficult to determine if one or more chemicals in a mixture makes an additive contribution to the overall toxicity or not.

The maximum signal to noise ratio is achieved by measuring the toxicity of binary mixtures with each chemical at an equitoxic concentration, i.e. where each chemical is present at the same fraction of a TU, and with the toxicity of the mixture expected to be equal to 1 TU, assuming concentration addition. This simplest mixture test avoids the problem associated with some complex mixtures where a small number of chemicals may account for the major portion of the overall toxicity and where consequently the ability to measure the contribution from the rest of the chemicals in the mixture is lost due to experimental noise. Clearly, as the number of chemicals in the mixture increases it becomes more difficult to determine whether or not a chemical is acting additively. Therefore when assessing complex mixtures, it may be valuable to use knowledge about the expected mode of action of the chemicals to make selected additional studies to assess if specific sub-mixtures act as predicted.

In practice, it is valuable to run a number of studies either to obtain several results for one replicate test mixture or to generate data on a series of specifically-designed mixtures where the ratio of the chemicals is varied. In the first case replicate tests enable the use of suitable hypothesis tests to be applied to the differences between the mean of the populations of the measured and predicted (assuming additivity or independent action) TU values of the mixture. Comparison of a series of such paired values can provide a statistical basis on which to conclude if the chemicals in the mixture act additively or not. For example, if the measured and predicted TU values are not statistically different at the 95% level of significance, then it is reasonable to conclude that the assumption about additivity is correct. In the second case the data are more likely to provide a weight of evidence approach. Application of statistics to determine additivity is likely to be complex and an assessment may be more dependent on subjective judgement.

The decision on whether the toxicity observed is to be expressed as more than additive or less than additive can therefore be based on a statistical approach or on simple judgement, for example a deviation of >30% from the expected additivity indicates antagonism or synergism.

Marking (1977) noted that the TU approach is not linearly distributed around 1.0. A modified system was developed to normalise concentration addition as a reference point (zero). Negative values (to -1.0) represent less-than-additivity, positive values represent more-than-additivity (to +1.0). Note that this Additivity Index (AI) model is still based fundamentally on the TU approach.

Könemann (1981) modified the scale to a mixture toxicity index (MTI) to include both non-interactive types of joint action (concentration and response addition) in a logarithmic form and to account for any number of substances in the mixture.

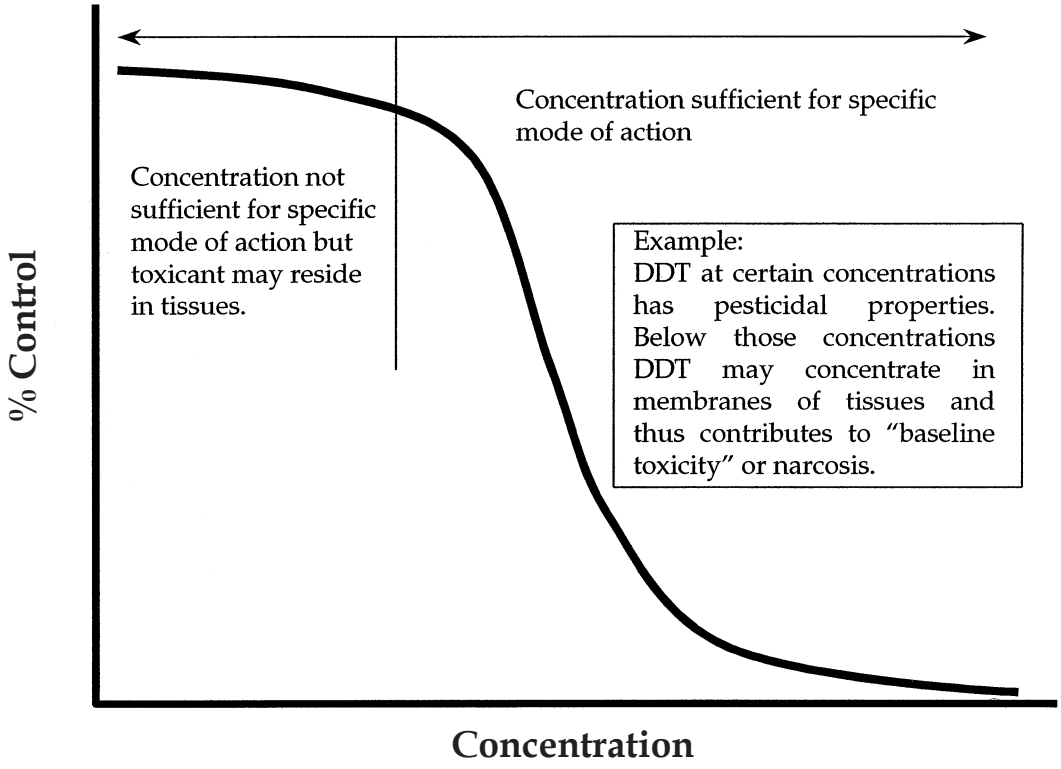
2.2 Modes of Toxic Action of Organic Substances

Because the mode of toxic action of an organic substance is crucial to its classification within a mixture (e.g. whether it has similar or dissimilar joint action) a summary of types of modes of action is presented below.

2.2.1 Non-specific modes of action: "Non-polar Narcosis", "Baseline Toxicity"

The concepts of baseline toxicity (Deneer *et al*, 1988; van Wezel and Opperhuizen, 1995; Verhaar *et al*, 1995; Van Loon *et al*, 1997) and critical body residue (CBR) (McCarty *et al*, 1992; McCarty and MacKay, 1993) state that chemicals present in tissues at concentrations below levels that are associated with specific modes of action may impart a narcotic mode of action despite any specific mode of action identified at higher concentrations (Figure 1). For example, an organochlorine insecticide present in tissues at 1/100th the LC_{50} may not be at a sufficient concentration to illicit a neurotoxic effect on fish, yet its presence in the fish may contribute to a generalised, narcotic mode of action. Hence, classifications of substances into specific modes of action are typically based on studies in which acute and/or chronic toxicity thresholds have been exceeded.

Figure 1: Illustration of the relationship of baseline toxicity from a chemical identified as having a specific mode of action



The CBR for non-polar narcosis is approximately 2-8 mmol/kg. Non-polar narcosis (Narcosis I), is considered reversible and due to general membrane perturbation (e.g. Veith and Broderius, 1990). McKim *et al* (1987) linked the apparent differences in different chemical "classes" to separate acute toxicity syndromes. Compounds eliciting a slightly more toxic response, such as phenols and anilines, were characterised as polar narcotics (Narcosis II) in the Fish Acute Toxicity Syndrome (FATS) scheme, with slightly lower CBRs of 0.5 to 1 mmol/kg. The mechanism was viewed as membrane depolarisation, leading to a more toxic response (Veith and Broderius, 1990).

2.2.2 Specific modes of action

Some substances have more "specific" modes of action, indicating more specific interaction with a target receptor (tissue, cell or molecular receptor) which leads to a toxic response. Examples of specific modes of action, again using the FATS classification, include acetylcholinesterase (AChE) inhibitors, oxidative phosphorylation uncouplers and substances causing respiratory irritancy.

2.2.3 QSARs

Aquatic toxicity data are not available for all substances. However, relationships between key physical-chemical parameters and toxicity have been established empirically for several series of substances, within and between chemical classes. Such Quantitative Structure-Activity Relationships (QSARs) have been derived for non-polar narcotics, polar narcotics, and for several specific modes of action (Veith *et al*, 1980). QSARs are accepted as a preliminary indication of toxicity in some national regulatory systems (e.g. US EPA, 1994). The European Union (EU) recently evaluated the use of QSARs for regulatory purposes and concluded that models for chemicals acting by non-polar and polar narcosis are valid in the log K_{ow} range of 1-6. (EEC, 1996). A separate ECETOC Task Force has examined the advantages and limitations of QSARs used in environmental risk assessment (ECETOC, 1998).

In the absence of experimental data, appropriate QSARs for individual substances, if available, can be used to estimate the aquatic toxicity of mixtures when basic physical-chemical properties are known or can be estimated. These data can be used to calculate the theoretical TU of the mixture (see also Section 6). The choice of the appropriate QSAR is critical to obtaining meaningful results.

2.3 Implications for the Toxic Effects of Mixtures

Within the above models, the following implications for the toxic effects of mixtures are apparent:

- all substances should contribute to "baseline" toxicity regardless of their individual concentrations;
- if a substance is below its own threshold for its specific activity, it is assumed not to contribute to this specific mode, but still to contribute to the "baseline" toxicity;

- specific modes of action are not additive across different modes of action (but are additive within their own class).

In summary, the consequences of these various hypotheses are as follows:

- for baseline toxicity (concentration addition) in the mixture (Table 1, A) no threshold exists, a proportional contribution can be summed even if individual concentrations are below the individual NOECs;
- for independent action of the individual substances in the mixture (Table 1, B), a threshold exists. No effects due to independent action are observed if the concentration of each individual substance in the mixture is below its own NOEC. However, these substances can still contribute to "baseline toxicity".

The review of the aquatic toxicology literature which follows (Sections 3 to 5) considers the theoretical actions of the different chemical types, and the consequences outlined above, and indicates that they are in fact observed under experimental conditions.

3. ACUTE TOXICITY IN THE LABORATORY

3.1 Acute Toxicity of Mixtures

There is a large body of literature addressing the acute toxicity of mixtures of substances to a wide range of aquatic invertebrates and fish (short-term tests with algae and bacteria are regarded as multi-generation, chronic tests and are dealt with in Section 4). Substances tested have included metals, surfactants, pesticides (herbicides, fungicides and insecticides) and "general chemicals". This grouping of substances is somewhat arbitrary but reflects the grouping that appears in the literature.

Testing of mixtures has ranged from the use of simple binary combinations of "related" substances to studies with complex mixtures of up to 50 substances from widely different groups.

This brief review is not intended to be an exhaustive analysis of the information available but is an examination of those publications with significant amounts of data considered to be useful in establishing an overview of the acute toxicities of mixtures.

The European Inland Fisheries Advisory Committee of FAO produced a report (EIFAC, 1987), reviewing the literature on "The toxic potential of several chemical substances in combination at low level" (some 192 publications were cited). This review concluded that " ...there is no evidence that mixtures of the common reactive substances, or of nonreactive substances, have markedly more than a concentration additive lethal joint toxic action, and for equitoxic mixtures of more than five substances the joint action is usually slightly less than additive. For all practical purposes, therefore, the possibility of supraaddition or synergism can be discounted for complex mixtures."

The bulk of the data on toxicity of mixtures is addressed in this extensive review (EIFAC, 1987) and the information collated below focuses primarily on papers published since 1986, although, where it was useful, older papers have been considered.

The data from the literature reviewed in this section are considered firstly for chemicals that fall within each of the following groups, metals, surfactants, pesticides and general chemicals and thereafter for complex mixtures of substances from these various groups.

3.2 Acute Toxicity of Metals

Wang (1987) reviewed the factors affecting the toxicity of metals to aquatic organisms, including the effects of mixtures of metals. He concluded that "interactions between heavy metals appear to be without pattern", although not all the data referred to were from acute toxicity tests. This lack of predictability of effects of mixtures of metals was true for binary and multiple mixtures. Despite this lack of predictability, in 21 out of 37 interactions analysed the effects were additive or less than additive.

Spehar and Fiandt (1986), in studies with six metals in fathead minnows (*Pimephales promelas*) and *Ceriodaphnia dubia*, concluded that for acute toxicity on fathead minnows, the effects were more than additive, but for *C. dubia* the effects were additive.

Long-term acute tests of cobalt and copper toxicity to rainbow trout indicated joint toxicity was time dependent. Cobalt-copper mixtures were antagonistic up to 96-h, but additive to "slightly synergistic" up to 14-d exposure (Marr *et al*, 1998).

Survival-time modelling of aluminium and zinc joint toxicity to Atlantic salmon (*Salmo salar*) indicated additivity (Roy and Campbell, 1995). Less than additivity was observed in the filtration rate of zebra mussels exposed to equitoxic mixtures of five metals (Cd, Cu, Ni, Pb, and Zn) (Kraak *et al*, 1999).

In the light of the above it is clear that prediction of the acute toxicity of mixtures of metals is presently not possible with any degree of accuracy. Where prediction of acute toxicity of metal mixtures is necessary, the assumption of additivity is probably the most balanced choice, unless there is clear evidence in the literature that mixtures of the metals under examination behave differently.

3.3 Acute Toxicity of Pesticides

Macek (1975) conducted a series of experiments on the acute toxic effects of binary mixtures of insecticide active ingredients on the bluegill sunfish (*Lepomis macrochirus*). Out of a total of 27 binary pairs tested, 10 showed greater than additive toxicity and 17 showed additivity; pairs were considered to show greater than additive toxicity if the effect was more than 1.5 times the toxicity expected from additivity. Only three pairs of insecticides showed effects more than three times the toxicity expected from additivity.

The acute toxicity of mixtures of formulated insecticides, fungicides and herbicides, to rainbow trout (*Oncorhynchus mykiss*) was examined by Matthiessen *et al* (1988). They found that of 11 combinations tested in binary mixtures, all demonstrated additive toxicity (i.e. were from 0.5 to 1.4 times the toxicity expected on the basis of additivity). Mixture toxicity of binary combinations of three herbicides and a surfactant to channel catfish, bluegill and crayfish (Procambarus spp.) ranged from additivity to less than additive responses (Abdelghani *et al*, 1997).

Deneer (2000) assessed the usefulness of the concept of "concentration addition" for describing the joint effect of pesticides on aquatic organisms, based on literature data from 1972 to 1998. For more than 90% of 202 mixtures in 26 studies, concentration addition was found to predict effect concentrations correctly within a factor of two. Deviations from additivity were most frequently found to be in mixtures with an organophosphorus ester, or a carbamate, with either another organophosphorus ester or a synthetic pyrethroid. Most of the surveyed studies dealt with acute toxicity.

Greater than additive responses for *Chironomus tentans* exposed to mixtures of atrazine and several organophosphate insecticides (trichlorfon, malathion, chlorpyrifos, methyl-parathion, and mevinphos) was observed by Pape-Lindstrom and Lydy (1997).

From the above it can be concluded that in acute toxicity tests, binary mixtures of herbicides, fungicides and insecticides tend to demonstrate additive toxicity, and that instances of significant synergistic effects are few.

3.4 Acute Toxicity of Surfactants

Acute toxicity tests with homologous series of surfactants used in commercial surfactants give a strong indication that additive toxicity applies. For example, the toxicity of a commercial alcohol ethoxylate can be calculated, assuming concentration addition, from a knowledge of the toxicity of each homologue and its content in the commercial mixture. This finding also applies to other types of nonionic and anionic surfactants including linear alkylbenzene sulphonates (LAS), alkyl ether sulphates and alkyl sulphates (Roberts and Marshall, 1995).

Lewis (1992) reviewed the information available on the toxicity of mixtures of surfactants and of mixtures of which surfactants were one component. Lewis concluded that the toxicity trends for mixtures containing a surfactant and either a pesticide or a metal were mixture-specific and therefore it is difficult to generalise or predict the toxicity of such mixtures. For example, combinations of copper and anionic surfactants were synergistic to trout but not copper and nonionic surfactant mixtures (Calamari and Marchetti, 1973). Some pesticide and LAS combinations were synergistic while others were not (Solon and Nair, 1970). In contrast to these rather unpredictable results the effects of mixtures containing oil and surfactants were consistently synergistic (Hokanson and Smith, 1971; Rehwoldt *et al*, 1974; Lavie *et al*, 1984) though it must be recognised that in these tests components were present in excess of their water solubilities and therefore the surfactant may have been acting in part as a potentiation agent.

Pantani *et al* (1990) examined the toxicity of an anionic surfactant and two nonionic surfactants in combination with copper and cadmium and the pesticides methyl parathion and methyl azinphos in tests with *Gammarus italicus*. They tested each metal and each pesticide with each surfactant in binary pairs and found additivity or less for all combinations tested.

Lewis and Perry (1981) examined the effects of four mixtures containing anionic, nonionic and cationic surfactants on bluegill (*Lepomis macrochirus*) and *Daphnia magna*. These acute toxicity tests indicated that the effects were in general antagonistic (72%) or additive (24%), depending on the combinations tested. Only a mixture of anionic and cationic surfactants showed greater than additive effects, and then only in tests with bluegills.

In summary, there is convincing evidence that surfactants, either in combination with each other, or in combination with metals or pesticides, rarely show more than additive toxicity, with regard to acute toxic effects. The exception to this is in tests with oils where components of the mixture were present in excess of their water solubility.

3.5 Acute Toxicity of General Chemicals and of Complex Mixtures

According to Warne and Hawker (1995), as the number of equitoxic components in a mixture increases, there is an increased tendency to observe additivity. The term describing this observation is the "funnel hypothesis". Importantly, this thought process transcends modes of action. For example, a mixture with 10 components each dosed at $1/10^{th}$ LC_{50} (a sublethal concentration, below acute mode of action), will be likely to show additivity. However, as the number of components decreases (toward binary mixtures) there is

increased variance in the observations. Some mixtures may show synergism, some may show antagonism. The literature is quite large that supports this hypothesis. For a "general chemical", where a narcotic mode of action can be assumed, then additivity is strongly supported. Complex mixtures that contain several components also illustrate additivity. Below, are a few of the more prominent publications and observations of mixture toxicity with general chemicals and complex mixtures.

Hermens *et al* (1984b) tested the toxicity to *Daphnia magna* of mixtures of 550 organic chemicals expected to have the same mode of action. The results clearly indicated additivity, as predicted. In 1982 Hermens and Leeuwangh reported on a series of acute toxicity tests with guppies (*Poecilia reticulata*) in which groups of 8 and 24 toxicants with diverse modes of action were used. They concluded that the results indicated additivity, despite the wide range of modes of action. This work was followed by another study, again determining acute toxicity to the guppy, but this time using 4 groups of chemicals, 11 chloroanilines, 11 chlorophenols, 9 reactive organic halides and 11 non-reactive, nonionised organic substances (Hermens *et al*, 1985a). In this study the toxicity of individual representatives of the groups showed a high variance but the joint toxicity tended to be additive or less. Experiments with mixtures of representatives of each of the groups gave less variable results and again the effects were additive, even when the mixture contained 33 chemicals.

Van der Geest *et al* (2000), using mortality of the mayfly (*Ephoron virgo*) as the endpoint, demonstrated that copper and diazinon act in a less than concentration additive manner and this finding was independent of the effect level on which the mixture was judged.

One of the few studies of the effects of mixtures of inorganic chemicals is that of Alabaster *et al* (1983) who reported on the toxicity of mixtures of cyanide and ammonia to Atlantic salmon (*Salmo salar*). It was concluded that mixtures of cyanide and ammonia were 0.6 to 1.25 times more toxic than expected, if additivity was assumed.

Hermens *et al* (1984a) determined the acute toxic effects of a series of 14 anilines in 3 different mixtures on the guppy (*Poecilia reticulata*). They concluded that in all 3 cases toxicity was additive.

Concentration addition and less than additive effects were observed in acute tests with *Daphnia magna* exposed to heterocyclic nitrogen containing compounds (Chen *et al*, 1996)

Using the fathead minnow (*Pimephales promelas*) Broderius and Kahl (1985) examined the effects of binary and multiple equitoxic mixtures of 7 groups of chemicals (7 alcohols, 4 ketones, 4 ethers, 3 alkyl halides, 3 benzenes, 3 nitriles, and 3 tertiary aromatics) on acute toxicity. They demonstrated additive joint action for the chemicals from the 7 different classes and for mixtures containing up to 21 chemicals.

Broderius *et al* (1995) in a further series of experiments examined the toxicity of binary mixtures to juvenile fathead minnows in 96h acute tests. They examined concentration and response addition and concluded that a chemical with a similar primary mode of action to that of a reference toxicant would display a concentration addition type of

action over the entire mixture range. Chemicals with different modes of action with steep concentration-response curves were generally less than concentration-additive but consistently more toxic than predicted by the response addition model. More than concentration-additive responses, indicative of interactive toxicity, were uncommon in their experiments. Forget *et al* (1999) studied the joint action of binary mixtures of metals and pesticides on the marine microcrustacean *Tigriopus brevicornis*. Their results indicate synergistic lethal effects in all cases and at the sublethal level, the presence of the three metals tested seemed to enhance the inhibitory effects of certain pesticides. The tests were however conducted with chemical concentrations one or two orders of magnitude higher than those found typically in the aquatic environment.

The acute toxic effects on the larvae of the clawed toad (*Xenopus laevis*) of mixtures of heavy metals (3), alcohols (13), amines (6), hydrocarbons (6) and halogenated hydrocarbons (5) alone and in combination were examined by de Zwart and Sloof (1987). When the groups of substances were tested individually the toxicity of the amines and heavy metals was more than additive. The halogenated hydrocarbons were additive and the alcohols and hydrocarbons less than additive. When various mixtures of the groups were tested they mostly acted in an additive fashion, and where this was not the case and synergism was seen, it was the result of the presence of an amine group.

Binary and complex mixtures of aromatics, halogenated aliphatics, alkanes, alcohols, ketones and esters exposed to two bacterial preparations indicated additivity (Xu and Nirmalakhandan, 1998). Whereas, the mixture toxicity of binary mixtures of non-polar and reactive (aldehydes) toxicants yielded additive to greater than additive effects in the Microtox assay (Chen and Chiou, 1995).

Additive or less than additivity was observed in 21 of 23 compounds found in detergent products. Up to 67% of the toxicity of the complex mixture was attributed to 2 components – sodium silica and surfactants (Warne and Schifko, 1999).

The phytoxic action of diuron in combination with copper or folpet on the duckweed (*Lemna minor*) resulted in additive to less than additive responses (Teisseire *et al*, 1999). Antagonism was observed in acute toxicity of phenanthrene and zinc to the sheepshead minnow (*Cyprinodon variegatus*) (Moreau *et al*, 1999).

The position with regard to the toxicity of complex mixtures was summarised by Broderius (1991) as follows: "it can be concluded from published work that, for most complex organic mixtures, the joint acute action of toxicants is either strictly additive or slightly less than strictly additive and that antagonistic or more than additive effects are not prevalent". More recently Broderius *et al* (1995), following further studies with fathead minnows, reinforced this conclusion, by stating "because our data set represents diverse chemicals from the inventory of discrete US industrial chemicals, it is concluded from previous research and 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 additive in their joint toxicity". These conclusions are supported by the present review.

3.6 Summary

For substances which are chemically related, or are known to have the same mode of action, additivity of acute toxic effects is general and well substantiated. However, some "groups" of substances when tested in relatively simple mixtures do not behave in a readily predictable manner (e.g. metals and some pesticides) but this is not surprising as these are not groups of substances assembled on the basis of their structural, chemical or physical properties.

There are only a few examples in the literature of synergism where the results are more than three times that predicted from additivity of acute toxic effects.

When large numbers of substances are present in mixtures at low concentrations relative to their individual acute toxicities, additivity of acute toxic effects is closely followed. This holds good even when the substances are not related chemically, or exhibit different modes of action when acting as acute toxicants alone.

4. CHRONIC TOXICITY IN THE LABORATORY

This section presents an overview of representative studies that have been reported on the chronic toxicity of mixtures. Rather than providing an exhaustive review, the intention is to discuss the key findings from a consideration of different types of mixtures.

4.1 Chronic Toxicity of Metals

The chronic toxicity of mixtures of metals to aquatic plants has been reviewed by EIFAC (1987). The report concludes that responses are inconsistent but all of the papers reviewed indicated toxicity in the range 0.2 to 2.3 times the toxicity predicted by concentration addition. A wider review of the chronic toxicity of metals to aquatic organisms by Wang (1987) concludes that there is no pattern in the toxicity of mixtures of different metals or metals and organic chemicals. This unpredictability not only appears to be dependent on the specific metals themselves but also on the test conditions and organisms tested. For example, a binary mixture of copper and zinc was synergistic to some species of marine algae but antagonistic to others (Braek *et al*, 1976).

Some additional examples of the range of antagonistic, additive and synergistic chronic toxicity of mixtures containing metals are given below.

Accumulation of some metals can be influenced by exposure to other metals. Doherty *et al* (1987) exposed the freshwater bivalve, *Corbicula fluminea*, to different concentrations of cadmium and then assessed biochemical changes and uptake of copper and zinc. The authors suggest that an intermediate concentration of cadmium (0.1 mg/l) could have stimulated the production of heavy metal binding proteins resulting in the observed lower accumulation of copper. Similar antagonistic interactions between other metal combinations and with other species have been reported (e.g. Speyer, 1980; Thomas *et al*, 1982, 1983a, b, 1985; Kay *et al*, 1986; Brown *et al*, 1986; Cuvin and Furness, 1988).

Synergistic toxic interactions of mixtures of metals to freshwater copepods and submerged aquatic plants have also been reported by Borgmann (1980) and Jana and Choudhuri (1984), respectively.

Eaton (1973) observed both greater than and less than concentration addition in the effects of mixtures of copper, zinc and cadmium on various endpoints in the reproduction and development of fathead minnows. It is difficult to draw any conclusions about additivity from these results since zinc was found to dominate the toxicity.

Spehar *et al* (1978) exposed the early life stages of flagfish to mixtures of zinc and cadmium. As with the findings of Eaton (1973) they concluded that zinc was much more toxic to larval fish than cadmium. This prevented an accurate assessment of whether both metals were acting by concentration addition.

Abram, cited in EIFAC, 1987, demonstrated that a mixture of nickel and chromium was 13 to 21 times more toxic than anticipated on the basis of additivity in 10-week LC_{50}

tests with rainbow trout. However, the concentrations used were very high and probably not relevant to the environment.

Stebbing and Santiago-Fandino (1983) demonstrated that the toxicity (11-d inhibition of colony growth) of mixtures of copper and cadmium were more or less concentration additive to the colonial marine hydroid, *Campanularia flexuosa*. However, this was the case at relatively low levels of growth inhibition. Mixtures causing high growth inhibition were associated with both antagonistic and synergistic action. These responses were attributed to differences in biological response (the response of a colony may reach steady state more quickly when exposed to low metal concentrations than when exposed to higher concentrations) rather than to interaction between the two metals. Similar variable responses of organisms may have been observed in tests of the effects of mixtures of zinc, mercury and lead on the growth of ciliates (Gray, 1974). Pre-exposure of the marine ciliate, *Uronema marinum*, to low concentrations of these three metals was observed by Parker (1979) to result in the loss of synergistic toxicity previously observed during a test without pre-exposure. These experiments on hydroids and ciliates suggest that there may be a delay before a steady-state response to a mixture of metals is achieved.

Dual combination toxicity of three metals (tributyltin chloride, dibutyltin dichloride and tin chloride) to bioluminescence to the marine bacterium *Vibrio harveyi* indicated additive to greater than additive responses (Thomulka and Lange, 1996).

Detoxification mechanisms such as metallothionein production, which can be induced on exposure to the relevant metal, may also cause different responses depending on the duration of exposure. A chronic test may be of sufficient duration for the toxic response to reflect the organism's ability to detoxify the metal. The duration of an acute test may be inadequate for acclimation to occur if there has not been any previous exposure.

Bioavailability is an important consideration in the toxicity of mixtures containing metals and organic substances that can form complexes with metals. Wang (1987) gives several examples of metal chelating chemicals (of low but measurable toxicity) greatly reducing the toxicity of various metals. It is questionable whether these are examples of antagonistic toxicity since the organism is exposed to a different metal species in the test medium to that in a test where the metal is the single toxicant. Although the general trend is for chemicals that can complex with metals to reduce metal toxicity, there are a small number of examples of synergistic toxicity.

In summary, it is not possible to make generalisations about the chronic toxicity of mixtures containing metals. Such mixtures can give responses across the entire range of interactions from antagonism to synergism. This may be largely due to different modes of action and differences in metal speciation in mixtures compared to single toxicant tests.

4.2 Chronic Toxicity of Pesticides

Hermanutz *et al* (1985) determined the chronic toxicity of two insecticides, malathion and endrin, to flagfish. Effects after 140 days on both survival and growth were additive

despite the likelihood that the insecticides had different modes of action (malathion is an AChE inhibitor; endrin stimulates the release of neural transmitters in the pre-synapse) (Matsumura, 1985).

Assuming additivity when the 95% confidence intervals of experimental and predicted toxicities overlapped, Faust *et al* (1994) found additivity in 66% of tests of the toxicity (24-h EC_{50}) of a variety of binary mixtures of pesticides with different modes of action to the green alga *Chlorella fusca*. The results showed that 24% were less than additive and 10% more than additive. Only two tests (5%, out of a group of 40 binary mixtures) did not fall within a factor of 2 of the predicted toxicity based on the assumption of additivity. Higher conformity with additivity (85%) was found in the toxicity to algae of 29 binary mixtures of herbicides.

Shabana and Abou-Waly (1995) investigated the toxicity (1 to 10-d EC_{50}) of two triazine herbicides on the growth of the cyanobacterium *Nostoc muscorum*. They found that three of the five mixtures tested were antagonistic but two mixtures, which caused the greatest growth inhibition, were considered to be additive. This variability in the type of mixture toxicity may be due in part to experimental difficulties encountered in maintaining constant exposure in algal toxicity tests. Additivity of 14 pesticides and 5 surfactants, via 137 binary mixtures, was found as the best overall fit of acute toxicity data with alga (Altenberger *et al*, 1996).

In summary, it appears that mixtures containing different pesticides mostly act additively even though they can have different modes of toxic action.

4.3 Chronic Toxicity of Surfactants

Lewis (1992) prepared a comprehensive review of the effects of mixtures containing surfactants and other substances, including metals, pesticides, other surfactants and municipal effluents, on freshwater and marine organisms. He reported that there were data indicating antagonistic, additive and synergistic effects from chronic exposure but the responses were not predictable.

Dyer *et al* (2000a) observed additive acute and chronic toxicity responses in *Ceriodaphnia dubia* exposed to four alcohol sulphate and alcohol ether sulphate surfactants.

4.4 Chronic Toxicity of General Chemicals

De Wolf *et al* (1988) observed concentration addition for mixtures of 10 and 25 alcohols and chlorohydrocarbons in chronic toxicity tests with *Daphnia magna* in which NOECs for growth and reproduction were determined. The chemicals in these mixtures were non-reactive, non-ionised organics with a baseline narcotic mode of action.

Herman *et al* (1990) found that the toxicity (8-d EC_{50}) of mixtures of six aromatic hydrocarbons to the green alga *Selenastrum capricornutum* was concentration additive. Some responses appeared synergistic but were considered to have been caused by unusual increases in sensitivity of the alga to high concentrations of some hydrocarbons.

Nirmalakhandan *et al* (1994) found the toxicity to a mixed bacterial culture (6-h EC_{50}) of a mixture of 50 diverse chemicals (aromatics, halogenated aliphatics, alkanes, ketones, esters and amines) to be additive.

The prediction of combined effects, based on the effects of the individual components of mixtures by using the pharmacological concepts of concentration addition and independent action, of a multiple mixture was determined in a bioluminescence assay with *Vibrio fischeri* (Altenburger *et al*, 2000; Backhaus *et al*, 2000). With 16 similar and specifically acting chemicals, Altenburger concluded that concentration addition is well predicted with both concepts. With dissimilarly acting chemicals Backhaus *et al* (2000), report that the independent action approach gives excellent results but concentration addition underestimates the EC_{50} of the mixture by only a factor of less than three. Thus the precise prediction of mixture toxicities depends upon a valid assessment of the similarity/dissimilarity of the mixture components. They concluded however that concentration addition may give a realistic worst case estimation of mixture toxicities for risk assessment procedures.

Additivity was found to apply to three different mixtures of i) non-reactive, non-ionised organic compounds ii) anilines and iii) reactive organic halogen compounds when tested in 14-d LC_{50} tests with guppies (Hermens *et al*, 1985b).

Hermens *et al* (1984b) also tested the chronic (16-d LC_{50}) toxicity of mixtures of chemicals considered to act by non-polar narcosis to *Daphnia magna*. They found that the toxicity of the mixtures was additive or close to additive. In another set of tests with a different mixture of chemicals (alcohols and chlorohydrocarbons) Hermens *et al* (1985a) found that the chronic toxicity to *Daphnia magna* was also additive.

Deneer *et al* (1988) tested mixtures of nine chemicals considered to act through different modes of action in *Daphnia magna* chronic toxicity tests. They found that for all the mixtures the 16-d EC_{10} for growth was less than additive. Earlier work by Hermens *et al* (1984b) with a similar mixture found that the degree of additivity was dependent on the sensitivity of the endpoint. Lethal effects were close to additive but inhibition of reproduction was much less than additive.

Deneer *et al* (1988) considered inhibition of the growth of *Daphnia magna* to be more sensitive than reproduction and found a lower degree of additivity for growth. They suggest that the differences in toxic action of the mixtures at different concentrations were due to the chemicals causing toxicity by different, multiple modes of action. Individual components of a mixture may have different primary (specific) modes of action but may also cause common secondary effects by one or more different mechanisms e.g. non-polar narcosis. At high concentrations more of the chemicals in the mix may exert these secondary effects in addition to their more specific mode of action. At lower individual concentrations of the chemicals and using more sensitive endpoints for the specific modes of action, the mixture is less likely to be additive, since the common secondary effects are expected to make a lower contribution to overall toxicity. Supporting this hypothesis are data from Niederlehner *et al* (1998), where reproductive responses

of *Ceriodaphnia dubia* were found to be more sensitive than mortality for three non-polar organic chemicals (benzene, tricholoroethylene, toluene) used in a tertiary mixture. Less than additivity was observed.

At even lower concentrations, i.e. below the concentration threshold for effects caused by a specific mode of action, chemicals may still contribute to the baseline narcotic toxicity of a mixture (McCarty and Mackay, 1993). However, in this case an effect would only be observed if a large enough number of chemicals was present, each adding a small contribution to an overall narcotic effect.

It follows that the larger the number of chemicals in the mixture and the smaller their individual contribution to the overall non-polar narcotic effect, the more likely it is that they will act additively. Such mixtures could contain many chemicals at very low concentrations, perhaps representative of environmental concentrations. In these cases extrapolation to effects of complex mixtures in the environment should be made with care since bioavailability may be significantly lower than when tested in laboratory media.

4.5 Discussion

Toxicokinetic modelling of mixtures of organic substances may help to explain the variability in the toxic action of mixtures, i.e. why some mixtures appear additive, synergistic or antagonistic at different times or in different tests. The key advantage of this approach is that the kinetics of toxicant uptake are taken into account (McCarty *et al*, 1992). A highly hydrophobic substance is less likely to partition rapidly into an organism compared to a substance of lower hydrophobicity (and higher solubility). An organism exposed to such a mixture may show an initial response dominated by the effects of the most rapidly accumulated substance, since this substance is likely to reach a toxic body-residue concentration faster (if the two substances are equally toxic). Such differential rates of uptake may be less influential in the longer time course of a chronic toxicity test since steady-state body concentrations are more likely to be achieved for substances with a wider range of hydrophobicities. Verhaar *et al* (1995) propose an approach that integrates QSAR with toxicokinetic modelling to understand potential effects from mixtures of chemicals with diverse physical/chemical properties and/or modes of action.

Differences in the time taken to reach some critical body residue may be less important if none of the substances in the mixture are at a sufficiently high concentration to cause an effect by themselves.

Considering the range of chemical mixtures described in the previous sections, it seems possible that all organic substances can act by baseline narcosis (i.e. general membrane perturbation which has been shown to be strongly dependant on the hydrophobicity of the substance) (Hermens *et al*, 1985a,b) even though some act by more toxic modes of action at low concentrations (e.g. Könemann, 1981). This might suggest that a mixture of many substances, each at a concentration below its threshold for causing toxicity

by a non-baseline mode of action, could act additively and cause a toxic effect by non-polar narcosis. This is because non-polar narcosis probably depends on the total quantity of non-polar substances present in the membrane but not on any specific chemical reaction with biological material (van Wezel and Opperhuizen, 1995; van Wezel *et al*, 1996).

It seems reasonable to assume that a mixture of substances with the same mode of toxic action will demonstrate concentration addition. In this case we can consider the substances in the domain of a specific QSAR for chronic toxicity as also acting additively, if the parameters in the relationship represent a specific mode of action. For example, a QSAR based on only one parameter such as log K_{ow} (as a surrogate for hydrophobicity) should be a reliable QSAR for baseline narcosis.

Of course, chronic toxicity tests are used to generate many different endpoints and for a given mixture a complete set of identical endpoints may not be available for each substance. As demonstrated by Deneer *et al* (1988) different endpoints are not necessarily additive which, in theory, creates a problem in estimating the toxicity of the mixture when additivity is assumed. In practice, however, the pragmatic use of the most sensitive endpoint for individual substances is reasonable, since the differences in toxicity between substances would usually be greater than the differences between endpoints.

4.6 Prediction of the Chronic Toxicity of Mixtures Based on Acute Toxicity

Consideration of toxicokinetics and modes of toxic action of mixtures gives an indication of whether it is possible to predict the effects of a mixture after long-term (chronic) exposure if the effects of short-term (acute) exposure are known.

The most straightforward case should be for a mixture of homologous substances with a non-specific mode of action. It seems likely that this type of mixture will act additively in both acute and chronic tests and therefore it may be possible to predict one from the other if the ratios between the sensitivity of the individual acute and chronic endpoints are known. The factors used in current risk assessment schemes could be applied to make conservative predictions. This is supported by Niederlehner *et al* (1998), who found that mixture toxicity predictions based on QSARs (K_{ow}-based) overpredicted both acute and chronic mixture effects of non-polar narcotic chemicals to *Ceriodaphnia dubia*.

It is more difficult to predict from the acute toxicity of the individual substances the chronic toxicity of a mixture containing substances with different modes of action, since these mixtures are more likely to act in a non-additive way and contain substances with different acute to chronic ratios, although, as reported here, such mixtures seem mostly to be close to additive. The chronic toxicity of mixtures of diverse substances is unlikely to be greater than predicted by additivity unless a single, relatively toxic substance, acting by a specific mode of action, is present at a much higher concentration than the others. Apart from this specific case, an assumption of additivity together with use of conventional "acute-to-chronic" application factors should give a conservative prediction of the chronic toxicity of mixtures.

5. BEYOND ROUTINE LABORATORY TESTS: MORE REALISTIC EXPOSURES

5.1 Introduction

The toxic effects of mixtures are of practical concern in the environment due to the exposure of aquatic communities to mixtures of substances from a variety of sources. The effects of such mixtures on environments have been investigated directly in field studies and in model ecosystems (microcosms and mesocosms) intended to simulate such environments. Complex effluents have also been studied with regard to the contributions of specific components to toxic effects. It may be expected that the results of such studies would provide further information regarding mixture toxicity.

Investigation of the toxicity of single substances in acute and chronic, single-species, laboratory test systems is sufficient to understand their inherent toxicity. In such controlled situations, exposure to the substance may be maintained at a constant concentration. Other factors that modify the toxic effects of a substance may also be controlled. The same principle is true for understanding the combined toxic effects of mixtures of substances under controlled laboratory conditions. In these studies it is important to determine the exact concentration of each component and to relate toxicity to the substances with no other interferences or contributions present. Studies of the toxic effects of mixtures of substances or complex effluents under more "environmentally realistic" conditions leads to a better understanding of the influence of modifying factors, changing concentrations and non-standard exposure durations. In principle, such studies do not add to the fundamental understanding of the toxicity of mixtures. They do however, add information as to the influence of exposure conditions on the combined toxic effects. The influence of factors which modify the exposure to substances in the environment is discussed in Section 5.2. This section also summarises the types of information from model ecosystems, field studies and effluent studies regarding mixture toxicity. A more detailed review of this topic is presented in Appendix 1. Analysis of these study results confirms that departure from additivity of toxic effects in the environment is due mainly to changes in availability or exposure concentration of the substance.

5.2 Factors Modifying the Toxic Effects of Mixtures

For substances discharged to receiving environments, it is rare that they react in the receiving environment thereby resulting in either increased or decreased toxicity. Any reactive substances in an effluent would be expected to have reacted before reaching the aquatic environment (an exception is where an effluent carrying one toxicant enters a river carrying another toxicant). For example, the addition of free chlorine to effluent containing ammonia results in the rapid formation of chloramines. The release to aquatic systems of readily hydrolysable substances results in the rapid conversion to the hydrolysis products; for example, acid anhydrides are rapidly transformed to the corresponding free acids.

A large number of physical and chemical factors can modify the exposure and availability of substances in the environment. Such factors are known to mitigate the uptake of

the toxic chemical species by the receptor organism. As with single substances, the presence of organic or inorganic complexing agents in a mixture can result in decreased toxic effects. Suspended solids, carbon dioxide content, redox potential, pH, dissolved oxygen, and temperature can affect speciation and/or bioavailability. For example, the presence of suspended solids and naturally-occurring dissolved substances decreases the bioavailability of cationic surfactants, thereby decreasing their toxic effects but not those of anionic and nonionic surfactants (Lewis, 1992). Consequently, mixture assessments based on total concentrations in media overestimate adverse effects to receiving water biology.

For example, Wildhaber and Schmitt (1996) conducted an analysis of the total TUs in Great Lakes sediments to laboratory-derived sediment toxicity tests from several taxa, including fish, zooplankters, benthic invertebrates, phytoplankters, macrophytes and bacteria. Approximately 1000 TUs in sediment pore water were required to illicit a >25% increase in adverse effects compared to control sediments. Many of the effects could be attributed to the toxicity of ammonia and iron, not the organochlorine, polycyclic aromatic hydrocarbons (PAHs), phthalate esters, or organo-metals.

The importance of the partitioning of PAHs to organic carbon in water and sediments was addressed by Di Toro *et al* (2000) and Di Toro and McGrath (2000), respectively.

The influence of modifying factors on observations of the toxic effects of pollutant mixtures or effluents is illustrated in the results of many of the field studies, model ecosystem studies and effluent studies presented in Appendix 1.

5.3 Model Ecosystems, Field Studies and Effluent Studies

Model ecosystems have often been employed to study the complexities of higher order effects on ecosystem functioning or of the integrated fate pathways of single substances. In single substance studies, the complexities of chemical behaviour and population interactions generally preclude the attribution of a single effect to a single chemical species. In such systems it is almost impossible to evaluate the relative contributions of components of mixtures to overall toxic effects. The information provided by these studies on the relative contribution of individual components of either complex or simple mixtures to overall effects is scant.

Even so, a recent study by Jak *et al* (1996) showed that metal mixtures acted additively toward freshwater zooplankton and plankton communities in enclosures. Importantly, they conclude "no large safety factors for extrapolation from laboratory toxicity data to water quality standards, concerning difference in conditions, are needed, and that simple rules can adequately cover the additional toxicity of a mixture of metals."

Generally no unexpected ecosystem effects or synergies are described in model ecosystem studies on mixtures and effluents. From the limited data available it appears that toxicities of mixtures are additive to less than additive in these systems and this outcome is considered more likely to be due to reduced exposure/bioavailability rather than any

violation of the principle of additivity (see Appendix 1 for a more detailed description of model ecosystem studies of mixtures).

A comparison of TUs from contaminated sediments from several locations in the Great Lakes to benthic community tolerance metrics was conducted by Wildhaber and Schmitt (1998). Bioavailable TUs as well as bulk sediment TUs were calculated based on water quality criterion and equilibrium partitioning models. Benthic community tolerance values per taxon were taken from two different sources (Hilsenhoff, 1987; Lenat, 1993). Highly significant correlations were observed between the tolerance values and TUs from both bioavailable and bulk values. Tolerance metrics for communities sampled from artificial substrates were also highly significantly correlated with ambient toxicity.

Dyer *et al* (2000b) assessed the relationship of fish tissue burdens of contaminants with fish community integrity (Karr's Index of Biotic Integrity, IBI) collected from 1010 sites throughout the state of Ohio, USA. Toxic units of 12 organic and 11 metal contaminants were based on regulatory-based protective limits (TU = USEPA water quality criterion bioconcentration factor). Summation of metals and organic TUs overpredicted adverse effects to individual fish and fish communities. However, addition of organic chemical molar units did not overpredict adverse effects, thus supporting the concept of baseline toxicity. The authors conclude that addition of molar units is appropriate, provided they are at concentrations below levels deemed protective of most species (e.g. 95%).

Biomimetic methods such as the use of solid phase microextraction (SPME) and/or semipermeable membrane devices (SPMD) for simulating body residues of complex organic mixtures has been advocated by many researchers (Verbruggen *et al*, 1999; Parkerton *et al*, 2000; Petty *et al*, 2000). These methods show promise with acute toxicity, however additional research is needed with chronic endpoints and field assessments.

The study of effluents and sites down-stream from effluents would be expected to provide information on mixture toxicity, since the effluents are complex mixtures. Furthermore, a considerable amount of work is being done in the USA to manipulate (for example, aerate, acidify, add EDTA) and fractionate toxic effluents to identify the toxic component ("Toxicity Identification Evaluation" or TIE). Effluent studies are considered in more detail in Appendix 1.

These studies display many of the modifying factors described in Section 5.2 and also present additional complications. A major problem is the temporal variability in the identity and concentration of the toxicant species in the effluents. Also, the manipulation process itself may give rise to changes in substance bioavailability.

Frequently effluents contain a single substance whose toxicity and concentration are sufficient to account for all of the observed toxic effects. However, a review of the literature did identify 18 studies where more than a single substance was believed to be the cause of the toxic effects. Of these, 13 were said to show additivity of toxic effects of the

components. Of the remaining 5 studies, 4 were explained by the authors as deviating from additivity due to either matrix interference with toxicity or the presence of additional unidentified substances. Only in one instance, concerning metal toxicity, were the results less than additive.

Field studies on the observed effects of effluent discharges on native organisms comprise too many unknowns or variables to assess quantitatively the relative contributions of particular substances. Effluent discharges are almost always as mixtures but the temporal and spatial variability of the release patterns and of the background contamination make exposure and effects difficult to relate. Nevertheless, there are no data from these types of studies that would indicate the toxicity of mixtures to be synergistic in the field.

Some of the studies on the source of toxicity in effluents demonstrate again that decreased exposure due to modifying factors complicates interpretation of results. However, the TIE studies in particular tend to support the conclusion that toxicity is additive.

Whilst these data are often limited and difficult to interpret from the standpoint of mixture toxicity, the evidence tends to support the concept of additivity, particularly taking into account the role played by bioavailability in many of the studies.

6. ASSESSING THE TOXIC EFFECTS OF MIXTURES OF SUBSTANCES IN THE ENVIRONMENT

6.1 Introduction

Previous sections have reviewed and summarised the literature concerning the toxic effects of mixtures during acute and chronic laboratory tests and under more environmentally realistic conditions. In general, acute and chronic toxic effects in mixtures have been found to be additive, though this is less true for mixtures containing metals.

Substances intentionally released to the environment can be regulated to be below their individual PNECs such that each should be present at levels which will not elicit even long-term toxic effects in the receiving environment. This procedure forms the basis of the EC environmental risk assessment procedure (EEC, 1996). However, many substances may be present in the environment at the same time, each substance being below its individual PNEC, but with the potential that as a mixture there may be a risk of toxic effects arising from additive toxicity.

Some practical methods are needed to determine if this potential for impact is expressed. This section outlines some approaches for assessing the risk of effects of mixtures of substances in the environment and discusses the advantages and disadvantages of each approach. On the regional level, the potential number of individual substances is greater due to multiple inputs, but potential concentrations are significantly lower, as each is regulated at the local level. In the following approaches, unless otherwise stated, each substance is considered to contribute proportionally to aquatic toxicity via a non-specific or "baseline" mode of action. Support for this approach is given in Section 5.

The approaches considered include the summation of individual risk quotients, the use of arbitrary "correction factors", and the use of environmental monitoring techniques.

6.2 PEC/PNEC Summations

In an ideal world, actual environmental concentrations ("ECs") and actual no effect concentrations ("NECs") would be available for every substance, and EC/NEC values could be summed and compared to the ratio of 1. In practice, availability of actual EC and NEC values is unlikely and estimation methods are needed. Some potential summation approaches are outlined below.

Approach 1: Toxic unit (TU) summation using actual environmental concentrations

Individual substances present in the environment would be identified and quantified. A QSAR-based toxicity value would be derived for each substance (NOEC or LC_{50}) based on the appropriate QSAR for that substance. The TU of each substance would be calculated based on its toxicity and its concentration and should incorporate what is

to be protected. For example, if the chronic effects of a substance are to be modelled, but only acute data or a QSAR is available, then an appropriate assessment factor needs to be employed to address chronic toxicity. Once the criteria are established, all TUs would be summed, and if greater than one, further evaluation would be considered (Section 6.4). The concept of using baseline toxicity as the foundation for additivity works best when PNECs are based on ecosystem protection, not acute or chronic toxicity endpoints where mode of action is quite relevant.

This approach has some distinct advantages, including:

- the number of substances to be included in the assessment is limited to those actually present;
- a relevant QSAR (acute or chronic) can be chosen, with use of appropriate application factors[1].

This approach may be practical in limited local situations, where the number of individual substances is likely to be low and their concentrations are quantifiable.

Some practical disadvantages limit the applicability of this approach. These include:

- identification and quantification of each of the substances present is required (time and cost intensive);
- for many substances, no (or an inadequate) analytical methodology may be available;
- reduced field exposure due to bioavailability factors would not be taken into account;
- if a substance's limit of quantification is higher than its toxicity value or if there are many substances present at low concentrations, a different approach may be needed. This may occur especially for assessments at the regional level;
- the toxicity of some compounds which are more rapidly metabolised than those used to derive the QSAR may be overestimated.

SUMMARY

Based on the TU concept, this approach is rigorous in theory, but often likely to be impractical to implement, due to difficulties in accurate measurement of the environmental concentrations of all substances present. Thus, in practice it may be resource intensive, costly and may provide limited additional knowledge on actual environmental risks. Nevertheless it has been used to a limited extent and for the specific purpose of identifying, empirically, the water quality required to characterise different types of fishery in an entire river basin (Alabaster *et al*, 1972).

1 If both the acute and chronic toxicity of mixtures can be considered to be additive, the application factor(s) necessary to predict the chronic toxicity of mixtures from their acute toxicity should be the same as those for individual substances. It also follows, in the absence of field data on the toxic effects of a mixture, that the application factors used to obtain a PNEC from acute or chronic toxicity data on a mixture should be the same for the mixture as for the individual substances.

Approach 2: PEC/PNEC summation with use of available risk assessment data

A related approach would employ available PEC and PNEC values to develop a risk assessment for a mixture of substances in the environment. Briefly, existing risk assessments - which should include substances of highest priority - would be reviewed. The calculated PEC and calculated PNEC for each substance, in the location or region to be assessed, would be used. All PEC/PNEC ratios would be summed, and if greater than one, further evaluation would be considered (Section 6.4).

This approach has some distinct advantages, including:

- makes use of available data; data on prioritised substances (those of most concern) would be available first;
- could develop various versions, using $PEC_{regional}$ or PEC_{local};
- at least initially, does not require environmental measurements;
- can selectively refine PEC and/or PNEC of individual substances;
- if individual PECs and PNECs are accurate, this method may work.

Some practical disadvantages limit the applicability of this approach. These include:

- assumes reliable risk assessments are available for each (important) substance;
- risk assessments may be available for high production volume substances only. On the other hand, this approach may overestimate the importance of some substances. It could be limited to the subset of substances actually present, but this would introduce many of the disadvantages of Approach 1;
- the calculated sum PEC/PNEC of a large group of substances is likely to be far worse than a realistic worst case, because individual risk assessments are not carried through to the determination of accurate PEC and PNEC values, but only until the PEC/PNEC < 1. Thus, available PEC and PNEC values will probably be overly conservative when summed;
- if based on a specific mode of action, individual PNECs may overestimate the contribution of each substance to the overall toxicity (which in this case is probably due only to their "baseline" toxicity contribution alone);
- reduced bioavailability in the field can significantly reduce field exposure and thus observed effects. This would rarely be taken into account if only laboratory-based PNECs are available.

SUMMARY

Although less rigorous in theory than Approach 1, this approach should include the highest profile substances, and should include more substances as databases expand. However, individual risk assessments will generally not be refined enough to provide realistic answers, but may result in very conservative estimates which do not indicate where the greatest uncertainties lie. In practice, this may be resource intensive and costly and provide minimal additional knowledge of actual environmental risks.

6.3 Use of a Correction Factor

Approach 3: Modification of individual substance assessments.

The potential impacts of mixtures of substances on the environment could be taken into account by modifying the current risk assessment procedure. As a basis for this approach, it can be assumed that each substance contributes to the overall toxicity. Rather than summing all inputs, however, a correction factor could be applied to each individual risk assessment.

Conventional risk assessment procedures would be employed to derive a PEC and a PNEC for each substance. For each substance, PEC/(PNEC times X), with X representing the number of substances expected to be present in the environment under consideration, would equal a "mixture toxicity PEC/PNEC" value. If this calculated "mixture toxicity PEC/PNEC" is greater than one, further evaluation would be considered (Section 6.4).

This approach has some distinct advantages, including:

- it can be applied to individual substances on a case-by-case basis, but taking into account all other compounds (if "X" is correct);
- its ease of use.

Some disadvantages limit the applicability of this approach. These include:

- X is unknown and will probably fluctuate greatly from site to site;
- not easily applicable at a local level;
- substances may be present well below their calculated PEC (not refined to realistic values once PEC/ PNEC < 1). Confirmation of accurate PECs would require all the resources listed for Approach 1;
- it is not likely that each substance will be present at a constant proportion to the total concentration. "Important" substances would be weighted the same as other substances;
- if based on a specific mode of action, individual PNECs may overestimate the contribution of each substance to the overall toxicity (which in this case is probably due only to their baseline toxicity);
- reduced bioavailability in the field can significantly reduce field exposure and thus observed effects. This is unlikely to be taken into account if only laboratory-based PNECs are available.

SUMMARY

The issues identified in Approach 3 also apply here. Adherence to this approach is dependent on satisfactorily defining the "X" correction factor per substance - so as to account for additivity from other substances. Defining an appropriate correction factor

would require evidence that a factor is needed i.e. greater than additivity based on baseline concepts is observed in the environment. Hence, correction factors may be applicable where mixture effects exceed baseline toxicity.

6.4 Environmental Monitoring

A different approach to assess the effects of mixtures of substances in the environment is to employ environmental monitoring. Various methods exist for biological or chemical monitoring of the aquatic environment for adverse effects.

Approach 4: Chemical monitoring to estimate "available" substance concentrations

Extraction techniques can be employed in the field to simulate uptake of substances by biota. These may include, but are not limited to, selective solvent extraction techniques, Empore™ disk, Semi-Permeable Membrane Devices, and solid phase extraction. (For further detail, the reader is referred to van Loon and Hermens, 1996; ECETOC 1999). The results may provide a surrogate measure of the amount of bioavailable substances in the environment (Verbruggen *et al*, 1999; Parkerton *et al*, 2000; Petty *et al*, 2000). A summed value, as a surrogate for the body burden of the mixture in an organism, can be compared to a toxic threshold level (critical body burden) which would be expected to elicit an effect.

This approach has some distinct advantages, including:

- identification of substances and specific analytical methods are not required;
- derivation of a "bulk" parameter (which, if appropriately calibrated, could provide information on reductions in water quality prior to the occurrence of any biological effects (early warning));
- ease of execution;
- "biomimetic" techniques should reflect biological uptake and thus take into account the bioavailability of substances.

Some disadvantages limit the applicability of the chemical summing approach. These include:

- practical implementation in the field (e.g. potential interferences, fouling);
- not fully validated (e.g. assumptions concerning bioconcentration, correlation to toxic effect levels);
- does not reflect metabolic capacity of exposed organisms, so may overestimate internal dosages at target sites and thus toxic response.

SUMMARY

Chemical monitoring methods may be attractive in terms of practical implementation (although interferences may need to be overcome). Whilst they are promising new approaches, they are not yet fully validated in terms of biological relevance.

Approach 5: Biological field monitoring to evaluate current environmental status

Biological field monitoring (e.g. of benthic fauna, fish communities) can be carried out at target sites, or spatial and/or temporal trends can be evaluated. Results can be compared across sites, with matching of habitat types; adverse effects are then identified based on comparison with characteristics of communities present at non-impacted sites or these may be predicted using RIVPACS (Wright *et al*, 1993). Where effects are observed, additional studies are needed to determine cause and effect relationships.

This approach has some distinct advantages, including:

- provides an integrated biological picture: ecologically relevant endpoints can be directly assessed;
- requires the identification of substances and the application of specific analytical methodology only if effects are observed;
- field monitoring is well-established;
- all modes of biological uptake are incorporated, including the ones not examined in laboratory studies or with chemical extraction techniques. Thus, true bioavailability is represented;
- results are not specific to chemical toxicity per se. While this can be viewed as a limitation, it ensures that the integrated impact of multiple stressors (habitat degradation, direct chemical toxicity, pH or ammonia shifts) are observed.

Some practical disadvantages limit the applicability of biological field monitoring. These include:

- if impacts are observed, it may be difficult to determine cause and effect;
- not specific to chemical toxicity (see also under advantages!);
- biological variability across sites may be high, obscuring impacts;
- once impacts are observed, they may be irreversible.

SUMMARY

Biological monitoring directly identifies affected locations, but is not predictive and additional investigations are needed to determine causes of any effects observed. While biological monitoring can be resource intensive, extensive databases already exist for some locations. Importantly, the integration of biological responses with physical and chemical factors, including habitat, is essential for the validation of mixture risk assessment methods.

6.5 Discussion on Application of Approaches 1-5

In the approaches listed in Sections 6.2 to 6.4, attainment of a certain value would trigger consideration of further action. The most efficient action would be determined on a case-by-case basis. For PEC/PNEC ratio approaches, action may include deployment of

chemical or biological monitoring methods to determine whether any effects are observed (biological) or expected (chemical "summing"). Specific chemical monitoring may be employed to ascertain whether important individual substances are present at expected levels. Additional research may be needed to investigate and confirm plausible cause and effect relationships. All of these actions would take place within a defined framework at the local level.

Certain classes of substances (e.g. aquatic herbicides) may be regulated specifically with regard to their use which may influence their occurrence at a local level.

Metals appear to act differently from organics. Given the available information, it is not recommended that the above approaches be used for mixtures of metals. The reader is referred to the EU Technical Guidance Document for risk assessment (EEC, 1996) for further details.

This report focuses on toxicity due to non-polar narcosis (Narcosis I). This appears to be an appropriate starting point, as most individual substances should be found at very low levels in the environment (baseline toxicity). Because the concentrations of substances should be well below their individual toxic thresholds they will contribute to the combined toxicity only via non-specific modes of toxicity (c.f. Section 2). An exception, i.e. mixtures acting by a specific mode of action, may be closely-related substances acting through the same receptor as target, which may sum to an amount greater than individual threshold values. Examples include Ah-receptor binding materials, uncouplers and AChE inhibitors. However, it should be noted that for receptor-mediated toxicity, the presence of compounds with varying affinities and activities may reduce the toxicity of some of the components present (due to receptor being occupied by less potent agonists or antagonists). For closely-related substances with a highly specific mode of action, combined effects should be examined more thoroughly.

A major concern is the potential for large, unexpected synergistic effects between low levels of biologically-active substances. Thus, the report of up to 1000-fold enhanced responses of binary mixtures of organochlorines for oestrogen receptor binding (Arnold *et al*, 1996) was closely monitored. In the interim, other laboratories and the original research group were unable to replicate these findings, resulting in retraction of the Science article (McLachlan, 1997). This area will receive continued, careful study, but to date there is little evidence available to indicate that significant synergism even rarely occurs. In contrast it has been observed that additivity is even a conservative assessment (Dyer *et al*, 2000b).

6.6 Conclusions

While Approaches 1 and 2 are currently unwieldy and somewhat impractical, the correction factor(s) to employ in Approach 3 is difficult to determine and drives the data generation portion of each individual risk assessment beyond a practical level. A more attractive solution might be to monitor the environment to obtain a first indication of whether the environment is degrading or that a problem does exist e.g. as proposed in the draft Directive establishing a framework for Community action in the field of water policy (EC, 1999). Approaches 4 and 5 outline several monitoring techniques

which might identify areas of degradation or decline in water quality, with subsequent action to identify and resolve potential problems. Extensive databases on historical trends in benthic and fish communities and water quality are increasingly becoming available at some regions and sites, whereas at others use of a chemical summing parameter method would be an excellent first step in pinpointing potential local problems. Biological monitoring provides the advantage of encompassing the impacts of ammonia, oxygen levels, and pH, which would not be captured by the chemical specific or bulk parameter approaches, but have proved to be some of the most severe environmental problems.

In effect, this section raises the question of whether the presence of mixtures in the environment should be considered a "chemicals regulation" issue in the first instance, or whether potential concerns should be identified via monitoring activities, with subsequent action at a local level to address any identified concerns. Ultimately, the final decision must be based on the practical ability to implement a reasonable, non-arbitrary, and data-based approach which can result in meaningful environmental improvements.

7. CONCLUSIONS AND RECOMMENDATIONS

7.1 Laboratory Exposures

In laboratory studies, the acute toxic effects associated with a mixture can in general be considered to be additive with respect to the acute toxicities of the individual components, particularly for large numbers of compounds dosed at equitoxic concentrations.

Additivity of toxic effects is also generally observed for the chronic toxicity of mixtures in laboratory exposures, particularly for large numbers of compounds. However, for mixtures of metals mixture toxicity appears to be more variable (e.g. ranging from antagonistic to greater than additive), depending on the organisms and the metals involved.

7.2 More Realistic Exposures: Model Ecosystems, Field and Effluent Studies

Data from model ecosystems, field studies and effluent studies are generally limited and difficult to interpret from the standpoint of the toxic effects of mixtures of substances. However, the evidence tends to support the basic concept of additivity, particularly when the role bioavailability can play in reducing toxic effects under environmental conditions is taken into account. Predicted mixture effects based on body residues from organisms exposed in the field indicate that additivity of substances below their PNECs (baseline toxicity approach) is sufficiently conservative for protection of aquatic resources.

7.3 Approaches to Assessing the Toxic Effects of Mixtures

Potential approaches to assessing the toxic effects of mixtures are outlined in Section 6. Three general approaches considered are:

- summation of (more or less) realistic PEC/PNEC values for each individual component in an environmental mixture;
- use of an additional "correction" factor on each individual risk assessment to account for the effect of mixtures;
- monitoring of environmental status to identify concerns prior to extensive refinement of assessments to incorporate effects of mixtures.

While future research may make the first type of approach more feasible, and the second less arbitrary, currently only the third is judged to be a viable option and is necessary for appropriate development of the first two approaches.

7.4 Recommendations

Based on a review of the literature and existing methodologies, this report outlines several potential approaches to the assessment of the impact of mixtures of substances in the aquatic environment:

- each potential approach has advantages and limitations. The practicalities and cost-effectiveness of each approach should be further evaluated;
- the need for development of additional alternatives should be evaluated;
- catchment and other integrated approaches should incorporate understanding gained in laboratory and mesocosm studies about the behaviour and effects of chemicals in the environment;
- in order to improve communication and understanding of research into the toxicity of mixtures, consensus should be reached on the terminology, statistical approaches and interpretation of different experimental designs.

GLOSSARY

Acute Toxicity
The harmful properties of a substance which are demonstrated within a short period (hours for e.g. algae to days for e.g. crustaceans and fish) of exposure.

Antagonism
Antagonism arises when the combined effect of two or more substances is smaller than the combined solitary effects of the substances.

Bioavailability
The ability of a substance to interact with the biosystem of an organism. Systemic bioavailability will depend on the chemical or physical reactivity of the substance and its ability to be absorbed through the gastrointestinal tract, respiratory surface or skin. It may be locally bioavailable at all these sites.

Chronic Toxicity
The harmful properties of a substance which are demonstrated only after long-term exposure in relation to the life of the test organism.

Critical Body Burden
The concentration of a substance in an organism at the time of death (or any other biological endpoint).

EC_{50} Value (median lethal concentration)
A statistically derived concentration which, over a defined period of exposure, is expected to cause a specified toxic effect in 50% of the test population.

Exposure
1) Concentration, amount or intensity of a particular physical or chemical agent or environmental agent that reaches the target population, organism, organ, tissue or cell, usually expressed in (numerical) terms of substance concentration, duration, and frequency (for chemical agents and microorganisms) or intensity (for physical agents such as radiation), and
2) process by which a substance becomes available for absorption by the target population, organism, organ, tissue or cell by any given route.

Hazard
The set of inherent properties of a substance or mixture which makes it capable of causing adverse effects in man or to the environment when a particular level of exposure occurs. c.f. risk.

LC_{50} Value (median lethal concentration)
A statistically derived concentration which, over a defined period of exposure, is expected to cause 50% mortality in the test population.

LOEC (lowest observed effect concentration)
The lowest test concentration at which the substance is observed to have a "statistically significant" and unequivocal effect on the test species.

NOEC (no observed effect concentration)
The highest tested concentration below the LOEC where the stated effect was not observed. The NOEC is usually connected with chronic effects.

Speciation
Determination of the exact chemical form or compound in which an element occurs in a sample, for example whether arsenic occurs in the form of trivalent or pentavalent ions or as part of an organic molecule, and the quantitative distribution of the different chemical forms that may coexist.

Synergism
Toxicological interaction in which the combined effect of two or more substances is greater than the simple sum of the effects of each substance.

ABBREVIATIONS

AChE	Acetylcholine esterase
Ah	Aryl hydrocarbon
AI	Additivity Index
CBR	Critical Body Residue
EC	(actual) Environmental Concentration
EC_{50}	Effect Concentration showing effects in 50% of a population
EIFAC	European Inland Fisheries Advisory Commission
FATS	Fish Acute Toxicity Syndrome
LAS	Linear Alkylbenzene Sulphonate
LC_{50}	Lethal Effect concentration in 50% of a population
LOEC	Lowest Observed Effect Concentration
MTI	Mixture Toxicity Index
NEC	(actual) No Effect Concentration
NOEC	(highest) No Observed Effect Concentration
QSAR	Quantitative Structure Activity Relationship
PEC	Predicted Environmental Concentration
PNEC	Predicted No Effect Concentration
TIE	Toxicity Identification Evaluation
TRE	Toxicity Reduction Evaluation
TU	Toxic Unit

APPENDIX 1. ENVIRONMENTALLY REALISTIC STUDIES

Model Ecosystems

There are few studies of model ecosystem responses to mixtures of chemicals and many of these provide no information on the joint toxic action of components, since the mixture is treated as a single substance. In a symposium on aquatic mesocosms, a single paper on mixtures (Crossland *et al*, 1992) studied the effects of a complex organic effluent, mainly chlorophenols, from a chemical plant on a model stream. The effluent was not evaluated as to its component chemicals but treated as a single toxic substance. This is a common approach to the application of mesocosms to such complex mixtures.

A similar approach, treating oil as a single substance rather than a mixture, is reported by Horvath *et al* (1980). He described subtle ecosystem effects but did not relate these to concentrations of specific component chemicals. In another mesocosm study on an oil derived from coal, Geddings *et al* (1984) found that at a low dose, the initial effects on zooplankton and metabolism of the ecosystem disappeared with time. Higher concentrations showed chronic effects which did not disappear until dosing stopped. The authors ascribed all of the time-dependent toxic effects to the phenol concentrations in the ecosystems.

In these types of studies of complex mixture toxicity in model ecosystems, separation and analysis of all component chemicals is needed to address the relative contribution to toxic effects of the particular components. (Effluent fractionation, which uses this approach of analysis for the toxic components is discussed below). Model ecosystems are ideal for investigating the effects of such complex mixtures, since they simulate a particular environment and model changes with time of the component chemicals in that environment without the need to measure individual concentrations. For this reason such detailed analyses are rarely made.

Some mesocosm studies have, however, provided evidence for the contribution of individual components to overall toxic effects of mixtures. These studies are predominantly of pesticide interactions. One such piece of evidence was published by Hoaglund *et al* (1993). They used a large tank mesocosm containing plankton and fish to investigate the combined effects of the pesticides atrazine and bifenthrin. The observed population impacts gave little evidence for interaction. When either pesticide was increased to high levels, its effects masked the effects of the other. The observed interaction effects at low levels also did not act synergistically. The authors discussed the published literature on pesticide mixture interactions and although a number of papers on single species tests of pesticide mixtures are available, found no other reports on ecosystem effects of combinations of pesticides.

Fairchild *et al* (1994) investigated the toxicity of a mixture of an herbicide, atrazine, and a pyrethroid insecticide, esenvalerate, in large mesocosms. They considered that the herbicide could effect the producer levels of an ecosystem whilst the insecticide could effect the consumer levels. It was supposed that these specific effects could lead to increased effects beyond those normally expected if the insecticide availability was influenced by adsorption on to the macrophytes. Atrazine did affect species composition of the macrophytes but did not influence the overall availability of the insecticide to

higher organisms. The absence of reduced atrazine concentrations was attributed to functional redundancy of the macrophyte community, so that overall biomass was not reduced, and to the rapid aqueous disappearance of the pyrethroid insecticide regardless of atrazine concentration. In a similar study of the effect of one toxicant on the soluble concentration of another, Robinson-Wilson *et al* (1983) demonstrated an increased availability of pentachlorophenol to fish in the presence of the herbicide simazine. Simazine effectively reduced dominant macrophyte species resulting in a reduction of the sorption of pentachlorophenol to biomass which in turn decreased survival of bluegill sunfish and largemouth bass.

These latter two studies point out another complexity of using model ecosystems to relate quantitatively the effects of a mixture on an ecosystem to the individual components of the mixture. There may be complex interactions within the ecosystem which affect the concentration of individual components. In order to understand the relative contribution of each component to mixture toxicity, the bioavailable concentration of each component must be known throughout the duration of the study. As described in Section 5.2 environmental factors may greatly influence availability. Reductions in toxicant availability due to toxicant interactions with each other or with the biomass are mitigating factors, rather than effects which are intended to be covered when evaluating joint toxic action of mixtures.

Model ecosystems could be useful in evaluation of the toxic effects of mixtures of chemicals upon ecosystem structure and function. A major use of such systems is to investigate the possibility of higher order ecosystem effects which would not be evident in single species tests. They are particularly useful in this regard since, in contrast to field studies, they are closed systems and environmental conditions can be controlled (National Research Council, 1981a,b). However, instability, inherent species interactions and cycling, responses to changes in environmental conditions, and many other factors, make interpretation of ecosystem effects difficult even for single chemicals. There are numerous examples of studies where single chemicals give rise to apparently conflicting results in ecosystem structure and function such as interactions between populations of organisms, energy transfer and productivity, diversity, (Taub *et al*, 1987; Kindig *et al*, 1983; Woltering, 1983; Burnett and Liss, 1990). In the light of these subtle and complex influences of only a single toxicant on higher ecosystem processes, where the effects may be in opposition at the same concentration for the same toxicant, it is unlikely that much evidence for the combined toxic effect of multiple chemicals may be investigated quantitatively in such systems.

Field Studies

There are more published reports of field studies dealing with mixture toxicity than there are from model ecosystem tests. Field observations and on-site testing studies have been carried out to address the concern that water quality standards for single chemicals may overlook the possibility that in the environment, a large number of pollutants individually may be below concern concentrations but still cause toxicity through joint action.

Lloyd (1986) summarised the results of 25 years of European research into the toxicity of effluents to fish. One of the main objectives of this work was to determine the extent to which water quality standards set for single chemicals are valid when other toxic substances are present. In nearly all instances the toxicity of complex effluents could be accounted for by their concentrations of five common pollutants (ammonia, phenol, copper, zinc and cyanide). In the few instances where toxicity was greater than the sum of the TUs of these pollutants, it was assumed that other toxic pollutants were present. Field studies on polluted rivers also showed that in general, the combined TUs for rainbow trout 48h LC_{50} was predictive of the presence of fish in the river at < 0.28 TU (Alabaster *et al*, 1972; Solbé, 1973).

Attempts to correlate the presence of fish with NOEC based TUs were less successful. Two complications were the confounding influence of low dissolved oxygen values in polluted rivers and the observation that fish populations are more affected by high level episodic discharges rather than continuous low level exposures. Lloyd (1986) observed that more-than-additive effects do not occur and, when considering water quality standards for mixtures of common pollutants, allowance need not be made for synergistic effects. Also, acute to chronic ratios are expected to be less for mixtures of common pollutants than for single pollutants if additivity between them is less than 0.3 TU. However, the pollutants being considered were unlikely to be acting through non-polar narcosis.

The toxicity of mixtures of metals has been addressed in numerous additional field studies. Hall *et al* (1988) attempted to correlate observed toxicities in striped bass pro-larval tests, both in the field and in water samples brought back to the laboratory (*in situ*) with analyses for priority pollutants. Toxicity in both types of tests could potentially be accounted for solely by the metals present. However, other toxicants may have contributed to the toxicity if the metal toxicity was less than expected based upon single metal laboratory studies. The authors state that the metal toxicity may have been reduced through a large variety of physical, chemical and biological factors influencing bioavailability.

More recently, Logan and Wilson (1995) analysed the data from the extensive studies by Hall *et al* (1988, 1992). They used a TU approach and statistical methods to evaluate contributions to toxicity from the individual pollutants measured. They found that the field results were in general agreement with the *in situ* toxicity measurements reported by Hall. Furthermore, the toxicity of all samples was adequately explained by just five metals (Al, Cd, Cr, Cu, and Zn) and the results were consistent with additive toxicity. This result was not considered to prove that the metals caused the toxicity, since their effects could be diminished by lower bioavailability and other unmeasured toxicants could be covariant and contributing to the toxicity.

A TU approach was also used by Kemble *et al* (1994) to investigate the toxicity of metal contaminated sediments. They performed chronic toxicity tests with a large number of test organisms on pore water and whole sediment samples from numerous sampling stations. The same samples were also analysed for 12 metals. Numerous samples with a total TU for chronic toxicity of greater than 1.0 showed no toxicity. A likely explanation of this lack of toxic effects is a lack of bioavailability. Some samples with total TUs less

than 1.0 did show toxicity, due to the presence of ammonia and hydrogen sulphide in the sediments. Organic toxicants could also have contributed.

Clements and Kiffrey (1994) investigated the effects of heavy metals in field experiments. They used on-site, rather than *in situ* testing to account for possible temporal changes in water quality. They found that the river showed strong spatial and temporal changes in metal contamination. They found no good correlation between laboratory toxicity tests and impacts on the benthic community and it was concluded that both types of tests were necessary. They also concluded that the spatial and temporal variability in water quality limits the usefulness of the sampling approach, while the field observation approach was limited by the variability in metal accumulation and metal toxicity to the various taxa. In another investigation of the toxicity of heavy metals to a polluted environment, Klerks and Levington (1989) found that organisms from the impacted areas showed resistance to metal toxicity. They concluded that such acclimation to toxicants "strongly alters the perception of the answers which typical laboratory toxicity studies give". The issues relating to bioavailability of metals and adaptation to metal toxicity severely limits the likelihood that field studies will provide definitive evidence for the additivity of their effects.

The major difficulty in relating toxicity to pollutant concentration in field studies is the analytical limitation that all toxicants can never be anticipated and analysed for. For example, Brunstrom *et al* (1992) found that the toxicity of the PAH fraction of sediment extract was too high to be explained by the toxicities of the 15 PAH compounds analysed. Further analysis indicated that PCBs and PCDD in the PAH fraction were primarily responsible for the toxicity. This result emphasises the problem in field studies that, if greater that additive toxicity is observed, there is always the possibility that some unknown substance caused added toxicity, rather than that the observation could be the result of synergy.

Effluent Studies and TIE Approaches

In studies in the USA it has been found that whole effluent toxicity correlates well with surface water impact. In one study, 43 comparisons between chronic toxicity to *Ceriodaphnia dubia* in effluents and standardised qualitative sampling of benthic macroinvertebrates downstream found 88% agreement (Eagleson *et al*, 1990). However, as discussed previously, modifying factors may greatly alter the toxic effects observed.

The US EPA's Technical Support Document (TSD) for Water Quality-based Toxics Control addresses the toxicity of complex effluents (US EPA, 1991a) and makes the following comments:

- the available information tends to indicate that the combined effects of individual acutely toxic agents are somewhat less than strictly additive;
- field studies of effluent toxicity and laboratory experiments with specific chemicals imply that synergism would be an extremely rare phenomenon which has not been observed during on-site effluent toxicity studies, and is not considered an important factor in the toxicological assessment of effluents;

- cases in which one effluent or pollutant parameter (such as total dissolved solids) ameliorated the toxicity of another effluent pollutant, antagonism, have been observed.

In the case of US EPA's Technical Support Document however, it was not made clear whether the toxicity observed was due to physical/chemical interactions between the chemicals in the effluent and/or receiving water or to true toxicological antagonism.

In summary, recommendations are made in EPA's implementation guidance that toxicity from complex effluents should be considered to be additive when performing a waste load allocation (Di Toro *et al*, 1991).

Toxicity Identification Evaluations (TIEs) are a part of the Toxicity Reduction Evaluation (TRE) associated with effluent evaluations for the US EPA's National Pollutant Discharge Elimination System (US EPA, 1991b,c, 1992, 1993a,b). Typically in a TIE, an effluent is subjected to a variety of physical and chemical treatments and aquatic toxicity tests are conducted with each manipulated sample. Phase I of this procedure contains methods to characterise, Phase II to identify and Phase III to confirm the suspected toxicants in the effluent.

Because effluents, surface waters and sediments are complex mixtures, an examination of published TIE results may offer insight on how some substances affect aquatic organisms when combined. However, the following must be considered as they can complicate the interpretation of a TIE:

- toxicity caused by substances which cannot easily be identified analytically;
- toxicity caused by identified compounds for which there are no published toxicity data;
- concentration/bioavailability of toxicants is affected by TIE manipulations (Hendriks *et al*, 1994; Munoz *et al*, 1994; Schubauer-Berigan and Ankley, 1991);
- characteristics of test organisms used (e.g. species, age, size, sex, health) may be different from those in the literature, creating an artificial difference between the calculated TU (using literature values) and observed TU (Wang, 1987; Lankford, 1990; Reece and Burks, 1985);
- differences in hardness in dilutions of the effluent during toxicity testing can alter toxicity (Di Toro *et al*, 1991).

In the literature surveyed, the chemical(s) causing toxicity are often determined by correlation of chemical concentrations, evaluation of symptoms, examination of matrix effects and comparison of species' sensitivity ("weight of the evidence" approach). Spiking a fractionated effluent alone often does not yield enough definitive information to identify the toxic agent(s).

Several of the TIE studies examined concluded that toxicity seen in an effluent was due to a single pollutant (Amato *et al*, 1992; Schubauer-Berigan *et al*, 1993; Munoz *et al*, 1994; Wells *et al*, 1994; Kszos *et al*, 1992). These studies will not be discussed as they do not

aid in assessing mixture toxicity. Because toxicity limitations on effluents is a permit driven process, the permittee stops examining effluent toxicity when the toxicity is low enough to "pass". It is difficult to say if only one component of the effluent is really responsible for all the toxicity in those instances.

A chemical plant effluent acutely toxic to *Daphnia pulex* was evaluated by Jop *et al* (1991). Approximately 70% of the toxicity was due to non-ionised ammonia. The remainder was thought to be due to 2-(4-morpholinyl)-benzothiazole with possible contribution from an unidentified benzothiazole derivative. However, when pure 2-(4-morpholinyl)-benzothiazole was tested in laboratory water, toxicity was much lower than when 2-(4-morpholinyl)-benzothiazole was spiked into the effluent. It is thought that a matrix interaction (possibly the unidentified benzothiazole derivative) in the effluent caused the discrepancy between spiked effluent and laboratory water toxicity.

Schubauer-Berigan and Ankley (1991) tested sediment interstitial water from a highly contaminated river for acute toxicity to *Ceriodaphnia dubia*. In Phase II, metals (Zn, Ni, Pb, and Cu) accounted for approximately 3 TU, ammonia for 3 TU, and non-polar organics for 5 TU. Whole pore water only had 6 TU. It is likely toxicity was not additive. Enough metals were present for toxicity, yet all toxicity could be accounted for by the ammonia present. Bioavailability and test matrices inherent to TIE procedures may have been a factor.

Reece and Burks (1985) examined a petroleum refinery wastewater. The most toxic fraction was steam volatile, base-neutral and aromatic organic in nature. Those peaks identified had a combined concentration of 1100 mg/l. The authors concluded that although the identified compounds were individually not considered acutely toxic to *Daphnia magna* at the mg/l level, they and the unidentified compounds could be acting in an additive or synergistic fashion to produce acute toxicity.

Drain water from rice fields was evaluated for acute toxicity to *Ceriodaphnia dubia* by Norberg-King *et al* (1991). Carbofuran and methyl parathion were identified as the toxicants. Add-back tests showed the toxicity to be additive.

Di Toro *et al* (1988) tested the assumption that toxicity is a measurable and additive property of various effluents. They selected 8 discharges into the Naugatuck River in Connecticut and tested for effects on *Ceriodaphnia* fecundity. The data indicated that the toxicities of the various outfalls were not additive. Possible reasons for the lack of additivity were that toxicity was not conserved (degrades) or that there were antagonistic effects. In a follow-up study, Di Toro *et al* (1991) identified copper as the primary toxicant. A strictly additive model failed to address the toxicity seen. A complicating factor was the interaction of copper toxicity with the changing hardness in the river and in the toxicity assays themselves.

Extensive toxicity and analytical testing was done on sediments and pore water from 13 sites in a contaminated river (Hoke *et al*, 1993). Potential toxicants determined by analytical means were non-ionised ammonia, bicarbonate, copper, zinc, naphthalene

and phenanthrene. Calculated acute TUs and measured TUs for *Daphnia magna* and *Ceriodaphnia dubia* were generally in good agreement for 12 of the 13 sites. In one site, the measured TU was much higher than calculated; one or more chemicals not measured was probably the cause.

The variability of toxicity in effluents is a very real problem (Weiss *et al*, 1992). An extreme example of variability was reported by Lewis *et al* (1988). In the middle of a study of the toxic fractions of an effluent, they found an increase in the toxicity in all samples including up-stream control water and dilution water. The toxicity was due to an episodic event up-stream from the effluent. Even disregarding that problem, they were not able to identify a specific toxic fraction and concluded that it is generally difficult to attribute toxicity of an effluent to a specific compound, either due to the presence of an unidentified compound or to a mixture effect.

Di Toro (1986) has addressed the difficult problem of temporal and spatial variability of toxicity in effluent streams and suggests a probabilistic approach towards exposure assessments in such situations. Spacie (1986) described an additional complication in that the biota affected by the pollutants also show spatial and temporal distribution. Such uncertainties certainly limit the possibility that strong quantitative data on the question of contribution of individual chemicals to mixture toxicity will be available from field studies. Also, even though such variability may be handled statistically, a frequent observation is that field studies are marred by large episodic events as described by Lewis.

Discussion

To summarise, in 19 of the TIE studies reviewed, more than one chemical was identified as responsible for aquatic toxicity. Among these 19 studies, 14 demonstrated additivity. In two, unidentified toxicants were used to explain the discrepancy, and in another two matrix effects interfered with a definitive answer. One study did not demonstrate additivity. The studies which found metals at toxic levels did not always find additivity. Metals should probably be considered as a special case when analysing the toxicity of a mixture. It should be noted that due to spatial and temporal variation in effluents, it is difficult, in the absence of extensive chemical analyses, to determine the toxic components of an effluent without collecting and fractionating a large number of samples.

Successful toxicant identification requires a complete understanding of the sensitivities of the test species used, dose-response curve for the toxicants, the influences of the effluent matrix on the toxicants and interactions among toxicants (Burkhard and Ankley, 1989). Nearly all of the studies discussed above demonstrated additivity. However, the majority of TIEs conducted in the USA are not available in the published literature. TIEs conducted at the Duluth EPA Research Laboratory have shown that toxic effects are often not additive and that toxicants are present in ratios such that the toxicity contribution of one might be diluted out in the range of the effluent effect concentration.

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